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Nonconjugative Plasmids Encoding Sulfanilamide Resistance

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Nonconjugative plasmids encoding sulfanilamide (Sa) resistance were demonstrated at a high frequency in *Shigella* and *Escherichia coli* strains resistant to sulfanilamide. These Sa plasmids were all compatible with the standard plasmids used in compatibility testing. The sizes of seven Sa plasmids were measured by electron microscopy and ranged from 1.79 to 2.08 μm, corresponding to 3.5 to 3.9 megadaltons.

Sulfanilamide (Sa) and its derivatives have been used as chemotherapeutic agents since 1940 in Japan against infections by both gram-positive and gram-negative bacteria and pioneered the era of chemotherapy as an effective antibacterial drug. Epidemiological surveys in Japan disclosed, however, the rapid appearance of Sa-resistant *Shigella* (8) and *Staphylococcus aureus* strains (7, 9, 10) after the introduction of Sa into practical medicine. Similarly, antibiotic-resistant strains have rapidly appeared since the introduction of these drugs, and the emergence of multiply drug-resistant strains has become one of the most important problems in practical medicine. This paper reports data of nonconjugative Sa resistance plasmids as one approach to the study of the formation of multiply drug-resistant plasmids.

MATERIALS AND METHODS

Bacterial strains. The strains used are stock cultures of this laboratory and were isolated from clinical specimens. *Escherichia coli* 58-161 Nal^r (resistant to nalidixic acid) and W3110 Rif^r (resistant to rifampin) were used as recipients for Sa resistance in conjugation experiments. *E. coli* C Rif^r was used as a recipient of Sa resistance in transformation. *Shigella flexneri* JS-E15 Sul^r (resistant to sulfanilamide) is a stock culture of this laboratory, whose determinant governing Sa resistance is located on the chromosome between *his* and *str* (streptomycin resistance) (12). Derivatives of *E. coli* K-12, J53 (*met pro*), J62 (*his try pro*), and CSH-2 (*met*) were used as the hosts of R plasmids for incompatibility testings.

R plasmids. Standard R plasmids for incompatibility testing were obtained from N. Datta, Royal Postgraduate Medical School, London. Rms306, a conjugative tetracycline (Tc)-resistant plasmid, was carried in *E. coli* 58-161 Nal^r and used to mobilize the transfer of the nonconjugative Sa plasmids.

Media. Brain heart infusion (BHI, Difco) broth was used for liquid cultures and for conjugal transfer of drug resistance. For the assay of Sa resistance and the selection of Sa-resistant transconjugants and transformants, a semisynthetic medium (S medium) was used, which 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 hydrochloride, and 2 g of glucose. Peptone-water (pH 7.2), used as a liquid culture for the determination of Sa resistance, consisted of 10 g of peptone, 5 g of sodium chloride, and 1,000 ml of distilled water. (S medium containing 1.2% of agar (S agar) was used for the determination of Sa resistance and for the selection of Sa-resistant transductants and transformants. S medium containing 1.0% lactose and 1.2% agar (SLac agar) was used for the slection of R^+ transconjugants in incompatibility tests.

Determination of Sa resistance. An overnight peptone-water culture was diluted 100-fold with fresh peptone-water, and 1 loopful of diluted culture was inoculated on a series of S agar plates containing serial twofold dilutions of Sa. The minimum inhibitory concentration of drug was scored after 18 h of incubation at 37°C.

Conjugal transfer of drug resistance. The conjugal transfer of drug resistance was followed by the method described previously (14).

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

Transformation of Sa resistance. The transformation procedure is essentially as described by Cohen et al. (2). *E. coli* C Rif^r was used as a recipient of Sa resistance. An overnight BHI broth culture of donor cells carrying an Sa plasmid (900 ml) was spun at 6,000 rpm for 15 min in a refrigerated centrifuge. The precipitate was washed once with Tris buffer [0.02 M tris (hydroxymethyl)aminomethane-0.02 M NaCl-1 mM ethylenediaminetetraacetate (pH 8.0)] and was used for the deoxy-

ribonucleic acid (DNA) preparation. The DNA preparation thus obtained was suspended in 2 ml of Tris buffer. *E. coli* C Rif^r was grown overnight in BHI broth at 37°C and used for the recipient. The recipient culture without the addition of the DNA preparation was used as a control.

