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Genetic and Biophysical Study of R Plasmids Conferring Sulfonamide Resistance in *Shigella* Strains Isolated in 1952 and 1956

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The conjugative plasmids determining sulfonamide resistance in five *Shigella* strains, each isolated from a different patient, have been characterized. One *S. flexneri* 2a strain, isolated in 1952, harbored an fi^+ plasmid of molecular weight 53×10^6, which specified synthesis of F-like pili and bore determinants for sulfonamide resistance (Su) and bacteriocinogeny (Col). This plasmid was compatible with plasmids of groups F_I, F_{II}, I_α, and P. A second *S. flexneri* 2a strain isolated in 1952 harbored an fi^- plasmid of molecular weight 59×10^6, bearing the Su determinant and compatible with all plasmids tested. This strain also harbored an fi^+ group-F_{II} plasmid of molecular weight 42×10^6, which bore the Col determinant and specified synthesis of F-like pili. Three *S. dysenteriae* 2 strains isolated in 1956 carried apparently identical fi^- plasmids of molecular weight 58×10^6, which bore the Su determinant, could form transconjugants in *Pseudomonas* but not in *Proteus*, and were incompatible with the P-group plasmid RP4.

During a study of the R plasmids harbored by a collection of *Shigella* strains (12) that had been isolated in Melbourne between 1952 and 1968, it was observed that the only R plasmids present among strains isolated before 1958 conferred resistance to sulfonamides alone.

In early studies of R plasmids among *Shigella* strains in Japan, transferable resistance to sulfonamides alone was not observed (41), although a high proportion of the *Shigella* strains isolated were resistant only to these drugs. Although R plasmids conferring only sulfonamide resistance (R_{Su} plasmids) have since been discovered in *Shigella* and other genera of the *Enterobacteriaceae* (8, 13, 36), the properties of such R_{Su} plasmids have not been studied in detail. This report presents studies on the genetic and biophysical properties of the R_{Su} plasmids in five *Shigella* strains isolated in 1952 and 1956.

MATERIALS AND METHODS

Bacteria and bacteriophages used. *S. flexneri* 2a strains SUM1 and SUM2 were isolated in 1952. Each carried transferable determinants for sulfonamide resistance and the production of a bacteriocin active on strains of *Escherichia coli* K-12. The *S. dysenteriae* 2 strains SUM5, SUM6, and SUM8 were isolated in August and September 1956, and carried transferable determinants for sulfonamide resistance alone. Each *Shigella* strain was isolated from a different patient in a Melbourne institution, and had been preserved until 1970 as a lyophilized culture. All of these strains were found to require nicotinic acid for growth in defined media; the *S. dysenteriae* strains also required tryptophan.

The *E. coli* K-12 strains used were all F^- multiple auxotrophs. Their strain numbers and markers relevant to this study were as follows: AB1450(Ilv^-Met^-), JP271 and its close relative JP990 (both $Ilv^-Nal^rPro^-$), JC411 (3) (Met^-), and JP249 ($Ilv^-Met^-Rec^-$, derived from JC411). The symbols Ilv^-, Met^- and Pro^- indicate requirement for isoleucine and valine, methionine, and proline, respectively; Nal^r indicates resistance to at least 50 μg of nalidixic acid per ml; Rec^- indicates incompetence for genetic recombination due to a mutation in the *recA* gene.

The serine-requiring *Pseudomonas aeruginosa* strain PAO2 (FP^- *ser*) and the *Proteus mirabilis* strain PM13 (which requires nicotinic acid for growth in defined media) were used in host range studies. Both of these strains had been used previously as plasmid recipients by Datta and Hedges (10). The marine pseudomonad PsBAL 31 was used to propagate phage PM2.

The phages used in genetic studies were the generalized transducing phage P1kc (28), the F-pilus-specific phage MS2 (31), and the I-pilus-specific phage If1 (34). The covalently closed circular double-stranded deoxyribonucleic acid (DNA) genome of the marine phage PM2 (16) was used as a standard for calibrating the contour lengths of plasmid DNA molecules.

Reference plasmids. Reference plasmids are listed in Table 1; each plasmid was introduced into the

TABLE 1. *Reference plasmids used*

Plasmid	Incompatibility group	fi	Phenotype determined[a]	References	Recipient	R^+ strain
F16	F_I	–	Ilv$^+$; derepressed for fertility and F-pilus synthesis; susceptible to fertility inhibition	24, 39	JP271 JP249	JP641 JP997
R1-16	F_{II}	+	Km-Nm; derepressed for fertility and synthesis of F-type pili; not susceptible to fertility inhibition	7, 10, 33	JC411	JP985
222/R4	F_{II}	+	Cm Sm Su Tc	24, 38	JP990	JP661
R64-11	I_α	–	Sm Tc; derepressed for fertility and synthesis of I-type pili	25, 27, 33	JP990 JP249	JP993 JP994
R144-3	I_α	–	Km-Nm Col Ib$^+$; derepressed for fertility and synthesis of I-type pili	10, 25, 33	JC411	JP986
RP4[b]	P	–	Ap-Cb Km-Nm Tc	10, 11, 40	JC411	JP987
R46	N	–	Ap Sm Su Tc	9, 10	JC411	JP988
S-a	W	–	Cm Km Sm Su	10, 23	JC411	JP989

[a] Symbols for phenotype determined: Ilv$^+$, no longer requires isoleucine or valine; Col Ib$^+$, produces colicin Ib. Antibiotic resistance: Ap, ampicillin; Cb, carbenicillin; Cm, chloramphenicol; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline. Where two symbols are joined by a hyphen, e.g., Km-Nm, both resistances are mediated by the one determinant.

