R-Factor Cointegrate Formation in *Salmonella typhimurium* Bacteriophage Type 201 Strains

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The genetic and molecular properties of the plasmids in *Salmonella typhimurium* phage type 201 isolates are described. Such strains are resistant to streptomycin, tetracycline, chloramphenicol, ampicillin, kanamycin, and several other antimicrobial drugs, and are highly pathogenic for calves. These strains have been encountered with increasing frequency since 1972 in West Germany and The Netherlands. We show that isolates of this phage type constitute a very homogeneous group with regard to their extrachromosomal elements. These bacteria carry three small plasmids: pRQ3, a 4.2-megadalton (Md) colicinogenic plasmid; pRQ4, a 3.4-Md plasmid that interferes with the propagation of phages; and pRQ5, a 3.2-Md cryptic plasmid. Tetracycline resistance resides on a conjugative 120-Md plasmid pRQ1, belonging to the incompatibility class H2. Other antibiotic resistance determinants are encoded by a nonconjugative 108-Md plasmid pRQ2. Transfer of multiple-antibiotic resistance to appropriate recipient strains was associated with the appearance of a 230-Md plasmid, pRQ6. It appears that pRQ6 is a stable cointegrate of pRQ1 and pRQ2. This cointegrate plasmid was transferable with the same efficiency as pRQ1. Other conjugative plasmids could mobilize pRQ2, but stable cointegrates were not detected in the transconjugants. Phage type 201 strains carry a prophage, and we show that phage pattern 201 reflects the interference with propagation of typing phages effected by this prophage and plasmid pRQ4 in strains of phage type 201.

Salmonella typhimurium is the most predominant serotype among *Salmonella* isolates in many countries. Phage typing is used for further differentiation of this serotype. In West Germany and The Netherlands, multiple-antibiotic resistance in natural isolates of *S. typhimurium* has been mainly confined to strains of one particular phage type, type 201. Isolates of this phage type have been encountered since 1972, and the majority of them originate from diseased calves (33). Phage type 201 strains cause severe salmonellosis in these animals, and therapy is hampered because these strains are resistant to almost all therapeutically useful antimicrobial agents.

During a study on the extrachromosomal elements involved in the interference with the propagation of phages (this phenotypic property is termed Phi), the observation was made that *S. typhimurium* of phage type 201 carried an antibiotic resistance transfer system with unusual properties. Mobilization of nonconjugative resistance plasmids by coexisting conjugative plasmids generally results in transconjugants in which both plasmids are unchanged and the plasmids remain as independently replicating entities (3, 24, 31). This was not the case with phage type 201 strains. In this paper we report that upon transfer of the multiple-antibiotic resistance, a stable cointegrate composed of two large plasmids was formed. This large cointegrate initially escaped our detection when we used several standard DNA isolation procedures. However, the alkaline denaturation method described here enabled us to detect these large molecules. The involvement of prophages and plasmids with regard to their phage inhibition properties is also presented.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Strain M346 harbors a 52-megadalton (Md) lactose plasmid, pRIL1. This plasmid originates from a natural isolate of *Salmonella typhi* which was obtained from S. Aleksic. The auxotrophic mutant BB53 was obtained after mitomycin C treatment of S2050. This strain was identical to S2050 with regard to its plasmids, transfer of resist-

