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Indirect Selection of Bacterial Plasmids Lacking Identifiable Phenotypic Properties

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A procedure is described that uses an indicator plasmid (pSC201) to identify cells in a bacterial population that have been co-transformed with a second plasmid lacking detectable phenotypic properties. Under appropriate conditions of indirect selection, between 50 and 85% of transformants carrying the indicator plasmid also contain the nonselected plasmid. A temperature-sensitive mutation in the replication functions of the indicator plasmid enables its elimination from doubly transformed bacteria. Using this procedure, we have isolated bacteria that carry only the small cryptic plasmid, P15A, of the *Escherichia coli* strain 15. This genetic element, which contains only 2,300 nucleotide pairs, is thus capable of functioning as a replicon independently of the two larger plasmids normally associated with it in *E. coli* 15 strains (Ikeda, Inuzuka, and Tomizawa, 1970).

Although it has been estimated that 50% of all naturally occurring strains of *Enterobacteriacae* may contain extrachromosomal replicons (9), studies have been restricted largely to those plasmids that express easily selectable or identifiable properties such as antibiotic resistance, colicin immunity, or conjugal fertility. Extrachromosomal elements that lack identifiable traits have been called "cryptic plasmids"; although some of these, such as the cryptic plasmid of *Salmonella typhimurium* LT2, are large enough to contain more than 200 genes (25), the only evidence of their genetic expression is a capacity for autonomous replication in bacterial cells. In part, the paucity of information about such plasmids is a consequence of the inability to introduce them experimentally into mutant bacterial strains suitable for particular genetic investigations and to propagate them separately from other plasmids that may coexist with them in their natural host.

This communication describes the novel use of a plasmid deoxynucleic acid (DNA) transformation procedure (5) to introduce replicons that lack selectable genetic properties into bacteria. The principle of indirect selection applied in these experiments was suggested during earlier investigations (4) involving co-transformation of *Escherichia coli* by two separate plasmid DNA species; in those studies, it was observed (unpublished data) that concurrent transformation of a bacterial cell population by a selected and an unselected plasmid occurred at a higher frequency than would be expected from the frequency of independent transformation by the two separate plasmids. Our subsequent investigations, which are reported here, have shown that particular cells in a $CaCl_2$-treated bacterial population become preferentially competent for transformation by plasmid DNA. Thus, a selectable plasmid can be used as an indicator or probe to identify cells that have also acquired a second plasmid, which otherwise would be nonselectable.

The current experiments have also employed the plasmid DNA transformation procedure for isolation of an indicator plasmid that is temperature sensitive (ts) in a replication function (Rep) which allows it to be eliminated easily from doubly transformed cells. The method used for the isolation of ts replication mutants (i.e., transformation of bacteria with mutagenized plasmid DNA) allows the cloning of single molecules of plasmid DNA, and thus is suitable for rapid isolation of a wide variety of recessive mutants of multicopy plasmids that previously could not be easily obtained. Together, the mutagenized ts indicator plasmid and the indirect selection procedure enable the propagation in an appropriate bacterial host of any transformable *E. coli* plasmid replicon lacking selectable phenotypic properties. They have been used here to isolate biologically the small cryptic plasmid (6) from *E. coli* strain 15, and to demonstrate that this minicircular genetic element, which contains only 2,300 nucleotide base pairs, carries the functions required for it to exist as an autonomous replicon.

MATERIALS AND METHODS

Most media, bacterial strains, and plasmids used in this study have been described previously (2-5). The ampicillin (Ap) resistance plasmid RSF1030 (5.5 $\times$ 10^6 daltons) was obtained from S. Falkow (11), the plasmid R6K was obtained from R. C. Clowes (18), and the *E. coli* TAU-bar strain from A. Ganesan (10). Mutagenesis of an *E. coli* C600 strain carrying the pSC105 plasmid (4) was accomplished using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine at a concentration of 100 μg/ml as described by Miller (20). This treatment produced 1% mutation of the strain to the galactose phenotype (as determined on MacConkey galactose plates [Difco]) and resulted in 40% survival of bacteria. The procedures employed for bacterial growth in L broth (24), isolation of covalently closed circular (CCC) plasmid DNA by detergent lysis and dye-buoyant density centrifugation (26, 27), transformation of *E. coli* by plasmids (5), isolation and use of the *Eco*RI restriction endonuclease (3, 8) and the *E. coli* DNA ligase (4), analysis of endonuclease-generated DNA fragments by electrophoresis in 0.8% agarose gels (3, 12, 23), and sucrose gradient centrifugation of plasmid DNA (26, 27) have been described. The *E. coli* DNA ligase was generously provided by S. Panasenko, P. Modrich, and I. R. Lehman (21).