[b] Although sent to us as RP4 by N. Datta, this plasmid may in fact be RP1 (40).

recipient strains shown in the table, to generate R^+ strains whose strain numbers are also indicated. These R^+ strains were used as reference plasmid donors in the experiments reported here.

Media. Minimal media were based on medium 56 of Monod et al. (37), but were used at half strength (designated 56/2). Defined media for the growth of *Shigella* and *E. coli* strains consisted of medium 56/2 supplemented with 0.2% (wt/vol) D-glucose, 0.1 μg of thiamine per ml, and the appropriate amino acids at 30 μg/ml. When required, nicotinic acid was added to 10 μg/ml. Defined media for growth of *P. mirabilis* PM13 consisted of medium 56/2 supplemented with 0.2% glucose, 0.05% tri-sodium citrate, and 10 μg of nicotinic acid per ml. For *P. aeruginosa* PAO2, defined media contained 0.2% glucose and 20 μg of DL-serine per ml in medium 56/2.

B medium consisted of medium 56/2 supplemented with 0.2% glucose, 0.25% (wt/vol) vitamin-free Casamino Acids (Difco), and 0.002 μg of thiamine per ml. When appropriate, nicotinic acid (5 μg/ml) and tryptophan (30 μg/ml) were added to enhance the growth of strains requiring these growth factors. Sulfonamide sensitivity and resistance could be reliably and readily tested in this medium.

Media in plates was solidified with 1.2% Oxoid agar no. 2. Soft-agar layers (3 ml) consisted of medium 56/2 containing 0.5% (wt/vol) Oxoid agar no. 2, except when used in plaque assays of phage Ifl; soft agar for this purpose contained only 0.25% Oxoid agar.

Other media used were nutrient broth no. 2 (Oxoid), Hirota broth (26), and Luria broth (30). Luria broth supplemented with 2.5×10^{-3} M $CaCl_2$, termed Z broth, was used for experiments with phage P1kc. Eosin methylene blue medium was prepared as described by Levine (29), except that lactose was omitted and 0.1% (wt/vol) glucose was added.

Media for the growth of the marine pseudomonad PsBAL 31 and for the propagation of phage PM2 were prepared exactly as described (16), from Difco ingredients.

The antibiotics employed and the concentration of each (micrograms per milliliter) generally used were: ampicillin (sodium salt), 25; chloramphenicol, 20; kanamycin (acid sulfate), 12.5 and 25; nalidixic acid (sodium salt), 30; neomycin (sulfate), 25; streptomycin (sulfate), 25; sulfathiazole, 100; and tetracycline (hydrochloride), 25. The concentrations used were chosen because they were found to prevent the growth of heavy inocula of sensitive strains while allowing resistant strains to grow and form single colonies after incubation for 18 to 48 h.

Chemicals and buffers. All chemicals were analytical reagent grade from commercial sources, and were used without further purification. Horse heart cytochrome *c*, either salt free from Fluka AG or type III from Sigma Chemical Co., was found to be suitable for the basic protein film technique. Phosphate and citrate buffers were prepared according to the formulae of Dawson et al. (15). TES buffer contained 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.01 M ethylenediaminetetraacetic acid (EDTA; disodium salt), and 0.2 M NaCl at pH 8 (38). Other Tris-EDTA buffers, used in preparing DNA for electron microscopy, were prepared as described by Davis et al. (14).

Culture conditions and conjugation procedures. Bacteria were grown and mated as described by Adelberg and Burns (1). Where transfer frequencies were to be determined, matings were sampled after 1 or 2 h. Transfer frequencies were calculated from the number of transconjugants formed per donor cell in the mating mixture, under conditions where the recipient was in excess. (Transconjugants are cells of the recipient strain that have received genetic material by conjugation.) The same procedures were followed in host range matings between R^+ *E. coli* strains and either *P. aeruginosa* PAO2 or *P. mirabilis*

PM13 (10). However, when formation of R^+ transconjugants could not be detected after the 2-h mating, mating mixtures and controls were incubated for 18 h before sampling, then diluted 1:20 into fresh broth and sampled again after incubation for a further 6 h.

Fertility inhibition and incompatibility grouping. For studies of fertility inhibition, strains carrying both the plasmid to be tested and either of the reference plasmids (F16 or R64-11) were constructed. The resulting plasmid "doubles" were then mated with a suitable recipient strain to determine the transfer frequency of both the test and reference plasmids. The ability of each double strain to be visibly lysed by, or to support the multiplication of, the appropriate pilus-specific phage was tested as described below.

Incompatibility between the *Shigella* plasmids and the reference plasmids that did not carry the Su determinant was investigated as follows. By mating overnight, the reference plasmid (bearing the Km determinant in a Pro^+ Met^- host) was transferred into the strain (Pro^-Met^+) carrying the resident *Shigella* plasmid (Su or SuCol). Km^rMet^+ transconjugants were selected. From each mating, five transconjugants were picked and streaked for single colonies on the same selective media. Eight of the resulting colonies from each transconjugant (giving a set of 40 colonies per mating) were screened for the presence of the Su marker of the resident plasmid.

As a control, incompatibility between the reference F_{II}-group plasmid 222/R4 (in a Pro^-Met^+ host) and the same panel of reference plasmids was investigated by the same method. The number out of 40 Km^rMet^+ transconjugants that had lost the Su marker (and others) of 222/R4 indicated the result to be expected for pairs of compatible or incompatible plasmids.

Scoring unselected markers. Antibiotic resistance and auxotrophic markers were scored by replica plating to appropriate media. Bacteriocinogeny was scored by killing patches grown overnight on nutrient agar replica plates with chloroform vapor, and then overlaying the plate with a soft-agar layer seeded with a suitable indicator strain. Bacteriocin-induced zones of inhibition in the resulting indicator lawn could be scored after overnight incubation.