Incompatibility experiments. Incompatibility experiments were carried out by the method described by Datta (3). *E. coli* C Rif^r strains carrying nonconjugative Sa plasmids were obtained by transformation with the plasmid DNAs obtained from the original *Shigella* and *E. coli* strains (see Table 4), and six strains were used as recipients for the transfer of the incompatibility standard plasmids. *E. coli* K-12 strains carrying the standard R plasmids were used as the donors. A 2-ml amount of a BHI culture of recipient cells and 0.5 ml of a donor BHI culture were mixed at the early stationary phase of growth. The mixed culture was incubated with gentle shaking at 37°C for 2 h, and appropriate dilutions were then spread on SLac agar containing 100 μg of Sa per ml and 100 μg of rifampin per ml. Twenty colonies that grew on the plates after 48 h of incubation at 37°C were cloned three times by using the same medium, and their drug resistance was examined. The stability of two plasmids coexisting in the same cell was examined after subculture for at least 10 generations in nutrient broth containing no drug.

Isolation of plasmid DNA for electron microscopy. Isolation of the Sa plasmid DNA followed the method described by Clowes (1). Briefly, bacterial cells were harvested in late exponential phase and were lysed by ethylenediaminetetraacetate, lysozyme, and sodium dodecyl sulfate. Covalently closed circular form DNA was obtained by ethidium-CsCl density gradient centrifugation. Dialyzed DNA was mixed with 0.01% cytochrome *c*, spread as a monolayer, dried, and shadowed by platinum.

RESULTS

The resistance patterns of *Shigella* and *E. coli* strains to Tc, chloramphenicol (Cm), streptomycin (Sm), and Sa are shown in Table 1. The isolation frequency of quadruply resistant strains was the highest, followed by singly, triply (Cm Sm Sa, Tc Sm Sa, Tc Cm Sa, and Tc Cm Sm), and doubly (Cm Sa, Tc Sa, Tc Cm, Cm Sa, Tc Sm, and Cm Sm) resistant ones. Conjugative resistance plasmids were most frequently found in triply and quadruply resistant strains. Among the singly resistant strains, Sa resistance was most frequently isolated, and strains with other single resistances were very few in number. The frequency of conjugative plasmids from singly resistant strains was very low, except for Tc-resistant plasmids (Table 2).

We randomly selected from our stock cultures 25 *Shigella* and 23 *E. coli* strains possessing nonconjugative Sa resistance and examined them for the presence of plasmids encoding Sa resistance. A total of 18 *Shigella* strains (72%) and 13 *E. coli* strains (56.5%) lost their Sa resistance during storage or after treatment with acriflavine. These results strongly suggest that the determinant governing Sa resistance in *Shigella* and *E. coli* strains is most often located on a nonconjugative plasmid. To further examine the cytoplasmic inheritance of Sa resistance, we selected five *E. coli* and five *Shigella* strains, from which Sa resistance had been eliminated spontaneously or by treatment with acriflavine (see above). A transmissible Tc plasmid (Rms306) was conjugally transferred to each of these strains. As shown in Table 3, the nonconjugative Sa resistance determinants were mobilized by the presence of the Rms306 plasmid, but the transconjugants that acquired only Sa resistance could not transfer their Sa resistance by conjugation. In contrast, the Sa Tc-resistant transconjugants were capable of transferring their Sa resistance, due to the concomitant presence of the conjugative Tc resistance plasmid. These results confirmed the presence of nonconjugative Sa plasmids in the *Shigella* and *E. coli* strains.

Contour length measurements. The nonconjugative Sa plasmids in *Shigella* and *E. coli* strains were transformed into *E. coli* C Rif^r (Table 4). Plasmid DNA was prepared for elec-

TABLE 1. *Isolation frequency of resistant strains*[a]

Resistance pattern[b]	*Shigella* (%)	*E. coli* (%)
Quadruple	75.7	64.2
Triple	4.7	5.7
Double	2.8	8.9
Single	16.8	21.2

[a] The results are based on surveys from 15,903 strains, including 12,453 *Shigella* and 3,450 *E. coli* strains.

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

TABLE 2. *Isolation frequency of R plasmids from singly resistant Shigella and E. coli strains*[a]

Bacteria resistant to:	*Shigella*		*E. coli*	
	Resistant strains (%)	R$^+$ strains (%)	Resistant strains (%)	R$^+$ strains (%)
Sa	97.8	5.6	82.6	1.8
Tc	2.1	34.6	1.4	11.1
Sm	0.1	0	2.9	0
Cm	0	0	0	0

[a] The results are based on surveys from the strains shown in Table 1. The demonstrated frequency of R plasmids from the strains carrying the indicated single resistance is shown in Table 1.