^{3}H-labeled CCC DNA (containing the P15A, P15B, and P15C plasmids [13]) of *E. coli* TAU-bar was obtained from stationary-phase cells grown in minimal glucose-Casamino Acids medium (7), containing 15 μg of uracil per ml, 3 μg of thymine per ml, 250 μg of deoxyadenosine per ml, and 8 μCi of [^{3}H]thymidine per ml. Total CCC DNA was isolated by detergent lysis and dye-buoyant density centrifugation (26, 27), and the P15A cryptic DNA was purified from this CCC DNA by preparative sucrose gradient centrifugation (SW41 rotor, 5 h, 39,000 rpm).

RESULTS

Isolation of ts replication mutant as an indicator plasmid. The pSC105 plasmid, which was previously constructed in vitro by linking a kanamycin (Km) resistance fragment of a large plasmid to the tetracycline (Tc) resistance plasmid pSC101 (2,4) was mutagenized with *N*-methyl-*N*-nitro-*N*-nitrasoguanidine. After 30-min of treatment with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine at 32 C, the mutagenized culture was sedimented, washed twice with an equal volume of sodium potassium phosphate buffer (0.1 M, pH 7.0), and used to inoculate 250 ml of L broth. Bacteria were grown for 16 h at 32 C to stationary phase; from these cells, CCC pSC105 plasmid DNA was isolated and used to transform *E. coli* cells at a concentration of 0.5 μg of DNA per 5 $\times$ 10^9 calcium chloride-treated bacteria. After the 42 C heat-pulse DNA uptake step of the transformation procedure (5), the mix was diluted 10-fold into L broth and incubated for 2 h at 32 C. Aliquots (0.1 ml) of cultures diluted to yield approximately 10^2 transformants per plate were spread on nutrient agar plates containing both Tc (20 μg/ml) and Km (20 μg/ml) and incubated at 32 C.

After sufficient incubation to allow the appearance of small colonies (approximately 16 h), the culture plates were shifted to a 45 C oven where they were incubated for an additional 24 h to permit enlargement of any colonies capable of growth at this temperature. Each colony that remained small was toothpicked onto two Tc-Km plates; one plate was incubated at 32 C and the other at 45 C to test for temperature sensitivity of growth in the presence of antibiotics. Single colony isolates of ts clones were tested for temperature sensitivity of resistance to each antibiotic, and those showing concurrent temperature sensitivity to both of them were considered to be putative ts replication mutants. However, all single colony isolates of clones showing any ts characteristics were toothpicked from 32 C plates onto nutrient agar plates lacking antibiotics and grown at 45 C for 24 h. Bacteria from the edge of the resulting colonies were purified at 32 C on nutrient agar plates (no antibiotics) and tested for the ability to grow on each antibiotic at this temperature. The conversion of initially antibiotic-resistant colonies to drug sensitivity by growth of the clone at 45 C in the absence of the drugs was taken as a further indication of a ts defect in plasmid replication.

Of 5,500 transformed clones examined, 1,000 smaller colonies were toothpicked to Tc-Km plates at 32 and 45 C. Of 16 colonies showing ts growth characteristics on antibiotic medium, six were temperature sensitive for only Tc resistance (tsTc^R) and eight were tsKm^R. Two were temperature sensitive for both Tc and Km resistance, and the resistance properties of these clones were lost by growth at 45 C. One of these bacterial clones was designated SC331, and the putative ts Rep mutant plasmid it carried (which had been derived from the pSC105 plasmid) was called pSC205.

