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Construction of Biologically Functional Bacterial Plasmids *In Vitro*

(R factor/restriction enzyme/transformation/endonuclease/antibiotic resistance)

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ABSTRACT **The construction of new plasmid DNA species by *in vitro* joining of restriction endonuclease-generated fragments of separate plasmids is described. Newly constructed plasmids that are inserted into *Escherichia coli* by transformation are shown to be biologically functional replicons that possess genetic properties and nucleotide base sequences from both of the parent DNA molecules. Functional plasmids can be obtained by reassociation of endonuclease-generated fragments of larger replicons, as well as by joining of plasmid DNA molecules of entirely different origins.**

Controlled shearing of antibiotic resistance (R) factor DNA leads to formation of plasmid DNA segments that can be taken up by appropriately treated *Escherichia coli* cells and that recircularize to form new, autonomously replicating plasmids (1). One such plasmid that is formed after transformation of *E. coli* by a fragment of sheared R6-5 DNA, pSC101 (previously referred to as Tc6-5), has a molecular weight of 5.8×10^6, which represents about 10% of the genome of the parent R factor. This plasmid carries genetic information necessary for its own replication and for expression of resistance to tetracycline, but lacks the other drug resistance determinants and the fertility functions carried by R6-5 (1).

Two recently described restriction endonucleases, *Eco*RI and *Eco*RII, cleave double-stranded DNA so as to produce short overlapping single-stranded ends. The nucleotide sequences cleaved are unique and self-complementary (2–6) so that DNA fragments produced by one of these enzymes can associate by hydrogen-bonding with other fragments produced by the same enzyme. After hydrogen-bonding, the 3′-hydroxyl and 5′-phosphate ends can be joined by DNA ligase (6). Thus, these restriction endonucleases appeared to have great potential value for the construction of new plasmid species by joining DNA molecules from different sources. The *Eco*RI endonuclease seemed especially useful for this purpose, because on a random basis the sequence cleaved is expected to occur only about once for every 4,000 to 16,000 nucleotide pairs (2); thus, most *Eco*RI-generated DNA fragments should contain one or more intact genes.

We describe here the construction of new plasmid DNA species by *in vitro* association of the *Eco*RI-derived DNA fragments from separate plasmids. In one instance a new plasmid has been constructed from two DNA species of entirely different origin, while in another, a plasmid which has itself been derived from *Eco*RI-generated DNA fragments of a larger parent plasmid genome has been joined to another replicon derived independently from the same parent plasmid. Plasmids that have been constructed by the *in vitro* joining of *Eco*RI-generated fragments have been inserted into appropriately-treated *E. coli* by transformation (7) and have been shown to form biologically functional replicons that possess genetic properties and nucleotide base sequences of both parent DNA species.

MATERIALS AND METHODS

E. coli strain W1485 containing the RSF1010 plasmid, which carries resistance to streptomycin and sulfonamide, was obtained from S. Falkow. Other bacterial strains and R factors and procedures for DNA isolation, electron microscopy, and transformation of *E. coli* by plasmid DNA have been described (1, 7, 8). Purification and use of the *Eco*RI restriction endonuclease have been described (5). Plasmid heteroduplex studies were performed as previously described (9, 10). *E. coli* DNA ligase was a gift from P. Modrich and R. L. Lehman and was used as described (11). The detailed procedures for gel electrophoresis of DNA will be described elsewhere (Helling, Goodman, and Boyer, in preparation); in brief, duplex DNA was subjected to electrophoresis in a tube-type apparatus (Hoefer Scientific Instrument) (0.6 × 15-cm gel) at about 20° in 0.7% agarose at 22.5 V with 40 mM Tris–acetate buffer (pH 8.05) containing 20 mM sodium acetate, 2 mM EDTA, and 18 mM sodium chloride. The gels were then soaked in ethidium bromide (5 μg/ml) and the DNA was visualized by fluorescence under long wavelength ultraviolet light ("black light"). The molecular weight of each fragment in the range of 1 to 200×10^5 was determined from its mobility relative to the mobilities of DNA standards of known molecular weight included in the same gel (Helling, Goodman, and Boyer, in preparation).