TABLE 1. *Bacterial strains*

Designation	Species[a]	Relevant properties[b]	Origin and/or derivation
Stm 1	*S. typhimurium*	Susceptible to the typing phages, phage type 1	Natural isolate (32)
Stm 1N	*S. typhimurium*	Resistant to Nal	Mutant of Stm 1 (32)
Stm 117-3	*S. typhimurium*	Resistant to Tc, Cm, Ap, Km; phage type 201	Natural isolate from a calf, 1973, The Netherlands
Stm 135-22	*S. typhimurium*	Resistant to Ap, Km, produces colicin E1; phage type 201-B	Stm 470 × Stm 117-3 × Stm 1N
Stm 216-18	*S. typhimurium*	Resistant to Tc, Ap, Cm, Km; lactose-positive, phage type 201	M346 × Stm 117-3
Stm 216-19	*S. typhimurium*	Resistant to Tc, Ap, Cm, Km; lactose-positive, phage type 201	M346 × Stm 117-3
Stm 470	*S. typhimurium*	Carries the conjugative plasmid pRI470	Natural isolate (32)
M136	*E. coli*	Resistant to rifampin and colicin E1	Mutant of C600
M216-25	*E. coli*	Resistant to Ap, Cm, Km; lactose-negative	Stm 216-19 × M136
M346	*S. typhimurium*	Lactose-positive, carries pRIL1	*E. coli* (pRIL1) × Stm 1N
S2049	*S. typhimurium*	Resistant to Tc, Ap, Cm, Km; phage type 201	Natural isolate from a calf, 1977, West Germany
S2050	*S. typhimurium*	Resistant to Tc, Ap, Cm, Km; phage type 201	Natural isolate from a calf, 1977, West Germany
S2057	*S. typhimurium*	Resistant to Tc, Ap, Cm, Km; phage type 201	Natural isolate from a calf, 1977, West Germany
S2060	*S. typhimurium*	Resistant to Tc, Ap, Cm, Km; phage type 201	Natural isolate from a calf, 1977, West Germany
BB1	*E. coli*	Resistant to Nal, rifampin, and colicin E1	Mutant of JC3272
BB4	*E. coli*	Resistant to Tc, Ap, Cm, and Km	S2050 × BB1
BB53	*S. typhimurium*	Resistant to Tc, Ap, Cm, and Km	Auxotrophic mutant of S2050
BB67	*S. typhimurium*	Resistant to Ap and Km; phage type 201-C	Derivative of S2050, obtained after ethidium bromide treatment
BB69	*S. typhimurium*	Resistant to Tc	Derivative of S2050, obtained after ethidium bromide treatment
BB70	*S. typhimurium*	Sensitive to Tc, Ap, Cm, and Km; phage type 201-C	Derivative of BB69, obtained after ethidium bromide treatment
BB75	*S. typhimurium*	Resistant to Tc, Ap, Cm, and Km	BB4 × BB70
BB76-5	*E. coli*	Resistant to Ap, Cm, and Km	Spontaneous Tc-sensitive variant of BB4
BB77	*E. coli*	Resistant to Tc	BB53 × BB1
BB119	*S. typhimurium*	Phage type 201-A	Stm 1 lysogenized by phage Sf2060
BB170	*E. coli*	Resistant to Ap, Cm, and Km	BB76-5 × J53-2
BB173	*E. coli*	Carries the H1 plasmid R27	N. Datta (12, 15)
J53-2	*E. coli*	Resistant to Nal; *met, pro*	N. Datta (12)
JC3272	*E. coli*	*lac, gal, his, trp, lys, str*	(1)
W3110NC	*E. coli*	Resistant to Nal and colicin E1	Spontaneous mutant of W3110N

[a] All *E. coli* strains are derivatives of *E. coli* K-12.

[b] Abbreviations: Nal, nalidix acid; Tc, tetracycline; Cm, chloramphenicol; Ap, ampicillin; Km, kanxmycin.

ance determinants, and other phenotypic properties.

Phage typing. The techniques of phage typing and the system used have been described previously (16, 17, 27).

Media. Nutrient broth, nutrient agar, minimal medium, L broth, and L agar have been described previously (23, 32).

Colicinogeny. Demonstration of colicinogeny was

carried out as described by Fredericq (14). Colicin typing was done with the use of standard colicin-producing strains and colicin-resistant mutants (13).

Antibiotic susceptibility test. Sensitivity tests for antibiotics were carried out by an agar diffusion procedure using either antibiotic disks (Oxoid Ltd.) or tablets (Rosco). Strains were tested for sensitivity to: ampicillin, carbenicillin, chloramphenicol, streptomycin, spectinomycin, neomycin, tetracycline, kanamycin, paramomycin, sulfamethoxazole, cephalothin, cefoxitin, cephaloxin, gentamicin, sisomycin, tobramycin, rifampin, trimethoprim, and nalidixic acid.

