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Molecular Nature of Two Nonconjugative Plasmids Carrying Drug Resistance Genes

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Two nonconjugative R-plasmids, N-SuSm and N-Tc, have been characterized. Both were of relatively small size (5×10^6 to 6×10^6 daltons) and present in multiple copies within their respective bacterial hosts. N-SuSm possessed a guanine plus cytosine content of 55%, whereas N-Tc was 49% guanine plus cytosine. Although these plasmids were inherently nontransmissible they could be mobilized by a large variety of transfer agents including Ent, Hly, and K88. The fi^- transfer factors tested were far more likely (about 200×) to mobilize these nonconjugative plasmids than were the fi^+ transfer factors tested. Although the mobilization phenomenon was not found to be associated with a detectable level of direct stable recombinational union between N-SuSm or N-Tc with a transfer factor, we were able to demonstrate a low level of recombination between these replicons and a transfer factor by P1-mediated transduction. The isolation of recombinants between transfer factors and nonconjugative plasmids presumably represents one means by which unitary molecular types of R-plasmids arise and by which existing R-plasmids may acquire new resistance determinants.

A number of investigations have dealt with the genetic and molecular nature of R-factors which are transferred as a single unit at conjugation and transduced by P1 as a single linkage group (12, 32, 36, 42). This picture is not always the case, however. E. S. Anderson and his associates established that the transfer genes and the genes determining resistance may sometimes behave as located on essentially independent elements (1-5). In the same vein, multiply antibiotic-resistant clinical isolates have been encountered that do not transmit their resistance genes by conjugation. If such cell lines are infected with some suitable transfer factor, however, the drug resistance genes are often found to be mobilized at a measurable rate (4, 5, 39). The discovery of the inherent independence of transfer factors and resistance determinants in some bacterial strains suggested one mechanism by which single unit R-factors could be formed by recombination. Support for the view that R-factors may be recombinational assemblages of a transfer factor and a nonconjugative plasmid (the term nonconjugative plasmid used throughout is defined as a plasmid which has not so far been shown to bring about the conjugal transfer of its own or other genetic material) carrying resistance determinants has been previously presented by this laboratory and by others (13, 15, 26, 32, 35).

R-factors, called class 1 by Anderson (6) and plasmid cointegrates by Clowes (10) are represented by certain fi^+ R-factors (about 65×10^6 daltons, 51% guanine plus cytosine [G plus C]), which behave as single units in *Escherichia coli* but dissociate into separate replicons after transfer to *Proteus*. In *Proteus* the dissociated R-plasmid elements consist of a transfer factor (about 50×10^6 daltons, 49% G plus C) and a nonconjugative plasmid (about 15×10^6 daltons, 56% G plus C) carrying the majority of the drug resistance determinant genes (12, 13, 16, 32). The smaller nonconjugative replicon which appears in *Proteus* replicates at a rate which rapidly outstrips the large transfer replicon. Unfortunately, no one has yet been able experimentally to manipulate the small nonconjugative drug resistance replicon from *Proteus* into another host.

The genetic system described by Anderson and others existing in all hosts examined as two independently replicating plasmids have been called class 2 plasmids (6) or plasmid aggregates (10). Milliken and Clowes (31) recently examined several typical class 2 R-factors described by Anderson and Lewis designated ΔA and ΔS. These typical representatives of class 2 plasmids were found to consist of a transfer

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factor, Δ, (about 61 × 10^6 daltons) and a large number of copies of the smaller replicon, S or A, (about 5.6 × 10^6 daltons) determining drug resistance. These plasmids were transferred independently at conjugation and transduced independently by phage P1 (6), and the mobilization of drug resistance by Δ did not appear to require covalent linkage. Thus, the "normal" behavior of the class 2 R-factors mimics to some degree the molecular and (mostly inferred) genetic properties of class 1 plasmids in *Proteus*.

In the present investigation the molecular nature of two nonconjugative plasmids which carry drug resistance genes similar to those studied by Milliken and Clowes (31) were examined and their mobilization by transfer factors was characterized. Such nonconjugative resistance plasmids have some properties similar to the dissociated drug resistance replicons seen in *Proteus* and can recombine with transfer factors to form a single unit of replication and transmission.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial host strains and the plasmids used in this investigation are listed in Table 1. Unless otherwise indicated the plasmids were resident in *E. coli* K-12 strain W1485.

Media. Complex media used were Penassay Broth (Difco), nutrient agar (NB) (Difco), L-broth, and L-agar (28). For labeling experiments the minimal media of Freifelder and Freifelder was used (20) and supplemented with 250 μg of desoxyadenosine per ml and 0.5 μg of thymine per ml.

Materials. Reagents and sources were as follows: Brij 58 from Atlas Chemical; sodium desoxycholate (DOC) from Fisher Scientific; Sarkosyl (sodium dodecyl sarcosinate) from the Geigy Chemical Co.; [*methyl*-^{3}H]thymine (18 to 23 Ci/mmol) from New England Nuclear Corp.; CsCl (technical grade) from Rare Earth Division, American Potash and Chemical Corporation; lysozyme, ethidium bromide (EtBr), streptomycin, tetracycline, chloramphenicol, nalidixic acid, and rifampin were from Calbiochem.

Mobilization of nonconjugative plasmids by transfer factors. The presence of nonconjugative plasmids was determined by a modification of the resistance mobilization test described by Anderson (1). Donor strains carrying a transfer factor and intermediate strains carrying the nonconjugative plasmid N-SuSm or N-Tc were grown for 6 h in Penassay Broth. Donors and intermediates were mixed (1:1) in fresh broth and incubated for an additional 2 h. The cultures were diluted 1:10 and a final recipient, strain SF185, was added. After overnight incubation the matings were diluted and plated on nutrient agar supplemented with 50 μg of nalidixic acid per ml and 50 μg of streptomycin or tetracycline per ml. The frequency of transfer was expressed as the number of resistant recipient cells per donor cell in the overnight culture.