RESULTS

R6-5 and pSC101 plasmid DNA preparations were treated with the *Eco*RI restriction endonuclease, and the resulting DNA products were analyzed by electrophoresis in agarose gels. Photographs of the fluorescing DNA bands derived from these plasmids are presented in Fig. 1*b* and *c*. Only one band is observed after *Eco*RI endonucleolytic digestion of pSC101 DNA (Fig. 1*c*), suggesting that this plasmid has a single site susceptible to cleavage by the enzyme. In addition, endonuclease-treated pSC101 DNA is located at the position in the gel that would be expected if the covalently closed circular plasmid is cleaved once to form noncircular DNA of the same molecular weight. The molecular weight of the linear fragment estimated from its mobility in the gel is 5.8×10^6, in agreement with independent measurements of the size of the intact molecule (1). Because pSC101 has a single *Eco*RI cleavage site and is derived from R6-5, the equivalent DNA sequences of

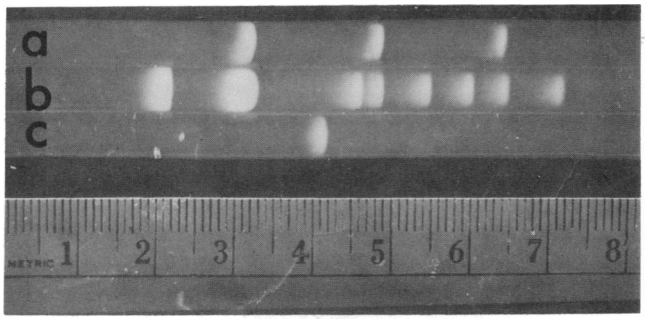


FIG. 1. Agarose-gel electrophoresis of *Eco*RI digests. (*a*) pSC102. The three fragments derived from the plasmid correspond to fragments III, V, and VIII of R6-5 (Fig. 1*b* below) as shown here and as confirmed by electrophoresis in other gels (see *text*). (*b*) R6-5. The molecular weights calculated for the fragments, as indicated in *Methods*, are (from *left* to *right*) I, 17.0; II & III (double band), 9.6 and 9.1; IV, 5.2; V, 4.9; VI, 4.3; VII, 3.8; VIII, 3.4; IX, 2.9. All molecular weight values have been multiplied by 10^{-6}. (*c*) pSC101. The calculated molecular weight of the single fragment is 5.8×10^6. Migration in all gels was from *left* (cathode) to *right;* samples were subjected to electrophoresis for 19 hr and 50 min.