Transfer of plasmids. Matings in liquid medium were carried out by mixing equal volumes of exponentially growing cultures and by incubating the mixture overnight either at 22 or at 37°C. Matings on filters were performed as described previously (32). Transconjugants were selected on L plates containing appropriate combinations of antibiotics at the following concentrations: nalidixic acid, 25 µg/ml; rifampin, 50 µg/ml; tetracycline, 25 µg/ml; ampicillin 400 µg/ml; chloramphenicol, 27 µg/ml; and kanamycin, 12.5 µg/ml. The transfer frequency for a given antibiotic resistance determinant is expressed as the number of resistant transconjugants divided by the number of donor cells. Transformation was carried out as described (32).

Demonstration of lysogeny and preparation of lysogenic strains. The techniques used were described previously (32). The temperate phages used for lysogenization were obtained from phage type 201 strains either by spontaneous release or by induction with mitomycin C (2 µg/ml).

Selection of antibiotic-sensitive mutants. Antibiotic-sensitive derivatives of S2050 were isolated by inoculation of about 10^3 cells in 1 ml of L broth supplemented with ethidium bromide (1.2 mg/ml). After overnight growth at 37°C, the bacteria were streaked onto L plates. About 500 colonies were checked for resistance to tetracycline, ampicillin, chloramphenicol, and kanamycin by means of replica plating. About 5% of the clones had lost one or more drug resistance markers.

Isolation of plasmid DNA. Plasmid DNA was isolated by a modification of the methods of Casse et al. (8) and Hansen and Olsen (19). Two milliliters of an overnight or an exponentially growing culture in L broth was centrifuged for 2 min. All centrifugations were done in an Eppendorf centrifuge (model 5412). The pellet was suspended in 1 ml of TE (50 mM Tris–10 mM EDTA, pH 8.0) and centrifuged again. The pellet was suspended in about 40 µl of TE, and 0.8 ml of lysis mix (1% sodium dodecyl sulfate in TE, pH 12.45) was added. The tube was gently inverted several times to ensure complete mixing. The lysate was incubated for 20 min at 37°C. Neutralization was achieved by the addition of 40 µl of 2 M Tris-hydrochloride (pH 7.0), followed by gentle inversion. After this step, the chromosomal DNA and proteins were precipitated by adding 120 µl of a solution of 20% sodium dodecyl sulfate in TE, followed immediately by the addition of 240 µl of 5 M NaCl. This mixture was incubated for 6 h at 0°C and centrifuged for 10 min. The plasmid DNA in the supernatant was precipitated by the addition of 0.3 ml of 50% polyethylene

glycol 6000 in TE at 0°C for 12 h. After centrifugation for 3 min, the pellet was suspended in 30 µl of sample buffer consisting of 3% Ficoll and 0.025% bromophenol blue. A 10-µl amount was analyzed on a 0.7%, 0.25-cm-thick agarose slab gel. Electrophoresis was done during 3 h at 150 V in electrophoresis buffer (0.09 M boric acid–0.09 M Tris–0.025 M EDTA, pH 8.2). The DNA bands were stained with ethidium bromide and photographed with a 302-nm transilluminator (Ultraviolets products, C-63, San Gabriel) with Polaroid type 665 film. Molecular weights were estimated by comparing the mobilities of the plasmids with those of known molecular weight ranging between 4.2 and 143 Md.

RESULTS

Epidemiology. The incidence of phage type 201 strains isolated in The Netherlands since 1971 is shown in Table 2. Isolates of this phage were first encountered in 1972, and from 1973 it was the most frequently encountered phage type among *S. typhimurium* strains isolated from calves. Since 1978 we have tried to trace the sources of human infections with *S. typhimurium* of phage type 201, and the majority of them were found to have been contracted by direct contact with fattening calves. The sensitivity of *S. typhimurium* isolates to ampicillin, chloramphenicol, tetracycline, and kanamycin was routinely tested. About 97% of all phage type 201 isolates were resistant to tetracycline, chloramphenicol, kanamycin, and ampicillin. Occasionally isolates were encountered that lacked resistance to one of these drugs.

In West Germany similar results have been obtained for *S. typhimurium* isolates of animal origin (data not shown).

