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Utilization of Two Distinct Modes of Replication by a Hybrid Plasmid Constructed *In Vitro* from Separate Replicons

(R-plasmids/DNA replication/restriction endonuclease/molecular cloning)

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ABSTRACT **A hybrid plasmid, pSC134, that codes for two distinct sets of replication functions has been constructed *in vitro* by ligation of *Eco*RI endonuclease-cleaved pSC101 and Col E1 plasmid replicons, and has been introduced into *Escherichia coli* by transformation. The replication properties of the pSC134 plasmid in DNA polymerase I-defective mutants or in the presence of chloramphenicol indicate that this hybrid plasmid can utilize the functionally distinct modes of replication specified by both of its parent replicons.**

Current information suggests that replication of various extrachromosomal DNA molecules (plasmids) in *Escherichia coli* may involve at least two distinct modes (1–3). Replication of the colicin E1 (Col E1) and cloacin DF13 plasmids and minicircular DNA from *E. coli* strain 15 does not require *de novo* protein synthesis, and is strictly dependent upon DNA polymerase I (4–9). In contrast, other replicons such as the fertility plasmid F, the antibiotic resistance plasmid R1, the Lemolysin plasmid Hly, and the colicin plasmids Col V and Col Ib have DNA synthesis requirements that closely parallel those of the *E. coli* chromosome; normal replication of these extrachromosomal elements can occur in DNA polymerase I-deficient cells, but requires continued protein synthesis (8, 10, 11).

Although *in vivo* recombination between different bacterial plasmids has been well documented (e.g., ref. 12), little is known about the functional interrelationships and mutual compatability of separate and possibly distinct types of replication mechanisms that may co-exist on a single plasmid molecule (13). Yet, information about the functions of such recombinant replicons is of fundamental importance to an understanding of plasmid evolution. The present report describes certain replication properties of a hybrid plasmid that has been constructed *in vitro* (14) by the joining of two different *Eco*RI restriction endonuclease-cleaved plasmid replicons. The plasmids Col E1 and pSC101 (15) were used as parent replicons in these experiments, since these plasmids have clearly distinguishable replication characteristics, and insertion of DNA at the single *Eco*RI cleavage site of each plasmid does not interfere with its replication (14, 16). Our results indicate that the pSC101–Col E1 constructed hybrid plasmid exists as a stable replicon in *E. coli*, where it can utilize the replication functions specified by *both* of its parent plasmid molecules.

Abbreviations: Col, colicin plasmid; *Eco*RI, the restriction endonuclease produced by *E. coli* strain RY13; Tc, tetracycline; Cm, chloramphenicol; ^{r}, resistant to specified drug; CCC-DNA, covalently closed circular DNA.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. SC181 is *E. coli* C600 r_K^--m_K^- (17); SC293 (TS214, ref. 9), kindly provided by D. R. Helinski, is a Thy$^-$ Mal$^+$ Col E1–K30 derivative of JC411 (His$^-$, *argA*, *metB*, Leu$^-$, Xyl$^-$, *lacY*, *strA*) that produces a temperature-sensitive DNA polymerase I (PolA$_{ts214}$). SC294 is a heat-cured Col$^-$ derivative of SC293. Col E1 was transferred to SC181 by conjugation with *E. coli* HfrH (Col E1), kindly provided by W. Goebel. Colicin E1-immune transconjugants were selected on nutrient agar containing 5 units/ml of colicin E1, which was isolated and assayed as previously described (18–20). Other plasmids were transferred into SC181 and SC294 by transformation (21). Covalently closed circular DNA was isolated by cesium chloride–ethidium bromide centrifugation of detergent lysates prepared from plasmid-containing cells essentially as described by Clewell and Helinski (22). *Eco*RI restriction endonuclease (23) was purified from *E. coli* strain RY13 (24) (kindly provided by H. W. Boyer) through the phosphocellulose column chromatography step (24). *E. coli* DNA ligase (25) was the generous gift of S. Panasenko, P. Modrich, and I. R. Lehman. The procedures used for construction of hybrid plasmids *in vitro* (14) and transformation of *E. coli* by plasmid DNA (21) have been described. Transformants were selected on nutrient agar plates containing tetracycline (20 μg/ml) and colicin E1 (5 units/ml). Analysis of fragments generated by *Eco*RI endonuclease by electrophoresis in 0.7% agarose slab gels has been described (26).