Visible lysis by pilus-specific phages was scored in a standard cross-streaking test (35) against either MS2 or If1 on eosin methylene blue agar plates. Plates for the test against If1 were incubated at 32 C.

Detection of F-like or I-like pili by phage propagation. The presence of sex pili on at least some of the cells of strains carrying repressed conjugative plasmids was determined as follows. The cells from 5 ml of mid-exponential Z broth culture were concentrated by centrifugation into 0.2 ml of fresh Z broth. About 2×10^7 plaque-forming units of either MS2 or If1 were added, and after 10 min at room temperature the infected culture was diluted to 10 ml with Z broth and incubated at 37 C overnight. Cells and debris were removed by centrifugation, and the supernatants were assayed for the appropriate phage on Z agar containing 500 μg of streptomycin per ml, to which the R^+ strain was sensitive. The indicator strain for MS2 was JP985 or JP997, and the indicator strain for If1 was JP986 or JP994. All these strains are streptomycin resistant.

In control experiments, the titer of phage recovered from R^- strains was essentially the input titer, whereas strains carrying the repressed plasmid 222/R4 supported an increase in the titer of MS2 of 5- to 20-fold.

Isolation of plasmid DNA. The following method was modified from the procedures of Nisioka et al. (38), Clewell and Helinski (5), and Freifelder et al. (19, 20).

Overnight cultures in B medium were diluted 1:20 into 10 ml of fresh medium and incubated with agitation for 2 h. Deoxyguanosine (2) and [6-^{3}H]thymidine were added to 10^{-3} M and 3.3×10^{-4} M (0.33 μCi/ml), respectively, and incubation was continued for 2 h. Cells were pelleted by centrifugation and washed three times with 10-ml volumes of TES buffer at 4 C. The washed cells were suspended in 0.5 ml of a 0.05 M Tris (pH 8) solution containing 25% (wt/vol) sucrose and 2×10^{-5} M *n*-dodecylamine (32), 0.1 ml of lysozyme solution (5 mg/ml in 0.25 M Tris, pH 8) was added and mixed very gently, and the suspension was incubated for 5 min at 0 C. EDTA, (0.2 ml, 0.25 M, pH 8) was then added, followed (after 5 min at 0 C) by 0.8 ml of lysing solution. Lysing solution was 2% (wt/vol) Sarkosyl L and 2×10^{-5} M *n*-dodecylamine in a solution containing 0.05 M Tris and 0.07 M EDTA (pH 8.1). After 30 min at 0 C, 0.4 ml of TES buffer was added, followed by 0.05 ml of Antifoam AF (0.1% [vol/vol] in TES buffer).

Each lysate was sheared by passing it, eight times, through a 21-gauge needle fitted to a 10-ml syringe whose plunger was driven at maximal hand pressure. At this stage the partially sheared lysate was frozen at −20 C, stored overnight, and thawed, and shearing was completed by an additional eight passages through a 21-gauge needle.

Density gradient centrifugation. To confirm the presence of plasmid DNA(20), sheared lysate (0.25 ml) was centrifuged through a 12-ml linear 5 to 20% (wt/wt) gradient of sucrose in 0.5 M NaCl, 0.01 M EDTA, and 0.3 M NaOH (pH about 12.3) at 34,500 rpm for 41 min at 22 C in the SW41 rotor of a Beckman Spinco L2-65 ultracentrifuge.

To separate plasmid DNA for electron microscopy, 0.8 ml of sheared lysate was added to 5.20 g of finely ground CsCl, followed by 1.6 ml of ethidium bromide (EB) solution (700 μg/ml in 0.1 M phosphate buffer, pH 7.4) and 3.0 ml of sterile water. After thorough but gentle mixing by inversion, the entire solution (about 6.3 ml) was placed in a polyallomer tube, overlaid with liquid paraffin, and centrifuged at 34,000 rpm (about $100,000 \times g$ at r_{av}) for 65 h at 20 C in the 50 Ti rotor of an L2-65 ultracentrifuge.

Gradients were fractionated by puncturing the bottom of the tube and collecting 0.6-ml (alkaline sucrose) or 0.2-ml (CsCl-EB) fractions. The radioactivity of 25-μliter samples of each fraction, applied to filter paper disks as described by Bollum (4), was determined in an Ansitron liquid scintillation spectrometer. The scintillation fluid used contained 0.5% (wt/vol) 2,5-diphenyloxazole and 0.02% (wt/vol) 1,4-bis-(5-phenyloxazolyl)benzene in toluene.

Fractions containing plasmid DNA were pooled and dialyzed extensively at 4 C against buffer containing 0.1 M Tris and 0.01 M EDTA (pH 8.2).

Electron microscopy of plasmid DNA. Plasmid DNA was mounted for electron microscopy by a modification of the formamide-basic protein film technique (14). A spreading solution was prepared that contained 20 μliters of DNA in 0.1 M Tris, 0.01 M EDTA (pH 8.2), 20 μliters of formamide, and 10 μliters of cytochrome *c* (200 μg/ml in 0.3 M Tris-0.03 M EDTA, pH 8.5). A 20-μliter amount of this spreading solution was allowed to run down a glass ramp onto the surface of a hypophase solution that contained 12% (vol/vol) formamide in 0.01 M Tris-0.001 M EDTA (pH 8.5). The spread film was allowed to stabilize for 45 s, and then fresh parlodion-coated grids were applied. These grids were then stained in 5×10^{-5} M uranyl acetate in absolute ethanol, rinsed in isopentane, and shadowed with platinum-carbon in a vacuum evaporator.