Phenotypic properties of phage type 201 strains. Ten independent type 201 isolates were arbitrarily chosen for sensitivity testing to 19

TABLE 2. *Incidence[a] of phage type 201 strains in The Netherlands*

Yr of isola-tion	Incidence (%) in:			
	Calves	Human	Pigs	Other
1971	<1 (0)	<0.01 (0)	<0.1 (0)	<0.01 (0)
1972	5 (5)	<0.01 (0)	<0.1 (0)	<0.01 (0)
1973	46 (89)	0.9 (45)	2.2 (13)	4.1 (43)
1974	72 (280)	0.7 (40)	1.8 (33)	5.6 (88)
1975	54 (174)	0.3 (16)	1.3 (23)	3.2 (37)
1976	20 (50)	0.01 (1)	0.2 (3)	0.3 (4)
1977	16 (60)	0.02 (1)	0.5 (9)	0.9 (9)
1978	32 (149)	0.05 (3)	1.2 (17)	3.4 (52)
1979	13 (74)	0.06 (3)	0.9 (8)	0.2 (4)

[a] The percentages represent the number of *S. typhimurium* isolates of phage type 201 versus the total number of *S. typhimurium* isolates from a given source. The number of phage type 201 isolates is in parentheses.

antimicrobial agents. All strains were resistant to ampicillin, chloramphenicol, tetracycline, kanamycin, streptomycin, carbenicillin, spectinomycin, neomycin, paramomycin, and sulfamethoxazole, and they were sensitive to trimethoprim, rifampin, nalidixic acid, and to the cephalosporins and the other aminoglycosides listed above. The drug resistance determinants were inherited quite stably, because the segregation percentage observed after growth in drug-free medium for more than 11 generations was less than 1%. The strains were also tested for the production of colicin, and all produced it. The colicin type of two strains, S2050 and Stm 117-3, was characterized as colicin E1. The colicin type produced by Stm 117-3 was confirmed by P. Fredericq (personal communication).

All 10 isolates were examined for the spontaneous release of phages using strain Stm 1 as indicator, and all strains did. These phages were able to lysogenize Stm 1, and all resulting lysogenic strains exhibited immunity to the phage isolated from strain Stm 117-3. This suggests that the prophages of the various type 201 strains are identical. The prophages of strains Stm 117-3 and S2060 were chosen as a representative and designated Sf 117-3 and Sf 2060, respectively.

Plasmid DNA was isolated from the 10 wild-type strains, and fie different plasmid species were detected (Fig. 1, track 2). The molecular masses were estimated to be 120, 108, 4.2, 3.4, and 3.2 Md, respectively. All but 2 of the 10 strains were identical with regard to the plasmid banding pattern. Strains S2049 and S2057 differed from the other strains only in the lack of the 3.2-Md plasmid (Fig. 1, track 3). The plasmids of strain S2050 were designated in decreasing size order as pRQ1, pRQ2, pRQ3, pRQ4, and pRQ5, respectively. For convenience we will use the same plasmid designations for plasmids from the natural isolate Stm 117-3.

Transferability of the drug resistance determinants. Matings of phage type 201 strains with the *Escherichia coli* K-12 recipient strains BB1 and W3110NC were performed in liquid medium at 37 and 22°C. As an example, the transfer frequencies for the different drug resistance determinants of the mating S2050 × BB1 are presented in Table 3. The tetracycline resistance transferred at a frequency of about 7×10^{-6} at 37°C and of about 5×10^{-5} at 22°C. At 37°C, transfer of the other drug resistance determinants was below the detection level of about 10^{-9}. However, at 22°C these markers transferred at a frequency of about 10^{-6}. When *S. typhimurium* strain Stm 1N was used as a recipient, similar results were obtained.

The drug resistance phenotypes of the transconjugants of the mating S2050 × BB1 that were selected on the various drugs are shown in Table 4. The tetracycline resistance transferred independently of the other markers. However, selection on ampicillin, chloramphenicol, or kanamycin generally resulted in transconjugants that were resistant to these three drugs as well as to tetracycline. Four transconjugants were tested with all other antimicrobial drugs mentioned above, and they were found to have the same resistance pattern as the donor S2050. When these transconjugants were again mated with *E. coli* K-12, the tetracycline, kanamycin, chloramphenicol, and ampicillin resistance determinants transferred as one linkage group, irrespective of the drug of selection. The transfer was now very efficient, and it was still temperature sensitive (Table 5). Similarly, the resistance of the tetracycline-monoresistant transconjugants could be further transferred, and the transfer frequency was also temperature dependent. These data suggest that in strain S2050 the tetracycline resistance determinant is encoded by a conjugative plasmid which exists physically independent of the other drug resistance determinants in S2050 and that these genes are linked to the tetracycline resistance plasmid after transfer of the multiple drug resistance determinants into the recipient. The transfer of all drug resistance determinants, except tetracycline, could be promoted at least 1,000-fold by