Sucrose gradient analysis of plasmid DNA was carried out in 5–20% neutral gradients containing 20 mM Tris·HCl, pH 8.0, 1 mM EDTA, and 1 M NaCl, as described previously (15). The internal sedimentation marker usually included in such gradients was ^{14}C-labeled Col D-CA23 DNA, which was isolated from *E. coli* K12 strain W3110 Sm^r(Col D-CA23). The observed sedimentation coefficient of 21 S for covalently closed circular Col D DNA is consistent with its molecular weight of 3.1×10^6, as determined by electron microscopy (K. Timmis and F. Cabello, unpublished data).

RESULTS

Construction of the Col E1-pSC101 Hybrid Plasmid. Covalently closed circular Col E1 and pSC101 plasmid DNA samples were separately cleaved to completion with *Eco*RI endonuclease, were mixed and ligated, and the ligation mixture was used to transform *E. coli* strain SC181. Since insertion of a DNA segment at the single *Eco*RI cleavage site of Col E1 interferes with colicin biosynthesis (16), transformants that expressed both tetracycline resistance (Tc^r) and

colicin E1 immunity were additionally screened for failure to produce colicin. Of 20 Tc^r, E1-immune clones that were examined, five failed to produce colicin. Covalently closed circular DNA (CCC-DNA) samples obtained from such isolates, or alternatively from colicin-producing bacteria that concurrently expressed both tetracycline resistance and colicin E1 immunity, were examined by agarose gel electrophoresis.

One representative sample of DNA (DNA A) (Fig. 1) obtained from a Tc-resistant colicin-immune, colicin-producing clone contains two separate CCC-DNA species that are indistinguishable from the circular pSC101 and Col E1 plasmids, indicating that this clone had been doubly transformed by separate plasmid replicons. Moreover, the increased intensity of fluorescence of the Col E1 DNA band relative to the pSC101 band, despite the smaller size of the Col E1 plasmid, suggested independent replication of the two DNA species. A second representative sample of DNA (DNA B), isolated from a clone which *failed* to produce colicin, but which expressed colicin immunity as well as tetracycline resistance, consists of a single circular plasmid species which is larger (i.e., migrates more slowly through the gel) than either of the two parent plasmids.* This plasmid DNA is cleaved by the *Eco*RI endonuclease into separate fragments having the size of linear pSC101 and Col E1 DNA. The relative intensity of fluorescence of these fragments is roughly proportional to the molecular weights of the pSC101 and Col E1 plasmids, suggesting that the two parent DNA species are represented in equimolar amounts in the hybrid plasmid (plasmid designation pSC134).

Sucrose gradient centrifugation of pSC134 DNA (Fig. 1, bottom) confirms the above interpretations. The CCC form of pSC134 plasmid DNA has a sedimentation coefficient of 32 S, which corresponds to a molecular weight of 10×10^6. The hybrid plasmid thus contains one copy each of the Col E1 fragment (molecular weight 4.2×10^6) and of the pSC101 plasmid fragment (molecular weight 5.8×10^6).

Transformation of *E. coli* by *Eco*RI-cleaved and lighted pSC134 DNA permitted recovery of plasmids which were indistinguishable from the pSC101 and Col E1 parent molecules.

The Col E1–pSC101 Hybrid Plasmid Can Replicate by Means of the Col E1 Replication Functions. A major difference in the replication characteristics of the pSC101 and Col E1 plasmids involves their respective requirements for protein synthesis; Col E1 replicates extensively in the presence of chloramphenicol (Cm) (relaxed replication†; ref. 4) whereas pSC101, like chromosomal DNA, does not (stringent replication control†; F. Cabello, unpublished observations). Accordingly, any replication of the pSC134 hybrid plasmid in the presence of Cm should occur by means of only the Col E1 replication functions. *E. coli* cells of strain SC181 (pSC134) grown to exponential phase in the presence of [^{14}C]thymidine, were