Preparation of cleared lysates. The lysis procedure has been described by Clewell and Helinski (8, 9). It essentially involved lysis of EDTA-lysozyme spheroplasts by Brij 58 and DOC. The lysate was then centrifuged at 48,000 × *g* for 25 min, removing 95% or more of the total chromosomal DNA. Most of the plasmid DNA is left in the supernatant fluid which is referred to as the cleared lysate. The cleared lysate was diluted 1:1 in TES (0.05 M NaCl, 0.005 M ethylenediaminetetraacetic acid [EDTA], and 0.03 M tris(hydroxymethyl)aminomethane [Tris], pH 8) before layering onto a 5 to 20% sucrose gradient.

Sucrose density gradients. Five to 20% neutral sucrose gradients contained 0.01 M KPO_4, 0.5 M NaCl, pH 7. Five to 20% alkaline sucrose gradients contained 0.5 M NaCl, 0.02 M EDTA, and 0.3 M NaOH, pH 12.5. Fractionation of gradients and counting of radioisotopes were carried out as previously described (17, 36).

Preparation of Sarkosyl lysates. Cells from 2-ml logarithmic cultures were uniformly labeled with ^{3}H-thymine and lysed according to method B of Young and Sinsheimer (43) as previously described (36), except that 1.2% Sarkosyl was substituted for sodium dodecyl sulfate.

Transformation. The transformation method is as described by Cohen et al. (11) except that when transforming for the N-SuSm plasmid, the transformation mixture was incubated overnight in L-broth to permit phenotypic expression before plating to nutrient agar supplemented with 25 μg of streptomycin per ml.

CsCl density gradients. ^{3}H-labeled plasmid deoxyribonucleic acid (DNA) was added to a concentrated solution of CsCl in TES buffer and the final density was adjusted to 1.710 g/cm^3 (refractive index, 1.4010). ^{14}C-lambda DNA was added as a density marker. The mixture was centrifuged for 40 to 60 h at 125,000 × *g* in a fixed angle type 65 rotor. Five- to 10-drop fractions were collected from the bottom of the tube. The refractive index of the fractions were determined and the density was calculated by the linear relationship between refractive index and density (18, 36).

Dye buoyant centrifugation. Material from Sarkosyl or Brij lysed cells was mixed with a concentrated solution of CsCl in TES buffer and ethidium bromide (final concentration, 250 μg/ml). The final density was adjusted to 1.6253 g/cm^3 (refractive index, 1.3925). The mixture was centrifuged for 40 to 60 h at 125,000 × *g* in a fixed angle type 65 rotor. Ten-drop fractions were collected from the bottom of the tube and 0.01-ml samples of each fraction were removed and counted as previously described (17). Material from the dense satellite bands containing covalently closed circular (CCC) DNA molecules was pooled, the EtBr was extracted with an equal volume of isopropanol and dialyzed.

Transduction. The P1 *vir* employed was obtained from L. Rosner. Transducing particles were prepared by propagation on strain SF187 by confluent lysis on agar (28). The titers of P1 lysates were 0.5 to 1.0 × 10^{10} plaque-forming units (PFU) per ml. Transduction assays were carried out at a low multiplicity of infection (MOI) (0.01) on strain W1485.

TABLE 1. *Bacterial strains and plasmids*

Stock no.	Mol wt[a] ($\times 10^6$)	Mol[b] fraction G + G	Phenotype	Genotype[c]	Source of derivation	Designation in text
A. Bacterial strains						
W1485				F^-, prototrophs		
SF185				F^-, prototroph *nal*-r	From W1485	
SF186				F^-, prototroph *rif*-r	From W1485	
B. Plasmids						
(i) Transfer plasmids						
FSF-1	62	0.49	*fi*$^+$ Pil		Classical sex factor of 58-161F$^+$	F
PSF-2	63	0.50	*fi*$^-$, ColIb-P9		*S. typhimurium* PG80 from P. Gemski	ColIb
PSF-3	ND[d]	ND	*fi*$^+$, K88		*E. coli* 08, K87, 88ab from H. Williams Smith (39)	K88
PSF-4	60	0.48	*fi*$^+$, EntA		*E. coli* P307 from H. Williams Smith (37)	Ent P307
PSF-5	21	0.41	*fi*$^-$, EntB		*E. coli* P95 from C. Gyles (37)	Ent P95
PSF-6	65	0.50	*fi*$^+$, Hly		From H. Williams Smith (38)	Hly
PSF-7	ND	ND	*fi*$^-$, Pil		*S. panama* SP1 from P. A. M. Guinee (21)	Δ47
RSF-8			*fi*$^-$, Sm, Sp, Cm		*S. panama* from P. A. M. Guinee (21)	
RSF-9	50	0.49	*fi*$^+$, Pil		Transfer factor of Rldrd19 (36)	RTF-1
FSF-2	95	0.50	*fi*$^+$, Pil *lac*		*E. coli* 200μ F-*lac*$^+$ from F. Jacob	F-*lac*$^+$
(ii) Nonconjugative plasmids						
RSF-1010			SuSm		*E. coli* strain 3 from P. Fredericq (19)	N-SuSm
RSF-1020			Tc		*S. panama* SP219 from P. A. M. Guinee	N-Tc
C. Derivative						
SF-187			*fi*$^-$, Sm, Sp, Cm, Tc		W1485 carrying RSF-8 & RSF-1020	
SF-188			*fi*$^+$, Hly, Su, Sm		W1485 carrying PSF-6 & RSF-1010	
SF-189			*fi*$^+$, Pil, SusM		W1485 carrying FSF-1 & RSF-1010	
SF-190			*fi*$^-$, ColIb, SuSm		W1485 carrying PSF-2 & RSF-1010	
SF-191			*fi*$^-$, SuSm		W1485 carrying RSF-1010 mobilized from *E. coli* 3	
SF-192			*fi*$^-$, Tc		W1485 carrying RSF-1020 mobilized from *S. panama*	

[a] The molecular weight of the plasmid species was determined by either sedimentation in sucrose gradients or by the examination of circular DNA in the electron microscope.