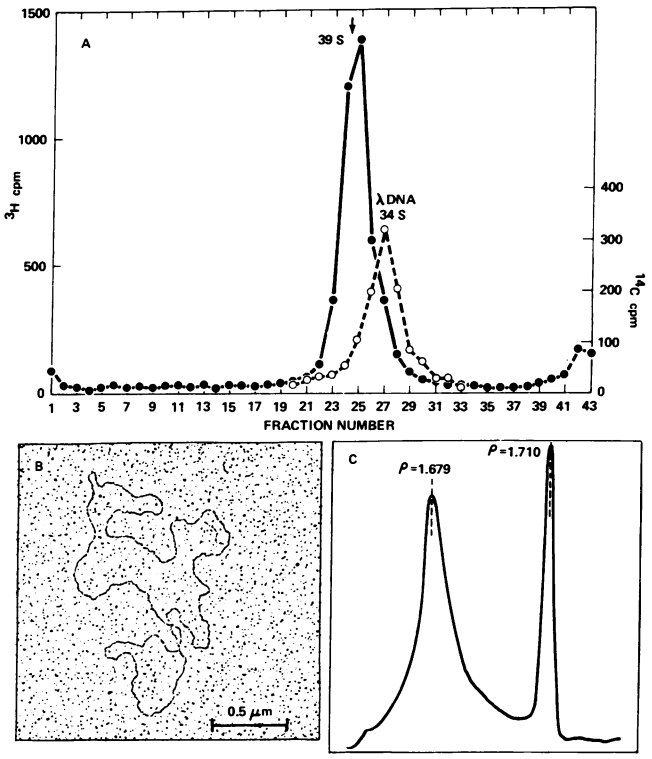


FIG. 2. Physical properties of the pSC102 plasmid derived from *Eco*RI fragments of R6–5. (*A*) Sucrose gradient centrifugation analysis (1, 8) of covalently closed circular plasmid DNA (●——●) isolated from an *E. coli* transformant clone as described in text. 34 S linear [^{14}C]DNA from λ was used as a standard (O- - -O). (*B*) Electron photomicrograph of nicked (7) pSC102 DNA. The length of this molecule is approximately 8.7 μm. (*C*) Densitometer tracing of analytical ultracentrifugation (8) photograph of pSC102 plasmid DNA. Centrifugation in CsCl (ρ = 1.710 g/cm^3) was carried out in the presence of d(A-T)$_n$·d(A-T)$_n$ density marker (ρ = 1.679 g/cm^3).

the parent plasmid must be distributed in two separate *Eco*RI fragments.

The *Eco*RI endonuclease products of R6-5 plasmid DNA were separated into 12 distinct bands, eight of which are seen in the gel shown in Fig. 1*b;* the largest fragment has a molecular weight of 17×10^6, while three fragments (not shown in Fig. 1*b*) have molecular weights of less than 1×10^6, as determined by their relative mobilities in agarose gels. As seen in the figure, an increased intensity of fluorescence, of the second band suggests that this band contains two or more DNA fragments of almost equal size; when smaller amounts of *Eco*RI-treated R6-5 DNA are subjected to electrophoresis for a longer period of time, resolution of the two fragments (i.e., II and III) is narrowly attainable. Because 12 different *Eco*RI-generated DNA fragments can be identified after endonuclease treatment of covalently closed circular R6-5, there must be at least 12 substrate sites for *Eco*RI endonuclease present on this plasmid, or an average of one site for every 8000 nucleotide pairs. The molecular weight for each fragment shown is given in the caption to Fig. 1. The sum of the molecular weights of the *Eco*RI fragments of R6-5 DNA is 61.5×10^6, which is in close agreement with independent estimates for the molecular weight of the intact plasmid (7, 10).

The results of separate transformations of *E. coli* C600 by endonuclease-treated pSC101 or R6-5 DNA are shown in Table 1. As seen in the table, cleaved pSC101 DNA transforms *E. coli* C600 with a frequency about 10-fold lower than was observed with covalently closed or nicked circular (1) molecules of the same plasmid. The ability of cleaved pSC-101 DNA to function in transformation suggests that plasmid DNA fragments with short cohesive endonuclease-generated termini can recircularize in *E. coli* and be ligated *in vivo;* since the denaturing temperature (T_m) for the termini generated by the *Eco*RI endonuclease is 5–6° (6) and the transformation procedure includes a 42° incubation step (7), it is unlikely that the plasmid DNA molecules enter bacterial cells with their termini already hydrogen-bonded. A corresponding observation has been made with *Eco*RI endonuclease-cleaved SV40 DNA, which forms covalently closed circular DNA molecules in mammalian cells *in vivo* (6).

Transformation for each of the antibiotic resistance markers present on the R6-5 plasmid was also reduced after treatment of this DNA with *Eco*RI endonuclease (Table 1). Since the pSC101 (tetracycline-resistance) plasmid was derived from R6-5 by controlled shearing of R6-5 DNA (1), and no tetracycline-resistant clone was recovered after transformation by the *Eco*RI endonuclease products of R6-5, [whereas tetracycline-resistant clones are recovered after transformation with intact R6-5 DNA (1)], an *Eco*RI restriction site may separate the tetracycline resistance gene of R6-5 from its replicator locus. Our finding that the linear fragment produced by treatment of pSC101 DNA with *Eco*RI endonuclease does not correspond to any of the *Eco*RI-generated fragments of R6-5 (Fig. 1) is consistent with this interpretation.