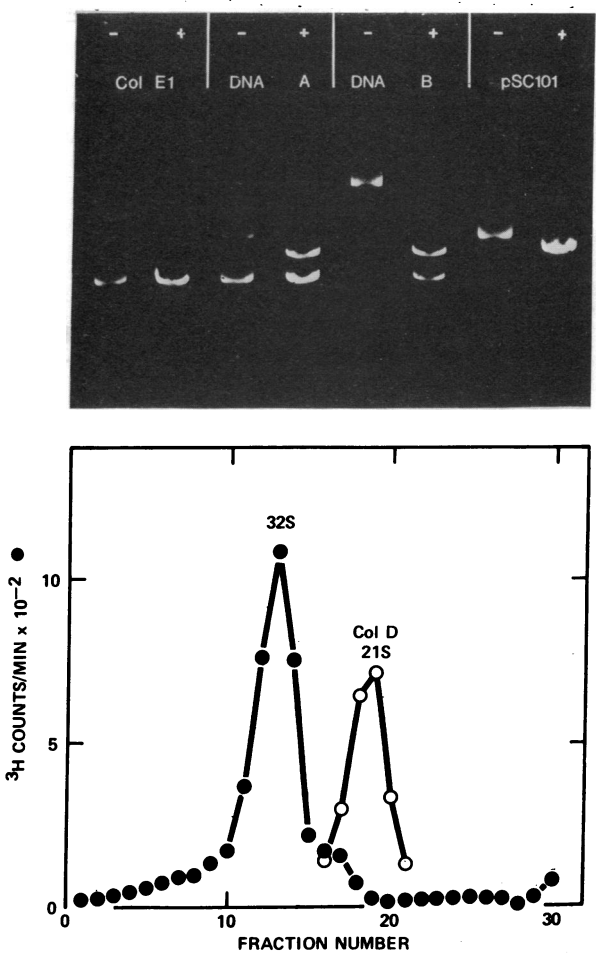

FIG. 1. Physical characterization of pSC134. (Top) Agarose gel electrophoresis analysis of plasmid DNA from two transformants. The Col E1 and pSC101 plasmids, and CCC-DNA (DNA A and DNA B) isolated from two bacterial clones that had been cotransformed for both Tc-resistance and colicin E1 immunity by a ligated mixture of *Eco*RI-treated Col E1 and pSC101 DNA, were separately cleaved by the endonuclease, as indicated in *text*. Reactions (50 μl) were terminated by the addition of 15 μl of a solution containing 5% sodium dodecyl sulfate, 25% glycerol, and 0.025% bromophenol blue. The mixtures were applied to a gel containing 0.7% agarose in TB buffer, pH 8.3 (0.09 M Tris; 2.5 mM EDTA; 0.09 M boric acid; ref. 26) and electrophoresis was carried out in a slab gel apparatus (31) at 50 mA and 100 V for about 150 min. Similar mixtures, but lacking *Eco*RI enzyme, were used as controls. The gel was stained for 30 min in TB containing 5 μg/ml of ethidium bromide, and the fluorescing bands which were visualized by long-wave ultraviolet light were photographed through a Wratten no. 8 yellow filter. Plus and minus signs refer to the presence or absence of endonuclease in each mixture. (Bottom) Sedimentation velocity centrifugation of pSC134. pSC134 (40 μl; ●) and Col D reference DNA (5 μl; ○) were applied to a 5-ml linear 5–20% sucrose gradient and centrifuged at 40,000 rpm for 120 min at 20° in the SW50.1 rotor. Fractions were collected and their radioactivity was measured as indicated in *Materials and Methods*.

* Agarose gel electrophoresis of uncleaved DNA is a general and rapid method of distinguishing recombinant DNA from separate plasmids present in doubly transformed bacteria. The procedure is particularly useful for this purpose when the ligation step may not be efficient (e.g., where DNA concentrations are low) or when one of the required DNA fragments cannot be selected in the transformation step.

† As used here, the terms *relaxed* and *stringent* do not apply to plasmids *per se*, but only the behavior of a plasmid in a particular environment. We consider replication of a plasmid to be stringently regulated under the particular conditions prevailing if its duplication is approximately coupled, directly or indirectly, with chromosome duplication. Conversely, relaxed replication is characterized by an increase in the ratio of plasmid to chromosomal DNA. Replication of any given plasmid may be either relaxed or stringent under different experimental conditions. Our definition assumes that copy number and mode of regulation of replication are independent characteristics.