[b] The mole fraction of G + C was determined in preparative neutral CsCl gradients of ^{3}H-thymine plasmid DNA purified by dye buoyant density equilibrium centrifugation. The G + C content was calculated relative to ^{14}C-thymine-labeled *E. coli* W1485 DNA which was taken as 1.710 g/cm^3, 0.50 mol fraction of G + C.

[c] Symbols and definitions of genotype and phenotype: Sm, Su, Tc, Sp, Cm, *nal*-r, and *rif*-r refer to resistance to streptomycin, sulfonamide, tetracycline, spectinomycin, chloramphenicol, nalidixic acid, and rifampin, respectively. Ent, Enterotoxin biosynthesis; Hly, production of hemolysin; K88, production of the surface antigen K88; Pil, gene affecting conjugal transfer; ColI, colicin I production; *fi*$^+$, plasmid phenotype operationally demonstrated as inhibition of either the conjugation or the pilus synthesis mediated by F; *fi*$^-$, the lack of the fi$^+$ character, regardless of mechanism.

[d] ND, Not done.

Electron microscopy. Spreading and staining of DNA were performed essentially as described by Kleinschmidt (24). The spreading solution contained 0.5 M ammonium acetate, cytochrome *c* (final concentration, 100 μg/ml), and DNA in 0.01 M Tris (final concentration, about 0.2 μg/ml). A sample (0.1 ml) of this solution was spread on a hypophase of 0.25 M ammonium acetate and examined in a Phillips 300 electron microscope. The negatives were projected and the molecular contours were traced on paper. Lengths of molecules with clearly defined contours were measured with a map tracer. The electron microscope was calibrated for a set of exposures at the same operational magnification by a grating replica. The conversion figure of 2.07×10^6 daltons per μm was employed to relate contour length to molecular weight (27).

RESULTS

Mobilization of N-SuSm and N-Tc by transfer plasmids. *E. coli* strain 3 resistant to streptomycin-sulfonamide received from the laboratory of P. Fredericq and a tetracycline resistant strain of *S. panama* SP219 received from the laboratory of P. A. M. Guinee were unable to transmit their drug resistance determinants at any demonstrable frequency ($<10^{-10}$ per cell) in standard conjugation experiments. If the strains were infected with a suitable transfer factor, however, the drug resistance of each strain was transmitted ("mobilized") at an easily demonstrable level. In both instances F-*lac*$^+$ was employed to mobilize drug resistance to strain W1485, and clones SF191 resistant to sulfonamide-streptomycin and SF192 resistant to tetracycline, but devoid of F-*lac*$^+$ (as evidenced by male phage resistance and lack of chromosome transfer), were isolated and used in all subsequent experiments.

The ability of several different transfer plasmids to mobilize the streptomycin-sulfonamide resistance determinants from strain SF191 in a triparental mating is shown in Table 2A. The results presented were calculated relative to the frequency of mobilization obtained with the classical F factor since the frequency of self-transfer by F was known to approach 100%. It may be seen that the frequency of mobilization relative to F was some 200-fold higher for *fi*$^-$ transfer factors, although the size and the G plus C content of the *fi*$^-$ transfer factors that were employed varied considerably (see Table 1). That the mobilization of the streptomycin-sulfonamide determinants was independent of the frequency of self-transfer by the transfer factors themselves is shown in Table 2B. SF191 containing F, Hly or ColIb were mated directly with strain SF185. The transfer frequencies for

TABLE 2. *Relative frequency of mobilization of the N-SmSu plasmid*

A. Transfer factor[a]	*fi*	Relative frequency of SuSm mobilization
F	+	1.00
RTF-1	+	0.85
Hly	+	0.36
K88	+	1.03
Ent P307	+	0.38
Ent P95	−	215.0
ColIb	−	539.0
Δ47	−	200.5

B. Strain[b]	Frequency of conjugation (%)	Frequency of N-SuSm mobilization	SuSm exconjugants receiving transfer factor (%)
SF189 (F, N-SuSm)	77	5.2×10^{-6}	79
SF188 (Hly, N-SuSm)	33.5	7.5×10^{-7}	85.5
SF190 (ColI, N-SuSm)	39.6	1.8×10^{-3}	100

[a] All plasmids in strain W1485 were grown at 37 C in Penassay Broth for 6 h with gentle agitation to about 2.5×10^7 cells/ml. An equal volume of W1485 bearing the indicated transfer factor was incubated with an equal volume of strain SF191 (N-SuSm) and incubated for 2 h at 37 C. At this time the final recipient, SF185 *nal*^{r}, was added at a volume equivalent to the donor strain and this mating mixture was incubated at 37 C overnight without agitation. Samples were plated onto MacConkey agar with added streptomycin and nalidixic acid (25 μg/ml each). Control mating mixtures of the donor strain and final recipient, the intermediate and final recipient and the donor and intermediate were also plated. The results, presented as the number of Su^r Sm^r nal^r colonies appearing, represent an average of at least five separate experiments.

[b] Donor cells containing the indicated transfer factors and N-SuSm were mated overnight with an excess of SF186 *rif*-r cells. The mating mixtures were plated on a medium containing 100 μg of rifampin per ml. The inheritance of F was scored by male phage sensitivity. The inheritance of Hly was scored by replica plating recipient colonies onto 5% sheep blood agar plates. ColIb production was scored by observing the zones of growth inhibition which developed around chloroform vapor-killed recipient colonies overlaid with 2.5 ml of soft agar containing strain W1485 and incubated at 37 C overnight. Identical samples of the mating mixture were also plated on media containing rifampin plus 25 μg of streptomycin per ml to score for those cells which received N-SuSm. These frequencies were calculated relative to the number of donor cells present at the time of mating. The plasmid$^+$ conjugates which received N-SuSm were also tested for the inheritance of F, Hly, and ColIb as described above.