Grids were examined in a Hitachi HS8 electron microscope, operated at 50 kV, at an instrumental magnification of ×7,500. At the beginning and end of each run, the constancy of the magnification was calibrated with a standard grid. Micrographs of circular molecules were copied, at constant magnification, on Kodak Kodalith 35-mm film, which was then projected onto a screen (4 by 3 ft [122 by 0.9 m]). Contours were traced on paper and measured with a Keuffel and Esser map-measuring device.

As a convenient circular DNA standard for calibrating contour lengths, phage PM2 DNA was chosen. This DNA is a double-stranded, covalently closed circular molecule with a contour length 3.02 ± 0.11 μm and a molecular weight of 6×10^6 (17). Phage PM2 was grown and purified as described by Espejo and Canelo (16), and the DNA from 10^{12} plaque-forming units in 1 ml of ST buffer was extracted with chloroform-isoamylalcohol (24:1, vol/vol). The phage DNA was banded in a CsCl-EB gradient, dialyzed, and mounted for electron microscopy as described above. A sample of 56 molecules of PM2 DNA was measured. Setting the mean of the observed contour lengths equivalent to 3.02 μm, a sample standard deviation of ±0.29 μm was obtained.

RESULTS

Frequency of transfer of the Su determinant between Shigella and E. coli K-12. Each *Shigella* strain was mated for 90 min with suitable *E. coli rec*$^+$ recipients to establish the transfer frequency of the sulfonamide resistance (Su) determinant. The Su$^+$ transconjugants from the *Shigella* parent strains SUM1 and SUM2 were screened for co-inheritance of the bacteriocinogeny (Col) determinant.

The *S. dysenteriae* strains SUM5, SUM6, and SUM8 each transferred their Su determinant to strain JP990 at a frequency of 10^{-5} per donor cell.

The results with the *S. flexneri* 2a strains SUM1 and SUM2 are shown in Table 2.

These results suggested that the Su and Col determinants of strain SUM1 were linked on the one plasmid, whereas the Su and Col determinants of SUM2 may have resided on separate plasmids. Su$^+$ transconjugants that had not also received the Col determinant remained sensitive to the bacteriocin produced by strain SUM2.

In an attempt to clarify the linkage relationships of the Su and Col determinants from strains SUM1 and SUM2, the Su determinant was transduced from *E. coli* Su$^+$ Col$^+$ transconjugants to the *E. coli recA*$^-$ strain JP249 by using phage Plkc. The resulting Su$^+$ transductants were screened for co-inheritance of the Col determinant. (Control conjugation experiments had shown that the Col determinants from both *Shigella* donors could be stably inherited and expressed in JP249.) All of 210 Su$^+$ transductants bearing the determinant from SUM1 were also Col$^+$, whereas none of 420 Su$^+$ transductants with the SUM2 determinant was Col$^+$. All of the Su$^+$ transductants examined, from both parents, could transfer the Su determinant in matings with JP990. These observations strongly supported the possibility that the Su and Col determinants were linked in SUM1 but not in SUM2. They also suggested that each Su determinant was linked to a conjugative plasmid.

Similar conclusions could also be drawn from the results of curing experiments performed by the method of Hirota (26) (but using quinacrine [22] as the curing agent). When *E. coli* Su$^+$ Col$^+$ transconjugants from SUM1 were grown overnight in the presence of quinacrine, 5% of the survivors lost the Su determinant, and in all of them the Col determinant was eliminated. None of over 500 Su$^+$ survivors lost the Col determinant. With *E. coli* Su$^+$ Col$^+$ transconjugants from SUM2, however, although none of 520 survivors lost the Su determinant, in 30% the Col determinant was eliminated.

TABLE 2. *Transfer and co-inheritance of Su and Col determinants*

Shigella donor	*E. coli* recipient	Transfer frequency for Su determinant[a]	Proportion of Su transconjugants that were also Col$^+$
SUM1	AB1450	2.9×10^{-4}	120/120
	JP271	3.2×10^{-4}	420/420
SUM2	AB1450	2.2×10^{-3}	20/210
	JP271	3.2×10^{-3}	95/210

[a] Number per donor cell.

The *Shigella* strains SUM1 and SUM2 and their Col$^+$ transconjugants in *E. coli* were mutually immune to each other's bacteriocin. Spontaneously occurring *E. coli* mutants resistant to one bacteriocin were always resistant to the other. However, no further characterization or identification of these bacteriocins was undertaken.

Further experiments with the *E. coli* Su$^+$ transconjugants of all five *Shigella* strains showed that each of the Su determinants could be stably inherited by the *recA*$^-$ strain JP249, regardless of whether conjugation or Plkc-mediated transduction was used to transfer them to that strain. For each Su determinant, conjugal transfer frequencies (per donor) or transduction frequencies (per pfu) were the same for both *rec*$^+$ (JC411) and *recA*$^-$ (JP249) strains.

Each *E. coli* Su$^+$ strain was able to form single colonies on B medium containing up to 120 to 140 μg of sulfathiazole per ml. The corresponding *E. coli* recipient strains were all unable to form single colonies on B medium containing more than 40 μg of sulfathiazole per ml.