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

Strains	Donor[b]	Recipient	Selection	Transconjugant		
				Transmission frequency	Drug resistance	Conjugal transferability of Sa resistance[c]
E. coli	GN2572 (Rms306)$^+$	58-161 Nal^r	Nal + Sa	6.8×10^{-6}	Sa (64/100)	−
					Tc Sa (36/100)	+
	GN2632 (Rms306)$^+$			7.2×10^{-7}	Sa (32/96)	−
					Tc Sa (64/96)	+
	GN3645 (Rms306)$^+$			8.3×10^{-7}	Sa (42/89)	−
					Tc Sa (47/89)	+
	GN3737 (Rms306)$^+$			2.9×10^{-6}	Sa (58/95)	−
					Tc Sa (37/95)	+
	GN4878 (Rms306)$^+$			4.5×10^{-6}	Sa (42/92)	−
					Tc Sa (50/92)	+
Shigella	JS13806 (Rms306)$^+$	58-161 Nal^r	Nal + Sa	8.8×10^{-7}	Sa (56/100)	−
					Tc Sa (44/100)	+
	JS13817 (Rms306)$^+$			3.5×10^{-6}	Sa (25/95)	−
					Tc Sa (70/95)	+
	JS13950 (Rms306)$^+$			4.2×10^{-6}	Sa (18/93)	−
					Tc Sa (75/93)	+
	JS13956 (Rms306)$^+$			2.3×10^{-6}	Sa (18/85)	−
					Tc Sa (67/85)	+
	JS13978 (Rms306)$^+$				Sa (28/93)	−
					Tc Sa (65/93)	+
	JS-E15 (Rms306)$^+$Sul$^{r d}$	58-161 Nal^r	Nal + Sa	10^{-9}		−

[a] S medium containing sulfisoxazole (50 μg/ml) and nalidixic acid (50 μg/ml) was used for selection.
[b] Donors were obtained by the conjugal transmission of the Rms306 plasmid to each strain carrying nonconjugative Sa resistance.
[c] Conjugal transferability was examined by the cross-brushing method with *E. coli* W3110 Rif^r.
[d] *S. flexneri* JS-E15 (Rms306)$^+$ Sul^r was obtained by the conjugal transmission of the Rms306 plasmid to JS-E15 Sul^r, whose determinant governing Sa resistance is located on the chromosome (12).

TABLE 4. *Transformation of Sa resistance by DNAs isolated from E. coli and Shigella donors carrying nonconjugative Sa resistance*[a]

Donor of Sa resistance	No. of Sa-resistant transformants per 10 μg of DNA used
E. coli GN2572	32
E. coli GN2632	57
E. coli GN3645	33
E. coli GN3737	36
E. coli GN4878	61
S. flexneri JS13806	51
S. sonnei JS13817	36
S. sonnei JS13950	588
S. flexneri JS13956	136
S. flexneri JS13978	56

[a] *E. coli* C Rif^r was used as the recipient.

tron microscopy, and electron micrographs of open circular molecules were enlarged, traced, and measured. The micrographs of the Sa plasmids are shown in Fig. 1. Details of contour length measurements are shown in Table 5. The seven Sa plasmids were measured by electron microscopy and ranged from 1.79 to 2.08 μm.

DISCUSSION

Conjugative resistance plasmids were first identified in Japan by the following properties (for a review, see reference 8): (i) transmission of resistance by mixed cultivation, (ii) interruption of the transmission with a fritted-glass disk to separate the two parental cultures, and (iii) spontaneous and induced loss of drug resistance from drug-resistant cells.

Nonconjugative resistance plasmids were discovered later in multiply resistant *S. aureus* strains by the irreversible loss of resistance and by genetic analysis of staphylococcal resistance (9–11, 13). Since then, gram-negative host bacteria carrying nonconjugative plasmids have been found, and the multiple resistance of clinical isolates has been found to be due mostly to the presence of either conjugative or nonconjugative plasmids. The finding of nonconjugative plasmids gives us a useful tool for the pursuit of