F, ColIb, and Hly as determined on non-selective media was 78, 39.6, and 33.5%, respectively. Yet, the frequency of mobilization of the streptomycin-sulfonamide resistance determinants was 5.2×10^{-6} and 7.5×10^{-7} for F and Hly, and 1.8×10^{-3} for ColIb. Thus, although transfer factors may vary in their capability to mobilize these determinants, it is apparent that mobilization does not appear to require a transfer factor possessing a special propensity for mobilizing drug resistance genes.

The tetracycline resistance determinant of *S. panama* was similarly mobilized by F-*lac*$^+$, ColIb and was specifically mobilized at high frequency by Δ47, an *fi*$^-$ transfer factor which was also derived from *S. panama*. The mobilization of the streptomycin-sulfonamide determinants of *E. coli* 3 and the tetracycline determinant of *S. panama* by a variety of transfer factors at conjugation was, of course, consistent with their designation as plasmids and they were called RSF-1010 (N-SuSm) and RSF-1020 (N-Tc).

Molecular nature of N-SuSm and N-Tc. As shown in Fig. 1, cleared lysates of strain SF191 showed a single homogeneous DNA peak which sedimented relative to ^{14}C-thymine λ DNA (34*S*) at 28 ± 1.5*S*. Samples of the 28*S* DNA fraction were pooled, dialyzed, and treated with low concentrations of deoxyribonuclease for a time calculated to introduce on the average one nick per molecule (7). This treatment resulted in a discontinuous transition from the 28*S* form to a form which sedimented at 20 ± 1.5*S*. This change in sedimentation properties was the

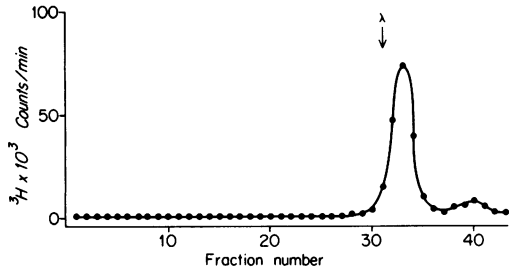

FIG. 1. *Sedimentation of N-SmSu in neutral sucrose. A 100-μliter amount of a cleared lysate of SF191 was diluted 1:1 in TES and layered onto a 5 to 20% neutral sucrose gradient. Centrifugation was carried out at 200,000 × g for 60 min at 15 C in the SW41 rotor of a Beckman model L2-50 ultracentrifuge. Twelve-drop fractions were collected and trichloroacetic acid was precipitated and counted. Linear monomers of ^{14}C-labeled λ DNA were added as a sedimentation reference.*

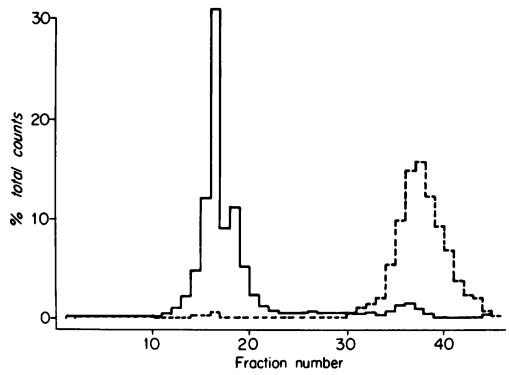

FIG. 2. *Alkaline sucrose gradient analysis of DNase degradation of N-SmSu DNA. The DNase reaction was performed essentially as described by Bazaral and Helinski (7). Samples were layered onto 17-ml, 5 to 20% alkaline sucrose gradients and centrifuged at 82,500 × g for 5 h at 20 C in the SW27 rotor of a Beckman model L2-50 ultracentrifuge. N-SmSu, no DNase treatment, ——; N-SmSu, 25-min DNase treatment, - - -.*

expected result for the transition of a twisted CCC molecule to an open circular form (OC). This conclusion is graphically shown in Fig. 2 which shows the sedimentation of the DNA isolation from a cleared lysate in a 5 to 20% alkaline (0.3 N NaOH) sucrose gradient before and after the introduction of a single-strand scission. There was a sharp discontinuous reduction (by almost 3.6-fold) from a rapidly sedimenting form characteristic of CCC molecules to a form which sedimented in a manner characteristic of OC or linear forms. Treatment of cleared lysates with Pronase failed to disclose the presence of a relaxation complex as has been reported by Helinski and his associates for Col E1 (8, 9, 22).

The identification of the 28*S* DNA fraction as CCC was further confirmed by the centrifugation of crude cell lysates in a mixture of cesium chloride and ethidium bromide. As shown in Fig. 3, approximately 9% of the total DNA complement of SF191 banded as a dense component characteristic of CCC. The material from this band was pooled and dialyzed and run on a neutral sucrose gradient where it sedimented uniformly at 25*S* ± 1.5. The sedimentation of CCC molecules at 25*S* after isolation from CsCl as contrasted to the value of 28*S* observed in cleared lysates has been a consistent finding. We interpret this difference as the loss of a protein component firmly attached to the CCC structure in cleared lysates but dissociated from the molecules (without a concom-

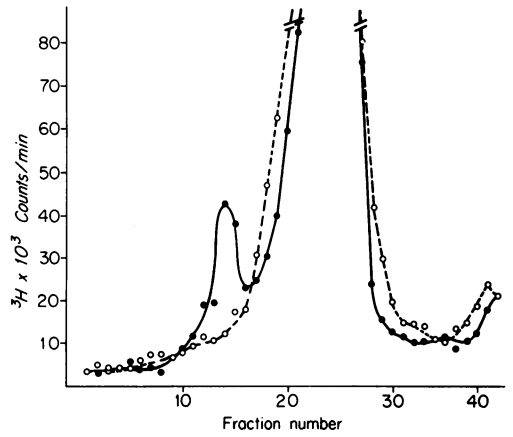

FIG. 3. *Dye buoyant equilibrium centrifugation of DNA isolation from E. coli K-12 N-SmSu and E. coli K-12 F⁻. A Sarkosyl lysed culture (0.5 ml) was mixed with EtBr and CsCl and centrifuged to equilibrium. Details of centrifugation are described in Materials and Methods. SF191 (N-SmSu) DNA, ——; W1485 F⁻ DNA, - - -.*

itant change in configuration) in CsCl (see ref. 22). Samples of both 25 and 28*S* material examined in the electron microscope revealed a single circular DNA species with an average contour length of 2.4 ± 0.2 μm (47 molecules measured). We concluded, therefore, that the SF191 strain harboring N-SuSm contained a single plasmid DNA species which was 5.0×10^6 daltons in size.