Fertility inhibition and determination of sex pili. The ability of the *Shigella* plasmids to inhibit the fertility of the F-merogenote F16 (i.e., the traditional fi response [31]) and of the derepressed I-like R plasmid R64-11 was investigated. In addition, whether each *Shigella* plasmid determined the production of either F-like or I-like pili in *E. coli* was investigated by testing the ability of *E. coli* Su$^+$ transconjugants to support the multiplication of the pilus-specific phages MS2 and Ifl. Included in these studies were strain JP643, a transconjugant that carried a spontaneously occurring fertility-derepressed mutant of the Su Col plasmid from *Shigella* strain SUM1, and strains JP646 and JP649, which were, respectively, Su$^+$ Col$^+$ and Su$^+$ (Col$^-$) transconjugants from strain SUM2. The results are shown in Table 3, which also presents the provisional designations of the *Shigella* plasmids and the strain numbers of *E. coli* transconjugants carrying them.

The frequencies with which each *Shigella* plasmid was transferred between *E. coli* strains suggested that each was subject to fertility repression. The SuCol plasmid pUM1 was fi$^+$ and determined the production of an F-like pilus, since it could support the multiplication of phage MS2. This was confirmed by studies on the mutant plasmid derived from pUM1 (i.e., pUM1 drd 1), whose properties were those expected of a fertility-derepressed mutant derived from a typical F-like fi$^+$ plasmid (31). The Col determinant from strain SUM2, but not the Su determinant, was associated with the fi$^+$ character and the ability to produce F-like pili (presumably constituting an fi$^+$ F-like conjugative Col plasmid, pUM3).

The Su plasmids pUM2, pUM5, pUM6 and pUM8 were all certainly fi$^-$, but they did not appear to specify the production of either F-like or I-like pili. A notable feature of the results with pUM5, pUM6, and pUM8 was their interaction with the reference plasmid R64-11. In each case, the transfer frequency of the Su determinant was increased about 100-fold in the presence of R64-11, whereas the transfer frequency for R64-11 markers was reduced between 10- and 25-fold. These donors were still sensitive to lysis by phage Ifl, and the basis for this change in transfer frequencies is not understood.

As is apparent from Table 3, none of the *Shigella* plasmids appeared to specify the production of I-like pili or to repress the fertility of the derepressed I-like plasmid R64-11. However, certain repressed I-like plasmids, whose derepressed mutants do produce I-like pili, apparently fail to propagate phage Ifl under conditions similar to those used here (27), and it is not known whether R64-11 is indeed subject to fertility inhibition in a manner analogous to F (33). Thus, these results do not exclude the possibility that the fi$^-$ plasmids are I-like.

Incompatibility grouping. To further characterize these *Shigella* plasmids, their incompatibility was investigated with reference plasmids of incompatibility groups F_I, F_{II}, I_α, and P (Tables I and 4). Incompatibility with group-N plasmid R46, or the group-W plasmid S-a, could not be tested because these reference plasmids carried the Su determinant.

The results obtained in control experiments with the group-F_{II} plasmid 222/R4 showed that displacement of the resident plasmid (i.e., 222/R4) was not detected when the entering plasmid was of a different incompatibility group, and also indicated the extent of displacement that might be observed after the entry of a plasmid belonging to the same incompatibility group.

The three fi$^-$ Su plasmids pUM5, pUM6, and pUM8, originally from *S. dysenteriae* strains, were each clearly displaced as resident plasmids by entry of the reference P-group plasmid RP4. They were not displaced by the three other reference plasmids and could thus be assigned to incompatibility group P.

Displacement of plasmids pUM1, pUM1 drd

TABLE 3. *Fertility inhibition and pilus-specific phage sensitivity conferred by Su plasmids*

Shigella parent	*E. coli* parent	*E. coli* transconjugant	Plasmids carried	Response to pilus-specific phage[a]				Transfer frequency[b] of determinant to AB1450 or JP249		
				MS2		If1				
				Lysis	Propagation	Lysis	Propagation	Su	Ilv	Tc
SUM1	JP271	JP610	pUM1[c]	r	+	r	–	2×10^{-4}		
		JP640	pUM1, F16	r	+	r	NT	2×10^{-4}	2×10^{-5}	
		JP642	pUM1, R64-11	r	NT	s	NT	2.8×10^{-4}		1.6×10^{-1}
SUM1	JP271	JP643	pUM1 drd 1	s	NT	r	NT	8×10^{-2}		
		JP644	pUM1 drd 1, F16	s	NT	r	NT	8×10^{-2}	2×10^{-2}	
		JP645	pUM1 drd 1, R64-11	s	NT	s	NT	8×10^{-2}		1.1×10^{-1}
SUM2	JP271	JP646	pUM2, pUM3	r	+	r	–	4×10^{-4}		
		JP647	pUM2, pUM3, F16	r	+	r	NT	2×10^{-4}	5×10^{-5}	
		JP648	pUM2, pUM3, R64-11	r	NT	s	NT	2×10^{-4}		2×10^{-1}
SUM2	JP271	JP649	pUM2	r	–	r	–	3×10^{-4}		
		JP650	pUM2, F16	s	NT	r	NT	4×10^{-4}	4×10^{-2}	
		JP651	pUM2, R64-11	r	NT	s	NT	2.6×10^{-4}		9×10^{-2}
SUM5	JP990	JP652	pUM5	r	–	r	–	5×10^{-5}		
		JP653	pUM5, F16	s	NT	r	NT	5.8×10^{-5}	4×10^{-2}	
		JP654	pUM5, R64-11	r	NT	s	NT	2.5×10^{-3}		8.5×10^{-3}
SUM6	JP990	JP655	pUM6	r	–	r	–	5×10^{-5}		
		JP656	pUM6, F16	s	NT	r	NT	4×10^{-5}	2×10^{-2}	
		JP657	pUM6, R64-11	r	NT	s	NT	4.1×10^{-3}		1.1×10^{-2}
SUM8	JP990	JP658	pUM8	r	–	r	–	3×10^{-5}		
		JP659	pUM8, F16	s	NT	r	NT	3×10^{-5}	2×10^{-2}	
		JP660	pUM8, R64-11	r	NT	s	NT	1.6×10^{-3}		1.5×10^{-2}
	JP271[d]	JP641	F16	s	+++	r	–		2.8×10^{-2}	
	JP990[d]	JP993	R64-11	r	–	s	+++			1.8×10^{-1}

[a] In cross-streak (lysis) tests, r indicates no visible effect of the phage on growth, and s indicates visible lysis. In propagation tests, – indicates no increase in titer, + indicates an increase in titer of between 5 and 20, and +++ indicates an increase of over 1,000-fold. NT, Not tested.