TABLE 3. *Transfer frequencies of drug resistance genes from S. typhimurium to E. coli K-12*

Mating	Temp (°C)	Transfer frequencies of resistance determinants to:			
		Tetracycline	Ampicillin	Chloramphenicol	Kanamycin
S2050 × BB1	37	7.4×10^{-6}	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.4 \times 10^{-9}$
S2050 × BB1	22	5.1×10^{-5}	3.2×10^{-6}	4.3×10^{-6}	4.2×10^{-6}
Stm 117-3 × W3110-NC	37	$<10^{-8}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$
Stm 470 × Stm 117-3 × W3110-NC	37	$<10^{-8}$	10^{-6}	10^{-6}	10^{-6}
Stm 216-18 × W3110-NC[a]	37	10^{-7}	10^{-5}	10^{-5}	10^{-5}

[a] Strain Stm 216-18 was constructed by introduction of pRIL1 into Stm 117-3. pRIL1 was transferred at a frequency of about 2×10^{-1}.

TABLE 4. *Resistance phenotype of transconjugants of the mating S. typhimurium S2050 × E. coli BB1*[a]

Selected marker	Phenotypes obtained[b] (%)			
	Tc	Tc, Ap, Cm, Km	Ap, Cm, Km	Tc, Ap, Km
Tc	100	<1	<1	<1
Ap	<1	85	4	11
Cm	<1	99	1	<1
Km	<1	93	4	3

[a] Abbreviations, see Table 1, footnote *b*.

[b] At least 100 colonies from each selective plate were checked for unselected markers by replica plating.

TABLE 5. *Transfer frequencies of drug resistance genes from E. coli transconjugants to the E. coli recipient J53-2*

Donor[a]	Drug resistance	Temp (°C)	Frequency of transfer
BB77	Tc	37	2×10^{-4}
BB77	Tc	22	4×10^{-3}
BB4	Tc, Ap, Cm, Km	37	1×10^{-5b}
BB4	Tc, Ap, Cm, Km	22	5×10^{-2b}

[a] BB4 and BB77 are transconjugants from S2050 and its auxotrophic derivative BB53, respectively.

[b] Virtually all transconjugants were resistant to tetracycline (Tc), ampicillin (Ap), chloramphenicol (Cm), and Kanamycin (Km), irrespective of the drug selected.

the conjugative plasmid pRI470 in triparental matings at 37°C. This was accomplished by the use of Stm 470 as a donor, Stm 117-3 as an intermediate, and either Stm 1N or W3110NC as a final recipient (Table 3). The majority of the transconjugants of the triple cross were able to further transfer the acquired resistance determinants. About 5% of the transconjugants were not, suggesting that the transfer factor, pRI470, is not necessarily linked to the resistance genes in the transconjugants.

Also, the conjugative lactose plasmid, pRIL1, was able to mobilize the ampicillin, chloramphenicol, and kanamycin resistances en bloc (Table 3). Mating experiments with the resulting transconjugants showed that the lactose genes of pRIL1 and the drug resistance determinants remained unlinked.

The temperature-sensitive transfer system of the phage type 201 strains suggests that the plasmid involved belongs to the incompatibility group H, because until now, only group H plasmids have been shown to exhibit this phenotype (30). This is in accordance with the observation that the *inc* H1 plasmid R27 is not stably maintained in transconjugants of S2050 or in tetracycline-sensitive mutants of S2050 (data not shown). Because F-*lac* was stably inherited in

S2050, we presume that the self-transferable plasmid in S2050 belongs to the incompatibility group H2 (29).