The pSC105 plasmid contains two *Eco*RI endonuclease-generated fragments, the larger of which corresponds to the pSC101 plasmid (4). The second fragment, which carries Km resistance, has not been found capable of autonomous replication (Chang and Cohen, unpublished data, and V. Hershfield, personal communication). The replicator fragment of the ts Rep pSC205 plasmid was isolated by *Eco*RI digestion of pSC205 DNA. The digested DNA was diluted 10-fold to a concentration of 6.0 μg/ml to

reduce intermolecular hydrogen bonding of the nuclease-generated fragments and treated with the *E. coli* ligase. The resulting mixture was used to transform *E. coli* strain C600 for Tc resistance at 32 C. Twenty Tc-resistant, Km-sensitive transformants were isolated, and all were found to have the ts Rep phenotype that had been observed for the pSC205 plasmid. The plasmid isolated from one of these transformant bacterial clones (designated SC332) was called pSC201. The plasmid DNA of pSC201 and pSC205 had molecular properties indistinguishable from the pSC101 and pSC105 plasmids, respectively, as determined by sucrose gradient analysis and agarose gel electrophoresis (results not shown).

The rate of loss of the ts Rep pSC201 and pSC205 plasmids from *E. coli* at 45 C is shown in Fig. 1. For both plasmids, the number of antibiotic-resistant bacteria equalled the total number of cells for approximately four generations; presumably, this initial period of stability of resistance represents the time required to dilute out the approximately six to eight copies (our laboratory, unpublished data) of the plasmids initially present in each bacterial cell. Once these multiple copies had segregated, inhibition of plasmid replication at 45 C was indicated by a rapid decrease in the frequency of antibiotic resistance cells. However, the slope of the segregation curve in this period was less steep than the theoretical segregation curve for a plasmid that is totally inhibited in its replication (see also reference 15).

Genetic and molecular evidence for selective loss of the pSC201 replicon at 45 C. An *E. coli* C600 clone containing a plasmid (pSC102) which carries resistance to Km, neomycin, and sulfonamides (4) was transformed to Tc resistance with pSC201 DNA at 32 C. Bacteria of the resulting clone, which contained both the pSC102 and pSC201 replicons, were grown to log phase and diluted to 10^4 cells/ml in L broth lacking antibiotics. The cells were grown to late stationary phase (5×10^9 cells/ml) at 32 or 45 C, and both cultures were assayed at 32 C for total cell count on nutrient plates, Km resistance (specified by the pSC102 plasmid) and Tc resistance (specified by the pSC201 plasmid). All cells grown at 32 C were resistant to both Km and Tc, but whereas all cells from the 45 C culture were Km resistant, only 0.05% of cells expressed Tc resistance, suggesting that preferential loss of the pSC201 plasmid occurred at 45 C. One colony was selected from each 32 and 45 C nutrient agar assay plate and was grown overnight in L broth in the presence of [^{3}H]thymidine. Plasmid DNA extracted from these cells was analyzed on neutral sucrose gradients (Fig. 2). As seen in this figure, the colony from the 32 C culture contained two DNA peaks having sedimentation properties characteristic of CCC DNA of the pSC201 (26*S*) and pSC102 (37*S*) plasmids, whereas the DNA of the 45 C colony showed only a single peak characteristic of the pSC102 plasmid.