[b] Transfer frequency (number) per donor cell in matings for 60 min. Transfer frequencies were the same for any plasmid to both recipient strains AB1450 (*rec*$^+$) and JP249 (*recA*$^-$).

[c] *Shigella* plasmids. pUM1 is the Su Col plasmid from strain SUM1; pUM2 is the plasmid bearing the Su determinant, and pUM3 is the plasmid bearing the Col determinant from strain SUM2; pUM5, pUM6, and pUM8 are the Su plasmids from strains SUM5, SUM6, and SUM8, respectively.

[d] Control strain.

1, pUM2, or pUM3 was not detected after entry of any of the four reference plasmids. In these cases, plasmid interactions in the reciprocal situation were examined. Thus, these *Shigella* plasmids were transferred into strains in which the reference plasmids were resident, and Su$^+$ Pro$^+$ transconjugants were selected. In the control reciprocal matings, the F_{II}-group plasmid 222/R4 displaced only R1-16 (also group F_{II}), as expected.

In crosses with the Su$^+$ Col$^+$ transconjugant, strain JP646, as donor, derived from the *Shigella* strain SUM2, only the F_{II}-group reference plasmid R1-16 was displaced and then only in Su$^+$ Pro$^+$ transconjugants which were also Col$^+$; R1-16 was not displaced by entry of the Su plasmid pUM2 alone. This result suggested that the fi$^+$ Col plasmid pUM3 could be assigned to the F_{II} incompatibility group.

Incompatibility between the *Shigella* plasmids pUM1, pUM1drd 1, and pUM2 and the reference plasmids of groups F_I, F_{II}, I_α, and P was not detected in any of these experiments.

Host range. In studies with strains belonging to a variety of gram-negative genera as recipients for R plasmids from *E. coli* donors, Datta and Hedges (10) revealed an apparent correlation between the host range of an R plasmid and its incompatibility group. Since their results with *P. aeruginosa* PAO2 and *P. mirabilis* PM13 were sufficient to define the host range for each of the plasmids tested, these strains were chosen as recipients to establish the host range of the *Shigella* plasmids. The ability of these plasmids and reference plasmids to form detectable transconjugants in these two recipi-

ent strains (and in *E. coli*, as a control) was determined (Table 5).

Like the reference F_{II}-group plasmid R1-16, the related *Shigella* plasmids pUM1 and pUM1 drd 1 both could form transconjugants in *Proteus* but not *Pseudomonas*. Like the I_α-group plasmid R144-3, the *Shigella* plasmid pUM2 appeared incapable of forming transconjugants in either of these hosts.

TABLE 4. *Incompatibility of Shigella plasmids with reference plasmids*

Recipient strain	Resident plasmid	No.[a] in which resident Su plasmid was displaced by entry of:			
		Group F_I F16	Group F_{II} R1-16	Group I_α R144-3	Group P RP4
JP610	pUM1	0	0	0	0
JP643	pUM1 drd 1	0	0	0	0
JP649	pUM2	0	0	0	0
JP646	pUM2, pUM3	0	0	0	0
JP652	pUM5	0	0	0	23[b]
JP655	pUM6	0	0	0	28
JP658	pUM8	0	0	0	27
JP661	222/R4	0	27	0	0

[a] Forty colonies were examined in each case. Each set of 40 colonies was derived from five original transconjugants.

[b] An additional three colonies, although being Km^+ Su^+ Met^+, were found not to carry the Tc or Ap-Cb markers of RP4.

The significance of the observed reductions in R plasmid transfer frequencies to the other hosts, compared to their transfer to frequencies *E. coli*, is not understood. Similar reductions were also observed by Datta and Hedges. In the case of the *Shigella* plasmids pUM5, pUM6, and pUM8, this reduction in intergeneric transfer frequency might account for the failure to detect transconjugants of these plasmids in *Proteus*. However, although the P-group plasmid RP4 formed transconjugants in *Proteus* at low frequency after mating for 2 h, transconjugants of pUM5, pUM6, or pUM8 in this host were not detected even after mating for 24 h. It was thus possible that, although RP4 and these three *Shigella* plasmids were incompatible, their host ranges were genuinely different.

Isolation of plasmid DNA. [^{3}H]Thymidine-labeled lysates of strains containing the *Shigella* plasmids (Tables 3 and 4) and of the host strains JP271 and JP990 were examined in alkaline sucrose and CsCl-EB gradients. All of the R^+ strains gave similar profiles for the distribution of [^{3}H]thymidine label in the gradients; representative gradient profiles (for lysates of strains JP643 and JP271) are shown in Fig. 1.