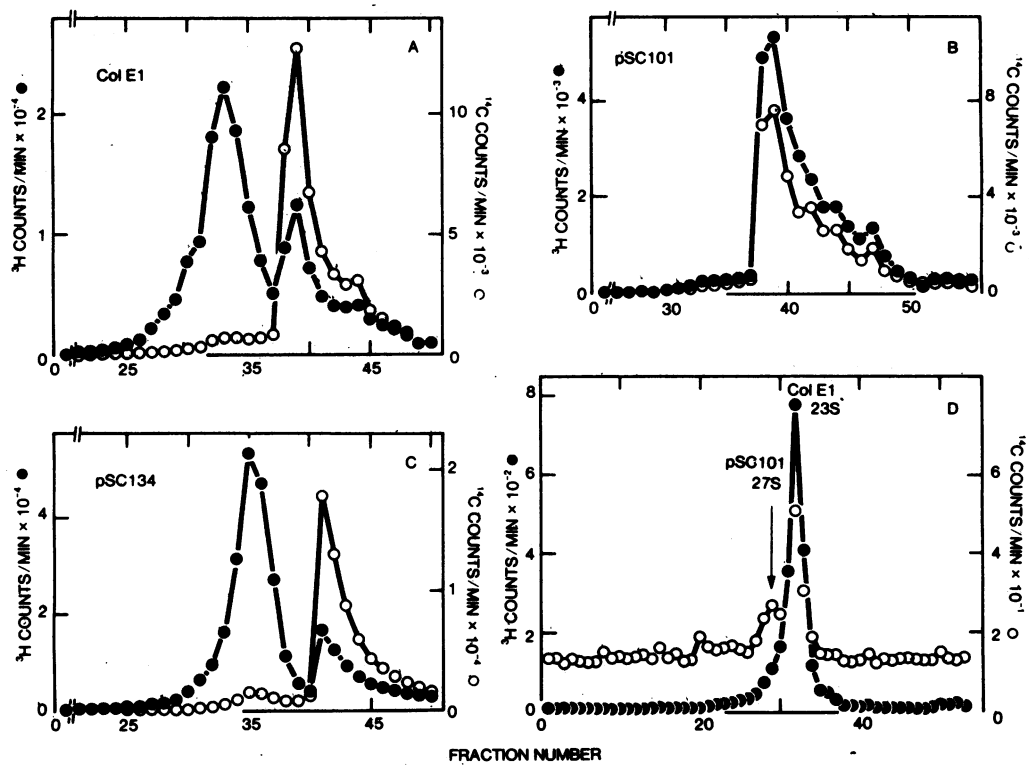

FIG. 2. Effect of chloramphenicol on the replication of Col E1, pSC101, and pSC134. Bacterial strains SC181 (Col E1), SC181 (pSC101) SC181 (pSC134), and the doubly transformed isolate referred to in Fig. 1A, SC181 (Col E1, pSC101), were grown in 25-ml cultures from a cell density of 1×10^7/ml to 2×10^8/ml at 37° in minimal glucose–casamino acids medium (32) containing 250 μg/ml of deoxyadenosine, 40 μg/ml of thymidine, and 1 μCi/ml of [2-^{14}C]thymidine. The cells were washed, resuspended in fresh medium containing 180 μg/ml of Cm but lacking radioactive thymidine, and were incubated again at 37° for 180 min. At this point 10 μCi/ml of [*methyl*-^{3}H]thymidine was added and incubation was continued for 60 min. Incorporation was stopped by addition of 20 ml of ice-cold TE (Tris·HCl 10 mM, pH 8.0; EDTA 1 mM) containing 20 mM KCN. Cells were washed once, resuspended in 2 ml of cold TE–KCN buffer, and lysed at 0° by the addition of 50 μl of 10 mg/ml of lysozyme and 50 μl of 10% Sarkosyl. The lysates were sheared by agitation for 5 sec by means of a vortex mixer; 6 ml of TE–KCN, 1 ml of 10 mg/ml of ethidium bromide, and 8 g of CsCl were added and the mixture was centrifuged at 40,000 rpm for 40 hr in the Beckman 50Ti rotor. Fractions were subsequently collected and the radioactivity in 50-μl aliquots was measured on filters as indicated in *Materials and Methods*. A, B, and C show the centrifugation profiles of Col E1, pSC101, and pSC134, respectively; density increases are from right to left. In the case of SC181 (Col E1, pSC101), the closed circular DNA was isolated from the CsCl–ethidium bromide gradient, and the two plasmid DNA species were resolved by sedimentation for 4 hr through a 12.4-ml 5–20% sucrose gradient at 38,000 rpm in an SW41 rotor (D).