Molecular properties of the R-plasmids in S. typhimurium 201 and its transconjugants. Initial attempts to isolate plasmid DNA from the multiply antibiotic-resistant transconjugants by the methods described by Clewell and Helinski (9) and Hansen and Olsen (19) failed in our hands. However, the alkaline denaturation method described above allowed the isolation of plasmid DNA from all transconjugants studied. Ten tetracycline-monoresistant BB1 and 10 tetracycline-monoresistant Stm 1N transconjugants of the mating with donor S2050 were tested, and all were found to carry a plasmid species that comigrated with pRQ1 on agarose gels (Fig. 1, track 4). This indicates that pRQ1 is a conjugative plasmid, encoding for tetracycline resistance.

Ten multiply resistant *Salmonella* transconjugants and 20 multiply resistant *E. coli* transconjugants were analyzed. Two of the latter ones carried two plasmids which comigrated with pRQ1 and pRQ2. However, all other 28 transconjugants examined carried only a single large plasmid species of about 230 Md. The large plasmid in one particular strain, BB4, was designated pRQ6 (Fig. 1, track 5). This suggests that pRQ6 might be a cointegrate between pRQ1 (120 Md) and pRQ2 (108 Md) and that pRQ2 carries all drug resistance genes, except tetracycline resistance. Direct evidence for the latter assumption came from plasmid analysis of ampicillin-, chloramphenicol-, and kanamycin-resistant transconjugants which were obtained after mobilization of these drug resistance determinants by pRI470 and pRIL1. All these transconjugants had pRQ2 in common. One transconjugant, Stm 216-25, carried pRQ2 as the only plasmid species (Fig. 1, track 6). The resistance determinants of Stm 216-25 could not be transferred by a biparental mating. These data indicate that pRQ2 is a nonconjugative plasmid encoding for the multiple antibiotic resistances, except for tetracycline resistance.

Previous studies on fused replicons have shown that these are incompatible with both of their progenitors (7, 21). If pRQ6 is a cointegrate of pRQ1 and pRQ2, one would expect pRQ6 to be incompatible with pRQ1 and pRQ2 or derivatives of these. To test this, we made use of BB70, an S2050 mutant which had lost all its drug resistances and concomitantly carried plasmids with higher relative mobilities than pRQ1 and pRQ2 (Fig. 1, track 7). We designate these deleted pRQ1 and pRQ2 plasmids pRQ7 and pRQ8, respectively. This mutant allowed us to
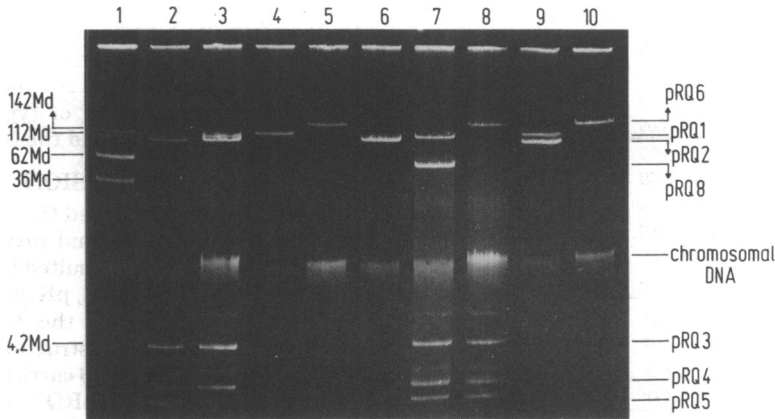
FIG. 1. *Agarose gel electrophoresis of plasmid DNA from the following strains: 2, S2050; 3, S2049; 4, BB77; 5, BB4; 6, M 216-25; 7, BB70; 8, BB75; 9, BB76-5; 10, BB170. Track 1 was loaded with the molecular weight standards TP116 (143 Md), R27 (112 Md), R1 (62 Md), RP4 (36 Md), and ColE1 (4.2 Md). The molecular weights were taken from reference 6. The designations refer to the covalently closed circular structures of the plasmids. The other visible bands represent open circular DNA.*

introduce pRQ6 by selection for multiple drug resistance. All 20 transconjugants tested were found to carry pRQ6 in addition to the three small plasmids, whereas pRQ7 and pRQ8 could not be detected (Fig. 1, track 8). This indicates that the presumed cointegrate pRQ6 displaced the resident plasmids pRQ7 and pRQ8, due to its incompatibility with both plasmids.