Each lysate of the R^+ strains showed a satellite peak in both alkaline sucrose and CsCl-EB gradients that was not present in lysates of the corresponding R^- strain; therefore, at least some of the DNA of the plasmids pUM1 drd 1, pUM2, pUM5, and pUM8 was

TABLE 5. *Host range of plasmids studied*

E. coli donor	Plasmid	Apparent frequency[a] of formation of drug-resistant transconjugants in:		
		E. coli JC411	*Ps. aeruginosa* PAO2	*P. mirabilis* PM13
JP610	pUM1	4×10^{-4}	ND[b]	5×10^{-8}
JP643	pUM1 drd 1	8×10^{-2}	ND	4×10^{-6}
JP646	pUM2, pUM3	4×10^{-4}	ND	ND
JP649	pUM2	3×10^{-4}	ND	ND
JP652	pUM5	6×10^{-5}	6×10^{-5}	ND
JP655	pUM6	6×10^{-5}	6×10^{-5}	ND
JP658	pUM8	4×10^{-5}	7×10^{-5}	ND
Controls[c]:				
Group F_{II}	R1-16	ca. 2×10^{0}	ND	1×10^{-4}
Group Ia	R144-3	ca. 2×10^{0}	ND	ND
Group P	RP4	3×10^{-3}	2×10^{-3}	7×10^{-7}
Group N	R46	4×10^{-3}	NT[d]	5×10^{-6}
Group W	S-a	4×10^{-4}	9×10^{-7}	5×10^{-7}

[a] Transfer frequency (number) per donor in matings for 2 h.

[b] Not detected in matings for 2, 18, or 24 h. Under the conditions used, the lowest detectable transfer frequency would have been 3×10^{-8} with PM13 and 8×10^{-8} with PAO2.

[c] Reference plasmids in JP990.

[d] NT, Not tested.

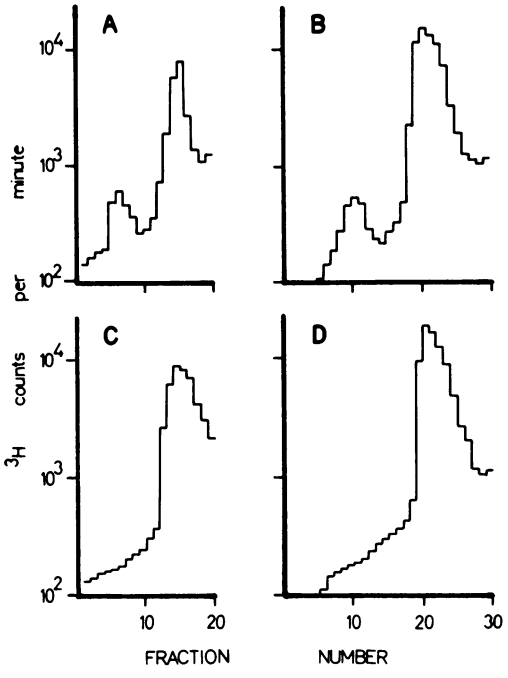

FIG. 1. *Representative density gradient profiles. [^3H]thymidine-labeled lysates of strain JP643 (A, B) or strain JP271 (C, D) were prepared and centrifuged. A, C, Alkaline sucrose gradients (0.6-ml fractions); B, D, CsCl-EB gradients (0.2-ml fractions). The satellite peak in the CsCl-EB gradient (B) of JP643 contained ^3H counts/min equivalent to 1.7% of the counts under the major (chromosomal) peak. In the other R^+ strains examined, the satellite peak amounted to 0.7 to 1.9% of the chromosomal peak in CsCl-EB gradients. Given the molecular weights determined for these plasmids (see Table 6), this suggests that these plasmids were not likely to be present in the cell in more than one or two copies per chromosome equivalent (6).*

present in the cells as covalently closed circular molecules. Although pUM3 alone could not be examined, the plasmid peak for the lysate of strain JP646 (which carried both pUM2 and pUM3) was larger relative to the chromosomal peak than the plasmid peak in the lysate of strain JP649 (pUM2 alone). This suggested that pUM3 DNA was also present as covalently closed circular molecules.

Contour length and molecular weight. Each plasmid peak from the CsCl-EB gradients was pooled and dialyzed, and the DNA was mounted for electron microscopy as described. In each set of grids from a plasmid preparation, about 5% of the DNA structures seen under the electron microscope had unequivocally circular and measurable contours. The number of circular molecules photographed and their mean contour length, with the sample standard deviation, are given for each plasmid preparation in Table 6. The distributions of the measured contour lengths are shown in Fig. 2.

The contour lengths and molecular weight values were all calibrated against and calculated from the respective values for PM2 DNA molecules. Although under certain conditions EB may significantly alter the contour length of DNA molecules (18), it appears that plasmid DNA recovered from CsCl-EB gradients is not subject to this effect (21, 38). Errors due to contour length distortion by EB in the above data would thus be small, and could be minimized by comparing populations of plasmid and PM2 molecules that had each been banded in CsCl-EB gradients and dialyzed in exactly the same way.

In the satellite DNA fraction derived from strains harboring the plasmids pUM1 drd 1 (JP643), pUM2 (JP649), pUM5 (JP652), and pUM8 (JP658), only one size class of circular DNA molecules was observed in each case. In the satellite peak from strain JP646, which from genetic studies was inferred to harbor the plasmids pUM2 and pUM3, two size classes of DNA molecules were observed, one being essentially the same size as the plasmid from strain JP649. This suggested that the shorter (21 μm) molecule was that of the Col plasmid pUM3. Thus, the conclusions reached from the genetic studies about the structural organization of determinants onto plasmids in these strains were amply confirmed.