A single clone that had been selected for resistance to kanamycin and which was found also to carry resistance to neomycin and sulfonamide, but not to tetracycline, chloramphenicol, or streptomycin after transformation of *E. coli* by *Eco*RI-generated DNA fragments of R6-5, was examined further. Closed circular DNA obtained from this isolate (plasmid designation pSC102) by CsCl–ethidium bromide gradient

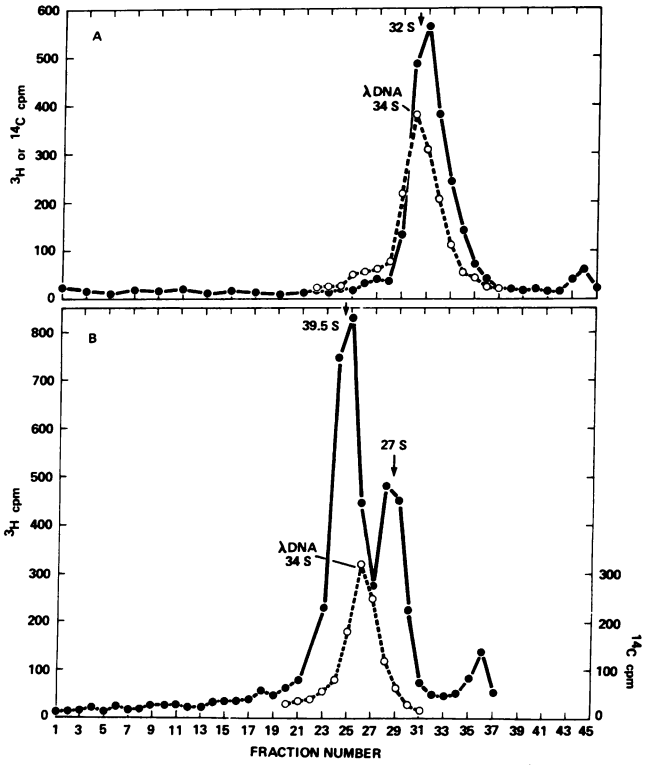


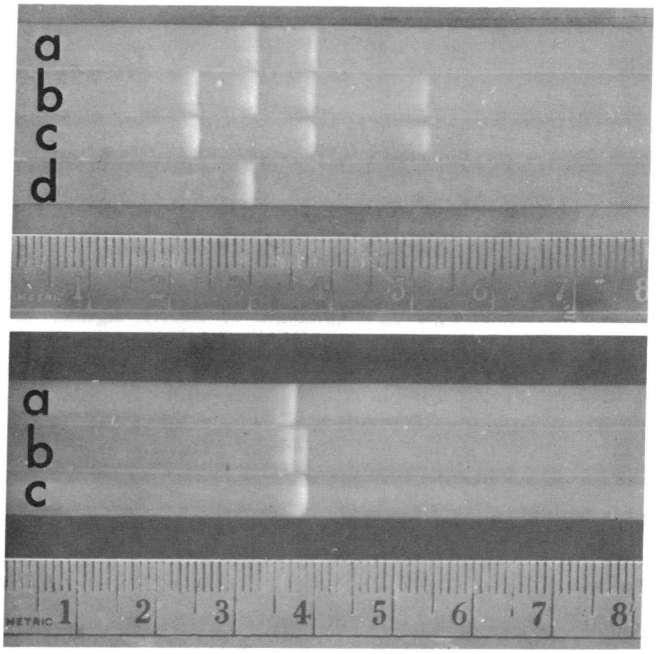
FIG. 3. Sucrose gradient centrifugation of DNA isolated from *E. coli* clones transformed for both tetracycline and kanamycin resistance by a mixture of pSC101 and pSC102 DNA. (*A*) The DNA mixture was treated with *Eco*RI endonuclease and was ligated prior to use in the transformation procedure. Covalently closed circular DNA isolated (7, 8) from a transformant clone carrying resistance to both tetracycline and kanamycin was examined by sedimentation in a neutral 5–20% sucrose gradient (8). (*B*) Sucrose sedimentation pattern of covalently closed circular DNA isolated from a tetracycline and kanamycin resistant clone transformed with an *untreated* mixture of pSC101 and pSC102 plasmid DNA.

centrifugation has an S value of 39.5 in neutral sucrose gradients (Fig. 2*A*) and a contour length of 8.7 μm when nicked (Fig. 2*B*). These data indicate a molecular weight

TABLE 1. *Transformation by covalently closed circular and EcoRI-treated plasmid DNA*

Plasmid DNA species		Transformants per μg DNA		
		Tetracycline	Kanamycin (neomycin)	Chloramphenicol
pSC101	covalently closed circle	3×10^5	—	—
	*Eco*RI-treated	2.8×10^4	—	—
R6-5	covalently closed circle	—	1.3×10^4	1.3×10^4
	*Eco*RI-treated	<5	1×10^2	4×10^1

Transformation of *E. coli* strain C600 by plasmid DNA was carried out as indicated in *Methods*. The kanamycin resistance determinant of R6-5 codes also for resistance to neomycin (15). Antibiotics used for selection were tetracycline (10 μg/ml), kanamycin (25 μg/ml) or chloramphenicol (25 μg/ml).