The stability of the presumed cointegrate structure pRQ6 was investigated by serial transfer of strain BB4 in drug-free liquid medium during about 55 generations. After plating, the antibiotic resistance pattern of 100 colonies was checked. All were identical to the parental strain BB4, except three strains. These lost their tetracycline resistance. Plasmid DNA was isolated from 10 strains, including the three tetracycline-sensitive ones. Nine of them carried a plasmid indistinguishable from pRQ6. However, one tetracycline-sensitive clone, BB76-5, carried two plasmid species with molecular weights of about 105×10^6 and 125×10^6 (Fig. 1, track 9). The multiple resistance of BB76-5 was retransferable by mating with *E. coli* strain J53-2, and the transfer frequency at 22°C was about 100-fold less efficient than that of BB4. The resulting transconjugants were found to carry a single plasmid species that comigrated again with pRQ6 (Fig. 2, track 10).

Phage inhibition by extrachromosomal elements in phage type 201. Earlier studies have shown that the phage sensitivity of bacteria can be influenced by plasmids and phages (2, 4). Previously we showed in a study on phage type 505 that the interference with propagation of typing phages is affected by the prophages and

plasmids present in the strains of this particular phage type (32). To investigate the role of the prophage and plasmids of phage type 201 in the inhibition of typing phages, we introduced the prophages Sf 117-3 and Sf 2060 and several combinations of plasmids from the strains S2050 and Stm 117-3 into Stm 1. Lysogenization of the phage-sensitive strain Stm 1 by the phages Sf 117-3 and Sf 2060 resulted in the inhibition of many of the typing phages. The phage patterns of such lysogenized strains were identical and were designated pattern 201-A (Table 6). Introduction of pRQ1, pRQ2, and pRQ6 into Stm 1 by conjugation did not affect the phage susceptibility of the host.

Plasmid pRQ3 was identified as a Phi⁻ colicin E1-encoding plasmid, because Stm 1 could be transformed by plasmid DNA from Stm 117-3 to colicinogeny. Such transformants carried pRQ3 as the only introduced plasmid, and no phage inhibition was observed. Transconjugants obtained by mobilization of the ampicillin, chloramphenicol, and kanamycin resistance determinants by the Phi⁻ transfer factor pRI470 (32) were found to contain pRQ2, pRI470, and various combinations of the small plasmids pRQ3, pRQ4, and pRQ5. These allowed us to determine the Phi properties of pRQ4 and pRQ5. Only when pRQ4 was present was phage inhibition observed, and the phage pattern obtained was designated 201-B (Table 6). The involvement of pRQ4 in the inhibition of typing phages was reinforced by the observation that elimination of pRQ4 from strain S2050 resulted in a variant, BB67, showing an increased phage susceptibility to the typing phages (phage pattern 201-C, Ta-

TABLE 6. Phage patterns of S. typhimurium observed in this study[a]

Strain	Desig-nation of phage pattern	Typing phages in routine test dilution adapted from wild-type phages:																																																Wild-type phages in routine test dilution				
		14-17																																		726			E1311									14-17											
		20	30	40	50	60	70	80	90	100	110	120	150	160	170	180	190	200	210	230	240	250	260	270	280	290	300	310	320	330	340	350	360	370	380	390	400	410	420	430	440	450	460	470	480	490	503a	510	520	530	500	501	502	503b	505
Stm 1	1	S	C	S	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	−	−	−	−	C
S2060	201	2	3	−	−	2	3	−	−	−	−	2	3	+	C	S	C	3	2	C	3	C	C	C	S	C	C	C	C	C	C	C	C	−	−	−	−	−	−	−	−	−	−	−	−	−	2	C	−	3	−	3	2	3	C
BB119	201-A	−	3	1	C	C	1	C	Sm	−	C	C	C	C	+	C	C	C	−	−	−	−	C	C	−	2	3	3	−	C	2	C	C	C	1	3	2	2	2	+	2	2	1	2	C	C	C	C	C	C	−	−	2	C	C
Stm 135-22	201-B	−	1	−	−	−	C	−	−	−	−	−	−	−	3	3	S	3	+	3	3	3	3	S	−	−	−	C	−	C	−	C	C	C	2	−	2	2	2	+	2	2	1	2	−	−	1	−	1	3	−	2	2	C	C
BB67	201-C	−	−	−	−	−	+	−	−	−	−	−	−	−	−	3	3	C	±	−	−	2	−	2	+	−	−	±	−	1	−	−	−	C	+	−	−	−	−	−	−	−	−	−	−	−	1	−	3	3	C	−	2	3	S	C