washed, treated for 3 hr with Cm (180 μg/ml) and were then "pulse" labeled for 1 hr with [^{3}H]thymidine. The bacteria were lysed with Sarkosyl, and the lysate was centrifuged to equilibrium in a CsCl-ethidium bromide density gradient. Cells carrying either Col E1 or pSC101, or carrying *both* plasmids concurrently, were used as controls.

As shown by Fig. 2 (A–C), 1–3% of the DNA isolated from bacteria carrying the pSC134 hybrid plasmid or either of its parent plasmids is in the closed circular form, when such cells are grown in the absence of Cm (^{14}C plots). Addition of Cm resulted in markedly increased incorporation of radioactive label into CCC-DNA in cells containing either the Col E1 (Fig. 2A) or pSC134 (Fig. 2C) plasmids; however, little replication of pSC101 plasmid DNA was observed in the presence of Cm (Fig. 2B) (^{3}H plots). Moreover, CCC-DNA synthesized in the presence of Cm by the bacterial strain carrying the hybrid plasmid was entirely 32S material as determined by sucrose gradient analysis (experiment not shown), indicating that replication of both the Col E1 and pSC101 segments of the pSC134 molecule occurs in Cm-treated cells.

Sucrose gradient analysis of plasmid CCC-DNA isolated from a bacterial clone that carries independent pSC101 and Col E1 replicons is shown in Fig. 2D. As shown by this figure, in the presence of Cm, negligible incorporation of radioactive label into the 27S pSC101 DNA was observed. Since this species comprised about 28% of the circular DNA synthesized in this doubly transformed clone in the *absence* of the antibiotic, these results suggest that co-existence of a replicating Col E1 plasmid in Cm-treated cells does not provide in *trans* a substitute for the Cm-sensitive component of the pSC101 replication machinery. Thus, replication of the pSC101 segment of the pSC134 plasmid in the presence of Cm must occur by means of a function provided *cis* by the Col E1 segment. The simplest interpretation of these results is that, under conditions precluding pSC101 replication, the entire hybrid molecule is replicated as a unit by the Col E1 replication machinery.

The Hybrid Plasmid Can Also Utilize the pSC101 Replication Functions. Since the Col E1 plasmid is unable to replicate in bacterial cells that lack functional DNA polymerase I (8), any replication of the pSC134 hybrid plasmid in DNA polymerase I-deficient bacteria must necessarily depend on the pSC101 replication machinery. In order to determine whether this machinery can accomplish replication of the entire

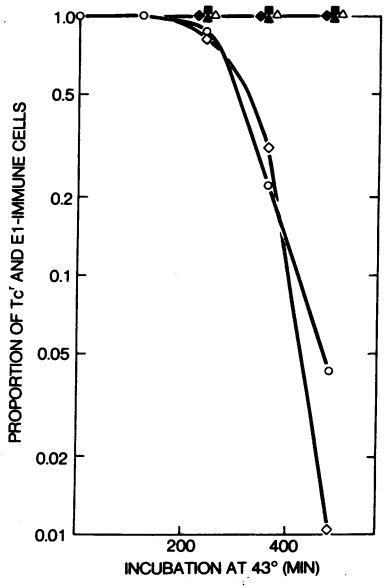

FIG. 3. Lack of segregation at high temperature of pSC134 by cells that produce a temperature-sensitive DNA polymerase I. SC293 (Col E1) (○), SC294 (pSC101) (■), SC294 (pSC134) (△, ▲), and SC294 (pSC101, Col E1) (◇, ◆) were grown at 32° in L-broth (33) containing 100 μg/ml of thiamine, 0.2% glucose, 3 μg/ml of thymine, and 100 μg/ml of trypsin (9) to a cell density of 6×10^8/ml. They were then diluted to 1×10^7/ml with prewarmed fresh medium and incubation at 43° was continued. At 0, 120, 240, 360, and 480 min after the shift to 43°, samples were removed and dilutions were plated on nutrient agar. After incubation of the plates for 36 hr at 32°, those containing between 60 and 120 colonies were replica-plated onto nutrient agar containing 20 μg/ml of Tc (filled symbols) or 5 units/ml of colicin E1 (open symbols). Clones on the master (original) plate that failed to grow on Tc-or colicin E1-agar were assumed to have lost pSC-101 or Col E1, respectively.