FIGS. 4 and 5. Agarose-gel electrophoresis of *Eco*RI digests of newly constructed plasmid species. Conditions were as described in *Methods*.

FIG. 4. (*top*) Gels were subjected to electrophoresis for 19 hr and 10 min. (*a*) pSC105 DNA. (*b*) Mixture of pSC101 and pSC102 DNA. (*c*) pSC102 DNA. (*d*) pSC101 DNA.

FIG. 5. (*bottom*) Gels were subjected to electrophoresis for 18 hr and 30 min. (*a*) pSC101 DNA. (*b*) pSC109 DNA. (*c*) RSF1010 DNA. Evidence that the single band observed in this gel represents a linear fragment of cleaved RSF1010 DNA was obtained by comparing the relative mobilities of *Eco*RI-treated DNA and untreated (covalently closed circular and nicked circular) RSF1010 DNA in gels. The molecular weight of RSF-1010 calculated from its mobility in gels is 5.5×10^6.

about 17×10^6. Isopycnic centrifugation in cesium chloride of this non-self-transmissible plasmid indicated it has a buoyant density of 1.710 g/cm^3 (Fig. 2*C*). Since the nucleotide base composition of the antibiotic resistance determinant (R-determinant) segment of the parent R factor is 1.718 g/cm^3 (8), the various component regions of the resistance unit must have widely different base compositions, and the pSC102 plasmid must lack a part of this unit that is rich in high buoyant density G+C nucleotide pairs. The existence of such a high buoyant density *Eco*RI fragment of R6-5 DNA was confirmed by centrifugation of *Eco*RI-treated R6-5 DNA in neutral cesium chloride gradients (Cohen and Chang, unpublished data).

Treatment of pSC102 plasmid DNA with *Eco*RI restriction endonuclease results in formation of three fragments that are separable by electrophoresis is agarose gels (Fig. 1*a*); the estimated molecular weights of these fragments determined by gel mobility total 17.4×10^6, which is in close agreement with the molecular weight of the intact pSC102 plasmid determined by sucrose gradient centrifugation and electron microscopy (Fig. 2). Comparison with the *Eco*RI-generated fragments of R6-5 indicates that the pSC102 fragments correspond to fragments III (as determined by long-term electrophoresis in gels containing smaller amounts of DNA), V, and VIII of the parent plasmid (Fig. 1*b*). These results suggest that *E. coli* cells transformed with *Eco*RI-generated DNA fragments of R6-5

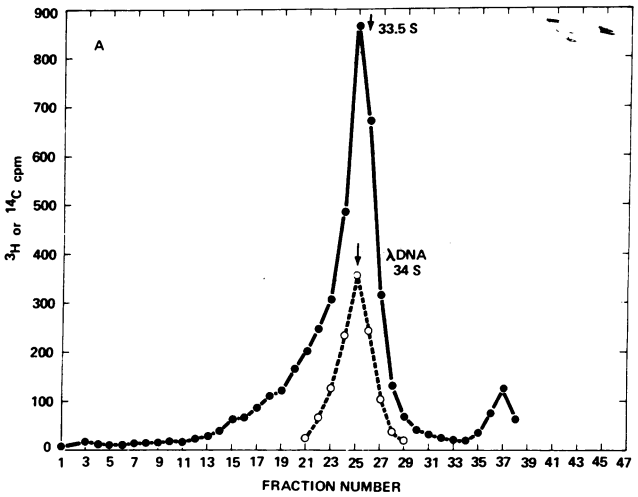


FIG. 6. Sucrose gradient sedimentation of covalently closed circular DNA representing the pSC109 plasmid derived from RSF1010 and pSC101.

can ligate reassociated DNA fragments *in vivo*, and that reassociated molecules carrying antibiotic resistance genes and capable of replication can circularize and can be recovered as functional plasmids by appropriate selection.

