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The innovative methodology described in this paper, along with the discovery of the Haemophilus influenzae restriction enzyme by Smith and Wilcox (see above), set the stage for recombinant DNA technology as we know it today. Cohen and colleagues were the first to construct a recombinant plasmid. They showed that ligation of two EcoRI-digested DNA fragments from separate antibiotic resistance plasmids resulted in a functional hybrid replicon when transformed into Escherichia coli C600. Furthermore, Cohen et al. showed that the recombinant plasmids retained the genetic nucleotides from both of the parental DNA sources. It should be pointed out that the demonstration of nucleotide sequence homology between parent and recombinant plasmid DNA was a much harder undertaking in 1973 than it is today. Indeed, to provide such proof, Cohen and colleagues had to show by electron microscopic analyses that heteroduplex formation occurred between pSC109 and each of its component plasmids. The Southern hybridization method and techniques to sequence segments of DNA were not published until 1975 and 1977, respectively (E. Southern, J. Mol. Biol. 98:503–517, 1975; F. Sanger, S. Nicklen, and A.R. Coulson, Proc. Natl. Acad. Sci. USA 74:5463–5467, 1975; and A. M. Maxam and W. Gilbert, Proc. Natl. Acad. Sci. USA 74:560–564, 1977). The process of cleaving DNA with an enzyme, joining the restricted fragments to form a plasmid, transforming the recombinant plasmid into a bacterial host, and permitting growth of the transformants with subsequent amplification of the recombinant plasmid, constitutes the bread and butter of molecular-cloning technology. As one of our committee members asked about the methods described in this paper, “What technique could have possibly had a bigger single impact on how we do molecular analyses of microbes today?”

Alison O’Brien

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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 reassocation 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 × 10⁶, 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, EcoRI and EcoRRI, 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 EcoRI 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 EcoRI-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 EcoRI-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 EcoRI-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 EcoRI-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 sulfanamide, 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 EcoRI 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 gels 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 (0.5 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 × 10⁶ 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 EcoRI 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. 1b and c. Only one band is observed after EcoRI endonucleolytic digestion of pSC101 DNA (Fig. 1c), 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 × 10⁶, in agreement with independent measurements of the size of the intact molecule (1). Because pSC101 has a single EcoRI cleavage site and is derived from R6-5, the equivalent DNA sequences of
the parent plasmid must be distributed in two separate EcoRI fragments.

The EcoRI endonuclease products of R6-5 plasmid DNA were separated into 12 distinct bands, eight of which are seen in the gel shown in Fig. 1b; the largest fragment has a molecular weight of $17 \times 10^6$, while three fragments (not shown in Fig. 1b) have molecular weights of less than $1 \times 10^6$, 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 EcoRI-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 EcoRI-generated DNA fragments can be identified after endonuclease treatment of covalently closed circular R6-5, there must be at least 12 substrate sites for EcoRI 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 EcoRI fragments of R6-5 DNA is $61.5 \times 10^6$, 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 pSC101 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 EcoRI 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 EcoRI 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 EcoRI 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 EcoRI endonuclease products of R6-5, whereas tetracycline-resistant clones are recovered after transformation with intact R6-5 DNA (1), an EcoRI 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 EcoRI endonuclease does not correspond to any of the EcoRI-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 EcoRI-generated DNA fragments of R6-5, was examined further. Closed circular DNA obtained from this isolate (plasmid designation pSC102) by CsCl-ethidium bromide gradient
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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 EcoRI 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. 2A) and a contour length of 8.7 μm when nicked (Fig. 2B). These data indicate a molecular weight 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. 2C). 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 EcoRI fragment of R6-5 DNA was confirmed by centrifugation of EcoRI-treated R6-5 DNA in neutral cesium chloride gradients (Cohen and Chang, unpublished data).

Treatment of pSC102 plasmid DNA with EcoRI restriction endonuclease results in formation of three fragments that are separable by electrophoresis in agarose gels (Fig. 1a); 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 EcoRI-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. 1b). These results suggest that E. coli cells transformed with EcoRI-generated DNA fragments of R6-5

<p>| Table 1. Transformation by covalently closed circular and EcoRI-treated plasmid DNA |
|---------------------------------|------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Plasmid species</th>
<th>Transformants per μg DNA</th>
<th>Kanamycin (neomycin)</th>
<th>Chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSC101 covalently closed circle</td>
<td>3 × 10^6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EcoRI-treated</td>
<td>—</td>
<td>2.8 × 10^6</td>
<td>—</td>
</tr>
<tr>
<td>R6-5 covalently closed circle</td>
<td>&lt;5</td>
<td>1.3 × 10^4</td>
<td>1.3 × 10^4</td>
</tr>
<tr>
<td>EcoRI-treated</td>
<td>—</td>
<td>4 × 10^4</td>
<td>—</td>
</tr>
</tbody>
</table>

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).
can ligate reassocciated DNA fragments in vivo, and that re-associated 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 EcoRI 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. 3B). The ability of two plasmids derived from the same parental plasmid (i.e., R6-5) to exist stably as separate replications (12) in a single 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 replications in Proteus mirabilis (8). Cotransformation of tetracycline and kanamycin resistance by the EcoRI 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. 3A) 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 EcoRI 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. 4d) while the smaller fragment corresponded to the 4.9 × 10⁶ dalton fragment of pSC102 plasmid DNA (Fig. 4e). 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-
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We 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 non-self-transmissible plasmid is cleaved at a single site by the EcoRI endonuclease (Fig. 5c). A mixture of covalently closed circular DNA containing the RSF1010 and pSC101 plasmids was treated with the EcoRI 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 EcoRI digestion (Fig. 5b) indicates that it consists of two separate DNA fragments that are indistinguishable from the EcoRI-treated RSF1010 and pSC101 plasmids (Fig. 5a and c).

Heteroduplexes shown in Fig. 7A 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 EcoRI-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 EcoRI-treated fragments increases the efficiency of new plasmid formation, recombinant plasmids are also formed after transformation by unligated EcoRI-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 EcoRI endonuclease.

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