[a] C, Confluent lysis; S, semiconfluent lysis; ±, +, 1, 2, 3, increasingly numerous plaques; −, no plaques; m, minute plaques.

ble 6). Lysogenization of pRQ4-carrying Stm 1 transconjugants by phage Sf 117-3 resulted in strains with phage pattern 201. These data indicate that phage pattern 201 reflects the interference with propagation of typing phages effected by the prophage and the 3.4-Md plasmid.

DISCUSSION

In this study we observed that mobilization of the nonconjugative plasmid pRQ2 by the conjugative plasmid pRQ1 resulted in the establishment of a 230-Md plasmid, pRQ6. The following observations suggest that the 230-Md plasmid, pRQ6, is a cointegrate structur composed of pRQ1 and pRQ2. (i) pRQ6 carries the resistance genes of both pRQ1 and pRQ2. (ii) The transfer of pRQ6 was temperature dependent as was that of pRQ1. However, the temperature sensitivity was more pronounced in the case of pRQ6, which might be related to its large size. (iii) pRQ6 was found to be incompatible with both of its progenitors.

Unfortunately, our attempts to derive a physical map of pRQ6, pRQ1, and pRQ2 have not yet been successful. The digestion of these large plasmids with several restriction enzymes resulted in so many bands, that no complete separation of all fragments was achieved. Hence, we cannot yet conclude that pRQ6 carries all the DNA of pRQ1 and pRQ2. However, the data suggest that the vast majority of the DNA sequences from both parental plasmids are present (unpublished data).

The phenomenon of plasmid cointegration observed resembles Hfr formation in E. coli K-12 and S. typhimurium. This recombination has been shown to take place at sites where the chromosome and the F-factor share homology by the presence of the transposable IS or γδ-sequences (5). Cointegration has been observed in E. coli (10) and S. typhimurium between the F-lac factor and the cryptic plasmid of strain LT2 (22, 26). These cointegrates could dissociate into apparently unaltered F-lac and LT2 plasmids, although genetic rearrangements in small parts could not be ruled out by the techniques used. Previous reports (11, 18, 25) showed that nonconjugative plasmids can be mobilized by conjugative ones when the latter carry transposable DNA elements that are able to jump onto the nonconjugative plasmid. The cointegrates observed could have arisen by recombination between the small homologous regions present in both plasmids. Alternatively, transposition itself might have resulted in the formation of cointegrates. In the recently presented molecular model (28) of transposition of translocatable elements, cointegrates between donor and recipient DNA are obligatory intermediate structures in the transposition process.

Hooykaas et al. (20) described the dissociation of cointegrates leading to the formation of a parental replicon and a derivative of the second plasmid that contained the transposable DNA element. A similar phenomenon has been observed for pRQ6. The dissociation products differed about 5 Md in size from the parental plasmids pRQ1 and pRQ2. This indicates that dissociation took place at sites different from those involved in cointegrate formation. However, we did not observe the increased transfer frequency of the dissociated plasmids as described by Hooykaas et al. (20).

Stable cointegrate formation does not seem to be a prerequisite for transfer of pRQ2 because pRQ1 and pRQ2 were occasionally found separate in the transconjugants using S2050 as donor. Furthermore, no cointegration at all was found after mobilization of pRQ2 by the conjugative plasmids pRI470 and pRIL1. We cannot rule out the possibility that unstable cointegrates were involved. Interestingly, recent observations indicate that the *inc* H plasmid R27 also forms stable cointegrates with pRQ2. This suggests that plasmids of the H incompatibility groups share the capacity to form stable cointegrates.

With regard to the presence and the nature of their extrachromosomal elements, strains of phage type 201 constitute a very homogeneous group of bacteria. Also, the epidemiological data presented suggest that strains of this phage type are descended from a clone which has spread since 1972 over several European countries causing severe salmonellosis, mainly in calves. Phage type 201 strains have been obtained from Belgium and Italy also (unpublished results). Thus far, however, no isolates with this phage type have been isolated in the United Kingdom (B. Rowe, personal communication).

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