pSC134 plasmid under conditions that prevent Col E1 DNA synthesis, we have studied maintenance of the hybrid plasmid in a bacterial host carrying a temperature-sensitive *polA* mutation. As seen in Fig. 3, neither the pSC101 nor pSC134 plasmid was lost from cells of strain SC294 ($PolA_{ts}$) when such cells were grown for an extended period at 43°. In contrast, under the same conditions, the Col E1 plasmid was lost from 95% of host cells that carried this plasmid, whether or not an autonomous pSC101 replicon was present concurrently. Since loss of Col E1 at 43° occurs in cells that carry both the autonomous Col E1 and pSC101 replicons, we conclude that the observed continued maintenance of the pSC134 plasmid at high temperature is not the result of a function provided *trans* by the pSC101 component of the hybrid molecule.

Additional support for this interpretation is provided by the Cm sensitivity of replication of the hybrid plasmid at 43°. If replication of the pSC134 plasmid at 43° in a temperature-sensitive PolA host resulted from use of residual DNA polymerase I (9) by the Col E1 replication apparatus, such replication would be expected to be Cm-*in*sensitive. Alternatively, if the replication at 43° is controlled by the pSC101 replication machinery, this replication should be inhibited by the antibiotic. As seen in Fig. 4, replication of the pSC134 hybrid plasmid in the $PolA_{ts}$ host (i.e., SC294) occurs in the presence of Cm and is relaxed at 32°, but does not occur to any significant extent at 43°. Since relaxed replication of the