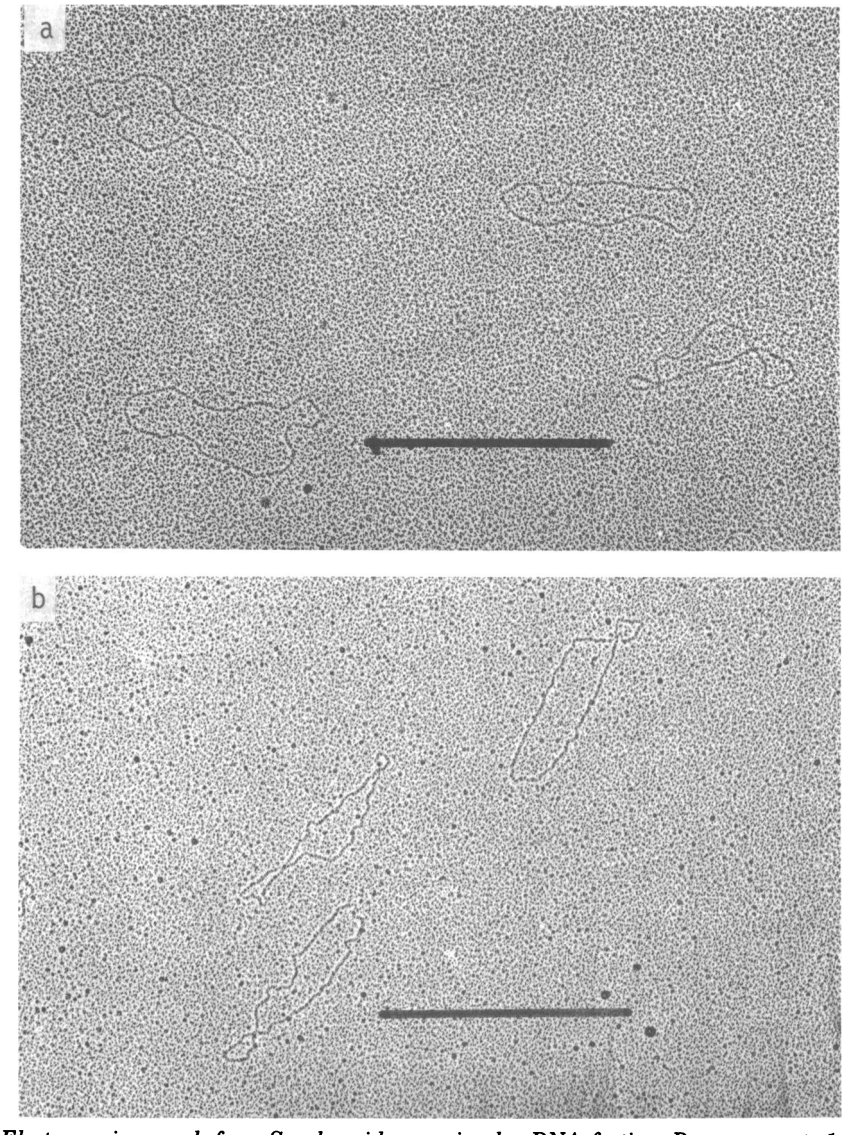

FIG. 1. *Electron micrograph from Sa plasmid open circular DNA faction. Bar represents 1 μm. (a) Sa plasmid rMS31 from E. coli GN2632; (b) Sa plasmid rMS36 from S. sonnei JS13950. Each Sa plasmid was transformed to E. coli C Rif^r. DNAs were prepared from the tranformants.*

TABLE 5. *Size of nonconjugative Sa plasmid DNA*[a]

Original host of Sa plasmid	Sa plasmid	No. of molecules measured	Contour length (μm)		Mol wt ($\times 10^{-6}$)[b]
			Mean	Standard deviation	
E. coli GN2632	rMS31	21	1.79	0.05	3.7
E. coli GN3645	rMS32	25	1.85	0.06	3.8
E. coli GN4878	rMS33	19	1.91	0.08	3.9
S. flexneri JS13806	rMS34	11	1.73	0.05	3.5
S. sonnei JS13817	rMS35	22	1.83	0.14	3.7
S. sonnei JS13950	rMS36	16	1.80	0.09	3.7
S. flexneri JS13958	rMS37	10	1.76	0.06	3.6

[a] The Sa plasmid was transformed to *E. coli* C Rif^r as shown in Table 4. *E. coli* C Rif^r carrying each plasmid was used for the determination of the size of plasmid DNA.

[b] Calculated on the assumption that 1 μm = 2.07×10^6 daltons (5).

the evolutionary processes of the formation of plasmids encoding multiple resistance.

After the introduction of Sa and its derivatives into practical medicine, bacterial strains rapidly acquired Sa resistance and were isolated at a high frequency from clinical specimens (7–10). Thereafter, the wide use of antibiotics such as Tc, Cm, and Sm led to the emergence of multiply resistant strains in addition to Sa resistance. It is known that multiple antibiotic resistance in clinical isolates is due mostly to the presence of conjugative plasmids, resulting in the rapid spread of multiple resistance (8).

It should be noted however that among strains singly resistant to Tc, Cm, Sm, or Sa, Sa-resistant strains were demonstrated most frequently in *E. coli* and *Shigella* strains. In contrast, the frequency of conjugative Sa plasmids is rather low in strains singly resistant to Sa.

The results described in this article have disclosed that nonconjugative Sa plasmids can be demonstrated at a high frequency in singly Sa-resistant *E. coli* and *Shigella* strains. The sizes of the Sa plasmids are found to be similar to those of nonconjugative plasmids in staphylococci. Most of the staphylococcal nonconjugative plasmids encode single resistance, and nonconjugative plasmids carrying multiple resistance are very few in number in staphylococci (9–11). It was found that Sa plasmids in *E. coli* and *Shigella* strains were all compatible with the standard R plasmids used for compatibility tests. The molecular weights of conjugative Sa plasmids ranged from 53×10^6 to 59×10^6, indicating a major difference between nonconjugative and conjugative Sa plasmids (4).

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