A mixture of pSC101 and pSC102 plasmid DNA species, which had been separately purified by dye–buoyant density centrifugation, was treated with the *Eco*RI endonuclease, and then was either used directly to transform *E. coli* or was ligated prior to use in the transformation procedure (Table 2). In a control experiment, a plasmid DNA mixture that had not been subjected to endonuclease digestion was employed for transformation. As seen in this table, transformants carrying resistance to both tetracycline and kanamycin were isolated in all three instances. Cotransformation of tetracycline and kanamycin resistance by the untreated DNA mixture occurred at a 500- to 1000-fold lower frequency than transformation for the individual markers. Examination of three different transformant clones derived from this DNA mixture indicated that each contained two separate covalently closed circular DNA species having the sedimentation characteristics of the pSC101 and pSC102 plasmids (Fig. 3*B*). The ability of two plasmids derived from the same parental plasmid (i.e., R6-5) to exist stably as separate replicons (12) in a single

TABLE 2. *Transformation of E. coli C600 by a mixture of pSC101 and pSC102 DNA*

Treatment of DNA	Transformation frequency for antibiotic resistance markers		
	Tetracycline	Kanamycin	Tetracycline + kanamycin
None	2×10^5	1×10^5	2×10^2
*Eco*RI	1×10^4	1.1×10^3	7×10^1
*Eco*RI + DNA ligase	1.2×10^4	1.3×10^3	5.7×10^2

Transformation frequency is shown in transformants per μg of DNA of each plasmid species in the mixture. Antibiotic concentrations are indicated in legend of Table 1.

bacterial host cell suggests that the parent plasmid may contain at least two distinct replicator sites. This interpretation is consistent with earlier observations which indicate that the R6 plasmid dissociates into two separate compatible replicons in *Proteus mirabilis* (8). Cotransformation of tetracycline and kanamycin resistance by the *Eco*RI treated DNA mixture was 10- to 100-fold lower than transformation of either tetracycline or kanamycin resistance alone, and was increased about 8-fold by treatment of the endonuclease digest with DNA ligase (Table 2). Each of four studied clones derived by transformation with the endonuclease-treated and/or ligated DNA mixture contained only a single 32S covalently closed circular DNA species (Fig. 3*A*) that carries resistance to both tetracycline and kanamycin, and which can transform *E. coli* for resistance to both antibiotics. One of the clones derived from the ligase-treated mixture was selected for further study, and this plasmid was designated pSC105.

When the plasmid DNA of pSC105 was digested by the *Eco*RI endonuclease and analyzed by electrophoresis in agarose gels, two component fragments were identified (Fig. 4); the larger fragment was indistinguishable from endonuclease-treated pSC101 DNA (Fig. 4*d*) while the smaller fragment corresponded to the 4.9×10^6 dalton fragment of pSC102 plasmid DNA (Fig. 4*c*). Two endonuclease fragments of pSC102 were lacking in the pSC105 plasmid; presumably the sulfonamide resistance determinant of pSC102 is located on one of these fragments, since pSC105 does not specify re-