The correspondence of the 5.0×10^6 dalton plasmid species with N-SuSm was shown by transformation studies. CCC DNA isolated by dye buoyant density centrifugation was extracted with isopropanol and dialyzed against TEN (0.02 M Tris, 1 mM EDTA, 0.02 M NaCl). Varying dilutions of the DNA (sterilized with chloroform) were added to a variety of drug-sensitive *E. coli* K-12 F⁻ cell lines in the presence of 0.03 M $CaCl_2$. After exposure to DNA and a short incubation at 43 C, the cells were diluted into L-broth to permit the expression of drug resistance and plated on a medium containing 25 μg of streptomycin per ml. Approximately 100 SuSm-resistant cells per μg of DNA were isolated. These clones were uniformly nontransmissible, and cleared lysates from five randomly isolated clones of three different strains showed a single DNA species which sedimented at 28*S* in neutral sucrose gradients.

Dissociated F-like R-factors in *Proteus* showed a 9×10^6 to 15×10^6 dalton plasmid which possesses a 0.56-mol fraction of G plus C (12, 18, 32). This plasmid type has been assumed (primarily by inference) to represent the drug resistance determinants of the R-plasmid (12, 16, 33). It seemed of some interest, therefore, to determine the mole fraction of G plus C possessed by the N-SuSm plasmid. Accordingly, ^{3}H-thymine-N-SuSm DNA was centrifuged to equilibrium in a CsCl density gradient to determine its buoyant density relative to added ^{14}C-thymine-λ bacteriophage DNA (1.709 g/cm^3; 0.49 mol fraction of G plus C). The buoyant density of N-SuSm in four separate experiments averaged 1.715 ± 0.0015 g per cm^3, which we calculated to be 0.55 ± 0.01 mol fraction of G plus C.

It would be expected that a single N-SuSm plasmid should constitute approximately 0.2% of the total DNA of a cell (assuming that the *E. coli* chromosome is 2.5×10^9 daltons). Reexamination of Fig. 3 reveals, however, that about 9% of the total DNA extracted from *E. coli* N-SuSm cells was isolated as 5.0×10^6 CCC molecules. It was apparent, therefore, that the N-SuSm plasmid must be represented as a multi-copy pool within host cells. The amount of DNA found as CCC molecules has been somewhat variable from experiment to experiment, ranging from 3 to 13% of the total DNA complement. On the basis of a 5.0×10^6 dalton size, this would correspond to 10 to 50 copies per cell. The molecular properties of the N-SuSm plasmid correspond very closely to those described by Milliken and Clowes for S (31), as well as possessing genetic properties essentially identical to those previously described by Anderson and his collegues for S and Δ-S (1-5). Anderson kindly provided us with an *E. coli* K-12 strain containing the S factor, which was identical to that examined by Clowes and Milliken (31). The S plasmid (5.9×10^6 daltons) and the N-SuSm (5.0×10^6 daltons) were slightly different in size. In preliminary DNA-DNA duplex experiments they showed about 90% nucleotide sequences in common. It is clear, therefore, that the nonconjugal plasmid S and N-SuSm are essentially identical. Barth (personal communication) has isolated a number of similar plasmids conferring streptomycin-sulfonamide resistance from several genera of enteric bacteria.

Studies with the N-Tc plasmid originally derived from *S. panama* have revealed certain similar characteristics in common with N-SuSm. Electron microscopy of N-Tc plasmid DNA isolated by cesium chloride-ethidium bromide centrifugation of cleared lysates of strain

SF192 revealed a single circular species of DNA with an average contour length of 2.9 ± 0.12 μm (57 molecules measured). Sedimentation of cleared lysates of N-Tc in 5 to 20% neutral sucrose gradients showed a single homogeneous component which sedimented relative to ^{14}C-λ DNA at 30 ± 1.5S and displayed a discontinuous transition to a 23 ± 1.5S sedimenting form after treatment with 2.5×10^{-4} μg of pancreatic deoxyribonuclease per ml consistent with a transition from a 30S CCC form to a 23S OC form. The calculated molecular weight for a DNA species having these sedimentation properties and contour length is 6.0×10^6 daltons. Centrifugation of several crude cell lysates of strain SF192 in cesium chloride plus ethidium bromide showed that from 4 to 7% of the total DNA complement banded as a dense component characteristic of CCC DNA molecules. It was apparent, therefore, that the N-Tc plasmid, like N-SuSm, must be represented in a multicopy pool (16 to 29 copies) within the SF192 host cell. A major point of divergence, however, was the finding that the buoyant density of ^{3}H-N-Tc DNA was 1.709 ± 0.0008 g/cm^3 which we calculated to be 0.49-mol fraction of G plus C. It may be noted that most genetic studies have shown that the Tc determinant of several single unit *fi*$^+$ R-factors is linked to transfer genes and that the G plus C content of the Tc gene has been previously inferred to be 0.49-mol fraction of G plus C (12, 18, 32).