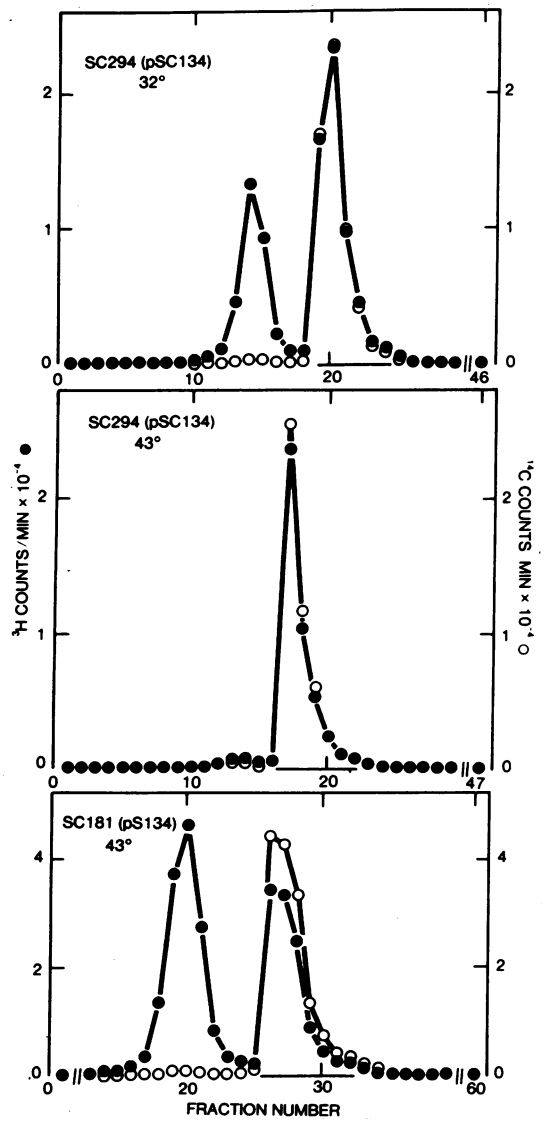

FIG. 4. Chloramphenicol inhibits pSC134 replication at high temperature in cells that produce a temperature-sensitive DNA polymerase I. *E. coli* strains SC294 (pSC134) and SC181 (pSC-134) were grown at 32° in L-broth containing 100 μg/ml of thiamine, 0.2% glucose, 3 μg/ml of thymidine and 1 μCi/ml of [2-^{14}C]thymidine (plus 250 μg/ml of deoxyadenosine, in the case of SC181) to a cell density of 2×10^8/ml. The cells were washed and resuspended in fresh medium containing Cm (180 μg/ml), but lacking radioactive thymidine. The culture was then divided into two parts, which were incubated for 2 hr at either 43° or 32°. Both parts were then labeled for 1 hr with 10 μCi/ml of [*methyl*-^{3}H]thymidine. The cells were lysed and the DNA analyzed by centrifugation in CsCl–ethidium bromide, as described in Fig. 2. For brevity, only the 43° plot for SC181 (pSC134) is shown. The 32° plot for this bacterial host was similar to the 32° plot observed for SC294 (pSC134).

pSC134 plasmid takes place at 43° in a wild-type host (strain SC181, Fig. 4 bottom), it appears that the observed Cm sensitivity of replication at high temperature in strain SC294 is a specific consequence of inactivation of the thermosensitive DNA polymerase I. Thus, replication of the pSC134 hybrid plasmid in the DNA polymerase I-deficient mutant is accomplished by the replication functions of its pSC101 component fragment.

DISCUSSION

Interference with expression of an identifiable genetic function of a plasmid by insertion of a DNA fragment of its *Eco*RI cleavage site is shown here to be a useful method for specific selection of conjoint molecules. In these experiments, all isolated clones that were concurrently Tc-resistant, colicin-immune, and colicin-producing contained independently-replicating pSC101 and Col E1 plasmids, whereas all similarly resistant and immune clones that *failed* to produce colicin contained hybrid plasmids. The general method for detection of molecular hybrids by inactivation of genes at an endonuclease cleavage site, which has been used here for isolation of the pSC134 plasmid, is potentially applicable for cloning various prokaryotic or eukaryotic DNA species that lack genetic and molecular properties suitable for their selection. The fortuitous location of the *Eco*RI cleavage site of Col E1 in a gene that is not essential for plasmid replication, and that expresses a readily detectable property (i.e., colicin production), makes this plasmid suitable for use in the selection of conjoint molecules by *insertional inactivation.*

The results reported here indicate that a hybrid plasmid containing two sets of operationally competent replication functions can be maintained stably in bacterial cells. Both the Col E1 and pSC101 replication mechanisms are capable of functioning in the hybrid plasmid: replication of the pSC134 molecular hybrid continues when replication of its pSC101 component segment is inhibited by chloramphenicol; alternatively, pSC134 can utilize its pSC101 fragment to replicate the entire hybrid plasmid when Col E1 replication is prevented, as in bacteria lacking a functional DNA polymerase I. When the hybrid plasmid utilizes pSC101 functions for replication, such replication is chloramphenicol sensitive.

Although we have shown that both sets of replication functions in the hybrid are operationally competent, it is not presently known whether normal replication involves both or one of the component replication systems of the plasmid, and whether such replication has a single or double origin.

Earlier studies carried out in a number of laboratories (27–30) have shown that certain naturally occurring plasmids consist in *E. coli* of cointegrated replicons, which can dissociate into separate autonomous plasmids in other bacterial hosts. Moreover, two structurally distinct compatible replicons have been isolated by cleavage of DNA from one of these plasmids (i.e., R6-5, ref. 14). However, it is not known whether both of the component replication systems of such plasmids are capable of functioning when they exist as part of a single plasmid molecule. Our present finding that a constructed hybrid plasmid can possess two distinct sets of replication functions implies that recombinant plasmids containing more than one set of replication functions may exist in nature. Moreover, the ability of the pSC134 hybrid plasmid to use alternative replication machinery when the normal functioning of one of its replication systems is prevented suggests a need for caution in interpreting the replication requirements of (possibly recombinant) naturally occurring plasmids in various DNA synthesis-defective mutant or in the presence of metabolic inhibitors.

Little is presently understood about factors that regulate the functional interrrelationships between different replication systems that may co-exist on a single plasmid molecule as a consequence of naturally occurring recombinational events. Such information appears to have important implications for an understanding of plasmid evolution, plasmid host-range relationships, and plasmid incompatibility. Hybrid plasmids constructed *in vitro* by the joining of separate, well-characterized replicons appear to be useful tools for studying these questions.

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