DISCUSSION

The results shown in Tables 3 through 6 summarize the properties of the plasmids found in five *Shigella* strains isolated in 1952 and 1956. Apart from the fact that they specified resist-

TABLE 6. *Contour lengths and molecular weights of Shigella plasmids*

Lysate of *E. coli* strain:	Plasmids present	No. of molecules measured	Mean contour length[a] ± sample standard deviation (μm)	PM2 DNA equivalent	Mol wt[b] (10^6)
JP643	pUM1 drd 1	26	26.5 ± 0.7	8.8	53
JP649	pUM2	19	30.9 ± 1.5	10.2	61
JP646	pUM2, pUM3	57 {23	30.0 ± 0.8	9.9	59
		{34	21.1 ± 0.6	7.0	42
JP652	pUM5	47	29.5 ± 0.8	9.8	58
JP658	pUM8	38	29.5 ± 1.0	9.8	58

[a] Standardized against molecules of PM2 DNA that have a mean contour length of 3.02 μm. The distributions of observed contour lengths are shown in Fig. 2.

[b] Calculated from the molecular weight of PM2 DNA, i.e., 6×10^6.

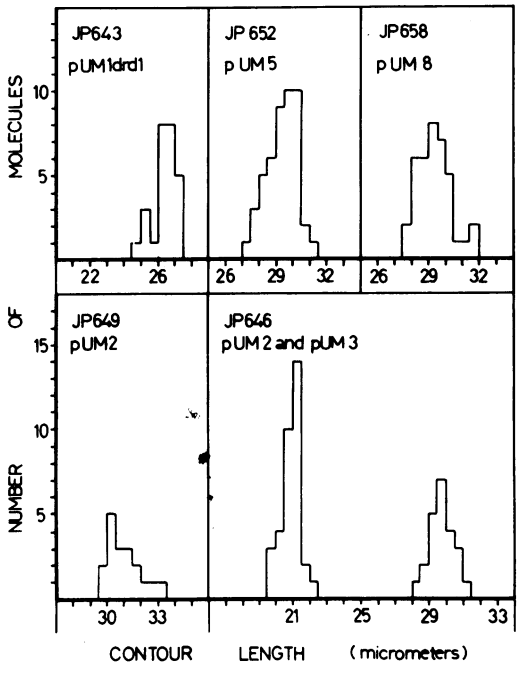

FIG. 2. *Frequency distributions of the contour lengths of circular DNA molecules (plotted to the nearest 0.5 μm). DNA from the satellite peak of CsCl-EB gradients was mounted for electron microscopy and measured. Contour lengths were calibrated against the mean contour length of phage PM2 DNA molecules (3.02 μm). Excluded from the calculation of mean contour length for circular DNA from strain JP646 was one molecule of contour length 58.5 μm. Not shown in the distribution for plasmid DNA from strain JP649 is one molecule of contour length 26.9 μm.*

ance to sulfonamides alone, these plasmids obviously do not form a homogeneous group. Although their sulfonamide resistance determinants rendered the strains of *E. coli* that carried them resistant to the same level of sulphathiazole, it is clear that the R_{su} plasmids from the 1956 *S. dysenteriae* 2 strains were not otherwise related to the earlier "R_{su}" plasmids found in the 1952 *S. flexneri* 2a strains.

The two *S. flexneri* 2a strains SUM1 and SUM2, isolated in 1952, were phenotypically indistinguishable in terms of serotype, drug resistance, and bacteriocinogeny. However, it is apparent that the Su and Col determinants harbored by each of these strains were carried on quite distinguishable plasmids. In strain SUM1, both determinants were carried on the one plasmid, pUM1. This plasmid was fi^+, specified F-like pili, and had the host range of F-like plasmids. However, although it can reasonably be assigned to the F-like plasmid family on the basis of these results, pUM1 was unexpectedly found to be compatible with plasmids of both the F_I and F_{II} groups, to which the majority of F-like plasmids belong (24).

By contrast, the Su and Col determinants in strain SUM2 were borne on separate plasmids. The Su plasmid pUM2 was fi^- and specified neither F-like nor I-like pili. It could not be assigned to any of the incompatibility groups, F_I, F_{II}, I_α or P, and its host range behavior was different from that of the group-N and group-W reference plasmids used.

Coexisting with pUM2 in strain SUM2 was the Col plasmid pUM3, a typical fi^+ group-F_{II} F-like plasmid. Although conjugal transfer of pUM3 alone was not investigated, it seems likely to be a conjugative plasmid, since it has been shown to specify the production of F-like pili and of a substance able to repress F-pilus synthesis.

The three 1956 plasmids (pUM5, pUM6, and pUM8), on the other hand, appeared to be very similar and possibly identical. Each was transferred between *E. coli* strains at essentially the same frequency, was fi^-, produced neither F-like nor I-like pili, and was incompatible with the prototype P-group plasmid RP4. Like RP4, these three plasmids could each form transconjugants in *Pseudomonas*, although, in contrast to RP4, transconjugants bearing these plasmids could not be detected in *Proteus*. Two of these three plasmids, pUM5 and pUM8, were found to have equivalent molecular weights. (The DNA of the third plasmid, pUM6, was not examined.)

A further similarity between pUM5, pUM6, and pUM8 as a group and the P-group plasmid RP4 was their interaction with the I_α-group plasmid R64-11. The transfer frequency of each R_{su} plasmid was increased about 100-fold by the presence of R64-11 in the same donor cell, and in such donors the transfer frequency of R64-11 itself was reduced about 10-fold. Similar interactions with R64-11 have been observed by Datta et al. (11) in their studies on RP4, but the basis for this interaction has not yet been explained.

The main departure shown by pUM5, pUM6, and pUM8 from the properties of the typical P-group plasmid RP4 was the apparent failure of all three to form transconjugants in *Proteus*.

In summary, five R_{su} plasmids obtained from strains of *Shigella* isolated in 1952 and 1956 have been shown to represent at least three different plasmid types. All three, however, have molecular weights roughly equivalent to

those found for the majority of conjugative plasmids (6).

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