Molecular nature of strains receiving a mobilized drug plasmid. The process of mobilization of nonconjugative plasmids such as N-Tc and N-SuSm might be imagined to involve a recombinational event with a transfer factor. To gain some view into the mobilization phenomenon, we examined the molecular nature of *E. coli* K-12 recipients which had received both N-SuSm and a transfer factor by conjugation. Figure 4 shows the plasmid DNA isolated from a cleared lysate of *E. coli* K-12 ColIb sedimented on a neutral sucrose gradient. Both CCC and a smaller amount of the OC forms of this plasmid can be identified as corresponding to 73S and 50S, respectively. Figure 4 also shows the plasmid DNA isolated from an *E. coli* strain which received both ColIb and N-SuSm by conjugation. It may be seen that one can identify both genetic elements as distinct DNA species within the cell of the latter strain. Clearly, the N-SuSm plasmid does not disappear and the 73 and 50S CCC and OC molecules of ColIb are still present. It should be noted, moreover, that the amount of N-SuSm DNA comprised the major proportion of plasmid DNA within the strain. There was, therefore, no indication of a major shift in the sedimentation properties of N-SuSm or of ColIb DNA within a cell which received both plasmids by conjugation. A total crude cell lysate of the strain which received both ColIb and N-SuSm by conjugation was centrifuged in a CsCl-ethidium bromide gradient and the dense component characteristic of CCC molecules was found to comprise 9.4% of the total DNA. An isolated sample of this CCC band was extracted with isopropanol, dialyzed against TES, and analyzed on a 5 to 20% neutral sucrose gradient. The numbers of ^{3}H-thymine counts sedimenting under the 73, 50, and 26S sucrose peaks were employed to estimate what proportion of the total plasmid DNA represented ColIb and what proportion represented N-SuSm. It was estimated that approximately 68% of the isolated plasmid DNA sedimented in the 26S N-SuSm band, whereas 24% of the plasmid DNA sedimented in the 75 and 50S bands; the remaining counts (8%) sedimented either between the 75 and 50S bands or in other regions of the gradient. These data indicate, therefore, that the amount of ColIb DNA present was equivalent to approximately one copy per cell, whereas the amount of N-SuSm DNA present was equivalent to approximately 12 copies per cell.

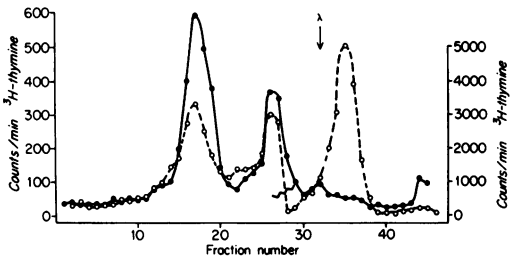

FIG. 4. *Sedimentation patterns in neutral sucrose of plasmid DNA isolated from E. coli K-12 ColIb and E. Coli K-12 SF190 (ColIb + N-SuSm). Centrifugation was performed as described in the legend to Fig. 1. E. coli K-12 ColIb,——; E. coli K-12 SF190, - - -. Please note that the left-hand scale refers to the counts per minute of ^{3}H-thymine-ColIb, whereas the right-hand scale refers to counts per minute of ^{3}H-thymine-N-SuSm. The change in scale for the E. coli K-12 SF190 gradient is indicated by the jagged line.*

Our experiments do not unequivocally rule out the possibility that some form of covalent linkage between a transfer factor and N-SuSm may be necessary for mobilization. Nonetheless, it seems clear that if covalent linkage (or other chemical bonding) did occur, it was not a stable union since one finds both genetic elements as

distinct entities and that the relative incorporation of the ^{3}H-thymine label into each plasmid type was consistent with there being a limited number of copies of the transfer factor and a multi-copy pool of the N-SuSm replicon. As concluded by Milliken and Clowes (31) from electron microscopy data for the ΔS system, certainly no more than 10% of the plasmid molecules could be recombinational products between the transfer factor and the nonconjugative plasmid. We have found completely analogous results with strains which received N-SuSm after mobilization with F, Ent-P95, and the Δ47 transfer factors. Additionally, the N-Tc plasmid behaved similarly, since mobilized N-Tc was found to remain distinct from ColIb, F-*lac*$^+$ and R-factors within *E. coli* plasmid$^+$ exconjugants after mobilization from *S. panama* (Fig. 5).

Recombination between the nonconjugative drug resistance plasmids and transfer factors. Although it seemed clear that the mobilization phenomenon was not associated with a direct, stable recombinational union between N-SuSm or N-Tc with a transfer factor, we attempted to demonstrate that recombination between these distinct replicons was possible. We presumed that transduction by phage P1 should be more selective for recombinants in the sense that transducing phage particles would be expected to carry only a single plasmid type as shown by Anderson and Natkin (6). A strain of *E. coli* K-12, SF187, was prepared carrying the transmissible *fi*$^-$ R-plasmid RSF-8 conferring resistance to Sm Sp Cm (68*S*, CCC; 46*S*, OC, 51 × 10^6 daltons) and the N-Tc plasmid (see Fig. 5). In conjugation experiments, this strain transferred Sm Sp Cm resistance at a frequency of approximately 5 × 10^{-3}/donor cell; Tc resistance was transferred at 10^{-5}/donor cell. Virtually all plasmid$^+$ exconjugants (99%) selected on plates containing tetracycline were also resistant to Sm Sp Cm. Cleared lysates of these exconjugants exhibited well-defined 68 and 30*S* components of CCC molecules and no discernable evidence of any molecular species which might be considered as recombinant molecules. P1 *vir* lysates were prepared from the strain harboring both plasmid types and were employed for transduction experiments with the drug-sensitive *E. coli* K-12 derivatives W1485. After phage adsorption (MOI 0.1), cells were plated on a medium containing chloramphenicol. Cells resistant to Cm were found at a frequency of 2 × 10^{-7}/adsorbed phage. Ninety-six percent of the Cm^r transductants were also Sm^r Sp^r but Tc^s, and were all able to transmit Sm Sp Cm by conjugation. We presumed that these transductants contained the complete R-factor and random clones examined by the cleared lysate technique showed only the expected 68*S* CCC molecular species. The remaining 4% of the transductant clones isolated on chloramphenicol plates also exhibited Sm Sp and Tc resistance (one clone was Cm^r, Tc^r, Sm^s, Sp^s). Cleared lysates of the transductant strains exhibiting Cm, Sm, Sp, Tc resistance showed single species of CCC DNA which sedimented variously at 63 to 65*S* (equivalent to about 44 × 10^6 daltons, Fig. 6). These clones transferred the three resistance determinants en bloc at a frequency of about 6 × 10^{-4}/donor cell. It appeared, therefore, that these transductants represented a recombinant class resulting from the interaction between the RSF-8 R-factor and the N-Tc plasmid. It seems evident, however, that this recombinational event cannot be interpreted in terms of a simple co-integration of the two plasmid types. Nor can it be concluded from these experiments that the recombinant classes were within the host cell prior to phage infection since one must entertain the possibility of a recombinational event mediated by phage recombinational genes. Preliminary evidence indicates, however, that virtually identical transmissible Cm Sm Sp Tc recombinants can be isolated by assiduous screening of plasmid$^+$ exconjugants.