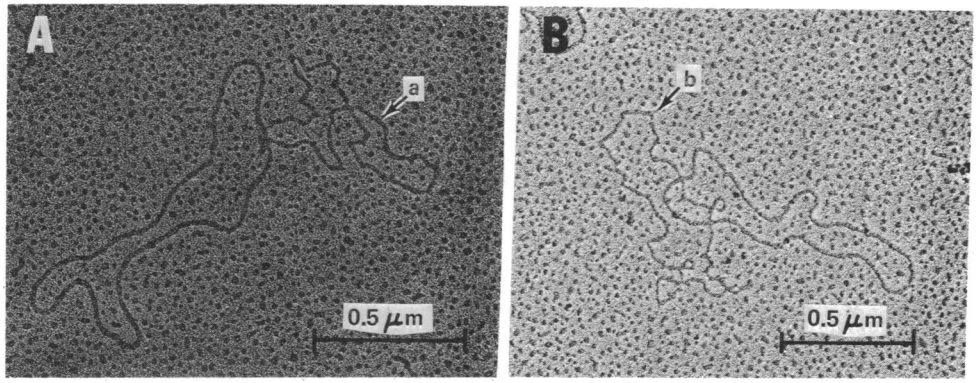


FIG. 7. (*A*) Heteroduplex of pSC101/pSC109. The single-stranded DNA loop marked by *a* represents the contribution of RSF1010 to the pSC109 plasmid. (*B*) Heteroduplex of RSF1010/pSC109. The single-stranded DNA loop marked by *b* represents the contribution of pSC101 to the pSC109 plasmid. pSC101 and RSF1010 homoduplexes served as internal standards for DNA length measurements. The scale is indicated by the bar on each electron photomicrograph.

sistance to this antibiotic. Since kanamycin resistance *is* expressed by pSC105, we conclude that this resistance gene resides on the 4.9×10^6 dalton fragment of pSC102 (fragment V of its parent, R6-5). The molecular weight of the pSC105 plasmid is estimated to be 10.5×10^6 by addition of the molecular weights of its two component fragments; this value is consistent with the molecular weight determined for this recombinant plasmid by sucrose gradient centrifugation (Fig. 3*A*) and electron microscopy. The recovery of a biologically functional plasmid (i.e., pSC105) that was formed by insertion of a fragment of another plasmid fragment into pSC101 indicates that the *Eco*RI restriction site on pSC101 does not interrupt the genetic continuity of either the tetracycline resistance gene or the replicating element of this plasmid.

We also constructed new biologically functional plasmids *in vitro* by joining cohesive-ended plasmid DNA molecules of entirely different origin. RSF1010 is a streptomycin and sulfonamide resistance plasmid which has a 55% G+C nucleotide base composition (13) and which was isolated originally from *Salmonella typhimurium* (14). Like pSC101, this nonself-transmissible plasmid is cleaved at a single site by the *Eco*RI endonuclease (Fig. 5*c*). A mixture of covalently closed circular DNA containing the RSF1010 and pSC101 plasmids was treated with the *Eco*RI endonuclease, ligated, and used for transformation. A transformant clone resistant to both tetracycline and streptomycin was selected, and covalently closed circular DNA (plasmid designation pSC109) isolated from this clone by dye–buoyant density centrifugation was shown to contain a single molecular species sedimenting at 33.5 S, corresponding to an approximate molecular weight of 11.5×10^6 (Fig. 6). Analysis of this DNA by agarose gel electrophoresis after *Eco*RI digestion (Fig. 5*b*) indicates that it consists of two separate DNA fragments that are indistinguishable from the *Eco*RI-treated RSF1010 and pSC101 plasmids (Fig. 5*a* and *c*).

Heteroduplexes shown in Fig. 7*A* and *B* demonstrate the existence of DNA nucleotide sequence homology between pSC109 and each of its component plasmids. As seen in this figure, the heteroduplex pSC101/pSC109 shows a double-stranded region about 3 μm in length and a slightly shorter single-stranded loop, which represents the contribution of RSF1010 to the recombinant plasmid. The heteroduplex formed between RSF1010 and pSC109 shows both a duplex region and a region of nonhomology, which contains the DNA contribution of pSC101 to pSC109.

SUMMARY AND DISCUSSION

These experiments indicate that bacterial antibiotic resistance plasmids that are constructed *in vitro* by the joining of *Eco*RI-treated plasmids or plasmid DNA fragments are biologically functional when inserted into *E. coli* by transformation. The recombinant plasmids possess genetic properties and DNA nucleotide base sequences of both parent molecular species. Although ligation of reassociated *Eco*RI-treated fragments increases the efficiency of new plasmid formation, recombinant plasmids are also formed after transformation by *unligated Eco*RI-treated fragments.

The general procedure described here is potentially useful for insertion of specific sequences from prokaryotic or eukaryotic chromosomes or extrachromosomal DNA into independently replicating bacterial plasmids. The antibiotic resistance plasmid pSC101 constitutes a replicon of considerable potential usefulness for the selection of such constructed molecules, since its replication machinery and its tetracycline resistance gene are left intact after cleavage by the *Eco*RI endonuclease.

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