When selection was performed for streptomycin-resistant transductants, the results were completely analogous to these described above. Selection for Tc resistance showed relatively few transductant clones (approximately 10^{-9}/adsorpted phage). None of these transductants were solely resistant to Tc and it appeared that the N-Tc plasmid was not transducible

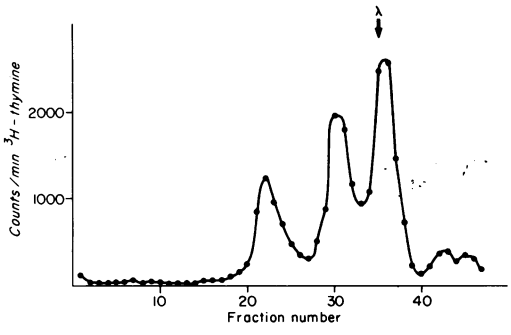

FIG. 5. *Sedimentation in neutral sucrose of a cleared lysate of E. coli K-12 SF187 (carrying N-Tc and RSF8, a transmissible R-factor). Conditions of centrifugation are those described in the legend to Fig. 1.*

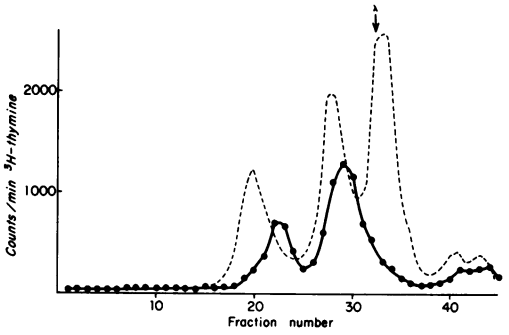

FIG. 6. *Sedimentation in neutral sucrose of a cleared lysate of an E. coli K-12 Cm^r Sm^r Sp^r Tc^r transductant clone. Conditions of centrifugation are those described in the legend to Fig. 1. The dashed line represents the sedimentation profile of the E. coli SF187 strain employed as the host for the preparation of the transducing lysates.*

under the conditions we employed. The majority of the transductant clones that did appear on tetracycline-selective media also carried Cm Sm Sp and were similar to the recombinants already described. A small proportion of the transductants were Cm^s Sm^r Sp^r Tc^r. Analysis of these transductants revealed that these resistance determinants were not detectably self-transmissible, but were mobilizable at low frequency by a suitable transfer factor. Cleared lysates prepared from these transductants revealed a single CCC DNA species of 42*S* which corresponds to a molecular weight of approximately 20×10^6 daltons (Table 3). Every Sm Sp Tc transductant examined thus far has displayed similar behavior, although roughly one-half were found to be unstable for the resistance genes. Thus, these studies, still preliminary to be sure, show that recombination can occur between the nonconjugative multi-copy drug resistance plasmid and self-transmissible plasmids.

DISCUSSION

Two independently isolated nonconjugative drug resistance plasmids have been shown to be small, multi-copy CCC DNA species. In the presence of a transfer factor, these nonconjugative plasmids may be mobilized. At present, one cannot precisely define the physical basis for the conjugal co-transfer of the N-Tc or N-SuSm elements in the presence of the transfer factor. Co-transfer appears to be independent of any physical association between the two, since the transfer factor and drug resistance plasmids were found as distinct molecules in both donor and recipient cells. Moreover, preliminary experiments suggest that the phenomenon is independent of the *rec* genes of the bacterial host. Some sort of transient covalent linkage cannot be ruled out, although it appears to be unlikely. Yet, the mobilization of the non-infectious N-SuSm and N-Tc plasmids by various transfer factors does not seem to be simply a passive phenomenon in which the noninfectious DNA, for example, accidentally enters a conjugation tube. Rather, we found that the mobilization phenomenon occurs about 200-fold more frequently in the presence of *fi*$^-$ transfer factors than the *fi*$^+$ transfer factors that we studied. Presumably, this difference reflects something other than just a difference in the nature of the conjugal tube, although the nature of the increased mobilization is unclear. It is not certain, furthermore, whether the mobilized nonconjugative plasmid is transferred as a single-stranded DNA molecule with a 5′-3′ orientation

TABLE 3. *Transduction and recombination of N-Tc and RSF-8*

A. Transduction of markers from SF187[a]

Markers	Frequency of transduction per adsorbed P1 phage	Frequency of conjugation by transductant
Cm Sm Sp	10^{-5}	3×10^{-5} to 5×10^{-3}
Tc	$0 (< 1 \times 10^{-9})$	
Cm Sm Sp Tc	2×10^{-7}	$3\text{–}5 \times 10^{-3}$
Sm Sp Tc	3×10^{-9}	$< 1 \times 10^{-9}$

B. Molecular species found in transduction classes

Phenotype	S value of CCC plasmid DNA present in Brij lysate
Cm Sm Sp Tc (SF187 donor strain)	68, 28
Cm Sm Sp	68
Cm Sm Sp Tc	63–65
Sm Sp Tc	42

[a] Strain SF187 (Cm Sm Sp Tc) contains plasmids RSF-8 and RSF-1020 and was used for the propagation of P1 *vir* as described in Materials and Methods. The P1 *vir* phage was adsorbed onto W1485 at a multiplicity of 0.1 and plated on media selective for one or more of the drug resistance markers. Ten each of the transductants of each class were mated with strain SF185 to determine the frequency of conjugal transfer of the transduced markers. The transductants employed in the mating experiments were also labeled with ^{3}H-thymine, lysed with Brij 58, and analyzed on 5 to 20% neutral sucrose gradients as described in the legend to Fig. 1. The frequency of conjugal transfer by strain SF187 was 3×10^{-3} to 5×10^{-3}/donor strain for Cm Sm Sp and 1.4×10^{-6} for Tc, respectively.

that seems characteristic of self-transmissible F and R factors.

The basic molecular features of N-SuSm and N-Tc show at least a superficial similarity to that of the Col E1 plasmid. All are noninfectious, about 5×10^6 daltons in size and are present as multi-copy pools throughout the growth cycle of the host cell. It has been noted that plasmids seem to be divisable into two distinct classes on the basis of whether their replication is under stringent or relaxed control (10, 25, 34). The distinction has been, at least in part, that larger plasmids ($>40 \times 10^6$ daltons) such as F, ColIb, and many R-factors replicate under stringent control and are present as but a few copies per cell. Presumably, such plasmids obey control mechanisms for replication and segregation that are like that of the bacterial chromosome itself. In contrast, Col E1, N-SuSm N-Tc, and the R-factor R6K (10, 22, 25) are considerably smaller ($<30 \times 10^6$ daltons) and replicate randomly as a multi-copy gene pool throughout the growth cycle of the cell. One assumes that segregation of these factors at cell division operates automatically because of the sheer weight of numbers. The smaller relaxed plasmid classes are, therefore, rather unique—almost like a controlled phage infection such as is seen with λ*dv* or certain N^- λ mutants (14, 29, 30). Clowes (10) has suggested that perhaps the control of plasmid replication was size related and that only smaller plasmids were relaxed. Small size in itself does not appear to be necessarily associated with a relaxed mode of replication, however, for we have characterized several naturally occurring transfer factors as small as 18×10^6 daltons that replicate under stringent control under normal laboratory growth conditions. Of course, R-plasmids that replicate under stringent control may contain a subunit replicon which is capable of relaxed replication. At any rate, while distinction into relaxed and stringent replication does seem to be highly correlated with molecular size, size alone (i.e., a simple genetic structure) does not necessarily dictate the mode of replication. Nonetheless, the analysis of the plasmid complement of a number of clinical isolates has shown that the small, multi-copy class of bacterial plasmids is quite common (unpublished observations).

Col E1 has been recently shown to possess several distinctive properties. Thus, in addition to a large number of copies and small size, Col E1 is genetically unstable in *polA1* mutants and continues initiation of CCC DNA synthesis in the presence of chloramphenicol for as long as 10 to 15 h (22, 23). It is interesting to note that our preliminary experiments indicate that these properties are not shared by either N-SuSm or N-Tc. Moreover, the virtual total lack of shared nucleotide sequence relationships between N-SuSm and N-Tc (unpublished observation) and their distinct G plus C contents suggests that they are of quite distinct ancestry despite their similar genetic and replicative behavior.

Although transfer factors do not ordinarily seem to form any sort of stable recombinational union to bring about transfer of either N-SuSm or N-Tc, one can demonstrate by P1-mediated transduction that occasional instances of recombination between transfer factors and nonconjugative drug resistance plasmids are possible. The recombinants isolated do not appear to be a simple fusion of the two replicons. Rather, recombinants are significantly smaller than the sum of the size of the two individual replicons. In this respect the recombinants resemble those reported between F-prime elements (33) as well as those we have isolated between incompatible plasmids (unpublished observations). Whether or not such recombinants would, nonetheless, dissociate into distinct replicons under suitable conditions as has been noted for certain naturally occurring R-factors remains to be seen. It is not altogether clear how often this kind of plasmid recombination may take place in nature, but it is, of course, a useful way to think of the accretion of drug resistance genes by transfer factors. Presumably, a recombinant between a nonconjugative drug resistance plasmid and a transfer factor would enjoy a distinct advantage in a mixed bacterial population under antibiotic selection.

The results of this investigation fully support the genetic observations of E. S. Anderson and his associates (1–5), as well as the molecular findings of Milliken and Clowes (31), and van Embden and Cohen (in press). It is clear that R-factors cannot be defined as any single kind of plasmid type, for virtually any transfer factor can mobilize a nontransmissible drug resistance plasmid such as N-SuSm or N-Tc. From a clinical standpoint it is interesting to see that transfer factors such as Ent, Hly, or K88 might be considered as R-factors in many circumstances.

It is perhaps most intriguing to consider the small nontransmissible plasmid class as the basic or raw material of drug resistance genes in bacterial populations. However, one could just as easily suggest that the smaller plasmids represent the breakdown product of a larger element. At the present time there appear to be at least three distinct kinds of genetic elements called R-factors that can be isolated from clini-

cal material. One type is a nondissociable single unit of replication and transmission. A second type is generally a single unit of replication and transmission but contains component replicons into which the R-factor may dissociate under certain physiological conditions. The third is the type discussed by E. S. Anderson and reported in this investigation, in which the transfer factor and drug resistance genes are represented by distinct replicons within the cell under virtually all conditions of growth. The isolation of plasmid recombinants, similar to those reported here, between a transfer factor and a nonconjugative plasmid carrying drug resistance determinants could presumably lead to either of the two types of unitary molecular types of R-factors.

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