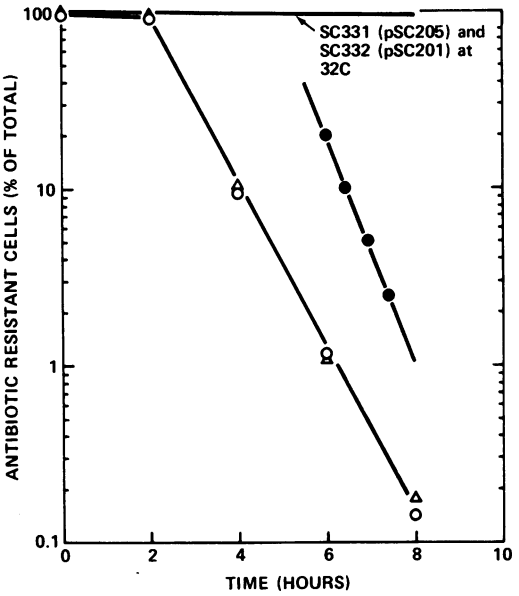

FIG. 1. *Segregation kinetics of pSC205 and pSC201 plasmids. E. coli C600 transformant clones SC331 and SC332 containing the pSC205 and pSC201 plamids, respectively, were incubated for 16 h in the presence of 20 μg each of Tc and Km per ml for pSC205, and 20 μg of Tc per ml for pSC201. These cultures were diluted 1:20 into L broth, and grown for 2 h at 32 C, and then each was diluted at zero time to approximately 10^4 cells/ml into two flasks containing 5 ml L broth prewarmed at 32 and 45 C. Samples (0.1 ml) from each flask were assayed at 2-h intervals for total cells (on nutrient agar plates) and for antibiotic-resistant cells (on Tc-Km plates for pSC205, and on Tc plates for pSC201). Results are plotted as percentage of antibiotic-resistant cells versus time. The percentage of resistant cells in cultures incubated at 32 C remained essentially constant (95 to 100%), and individual points are not shown above. The generation time of cells incubated at 32 C was 37 min, and was 28 min for the cells incubated at 45 C. Symbols: Δ, SC331 at 45 C; O, SC332 at 45 C; ●, theoretical segregation curve if replication is completely inhibited at 45 C. This curve was determined by considering a 50% decrease in the proportion of cells containing plasmid resistance markers per generation time (28 min). To avoid confusion with the actual data, this curve is displaced to the right of the measured curves.*

Indirect selection using the pSC201 indica-

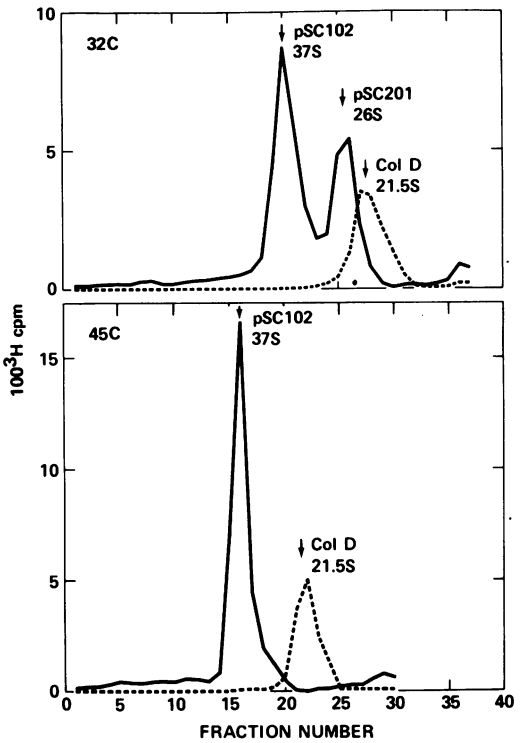

FIG. 2. *Molecular evidence for selective loss of pSC201 plasmid at 45 C. E. coli C600 cells containing pSC201 and pSC102 were incubated overnight in L broth at 32 and 45 C and assayed on nutrient agar plates. A single colony from each 32 and 45 C nutrient agar assay plate was inoculated into 250 ml of L broth containing 3 μCi of [^{3}H]thymidine per ml. After 16 h of incubation at 32 C, cells were harvested and lysed with Brij detergent, and CCC DNA of the cleared lysate was isolated by cesium chloride-ethidium bromide centrifugation as indicated in Materials and Methods, applied to a 5-ml linear 5 to 20% sucrose gradient, and centrifuged at 40,000 rpm for 100 min at 20 C in a Beckman SW50.1 rotor. A 10-μl volume of ^{14}C-labeled ColD DNA (21.5S, D. J. Kopecko, personal communication) was added to each gradient. Upper CCC DNA (30 μl) originating from 32 C assay plate; lower CCC DNA (50 μl) originating from 45 C assay plate.*

tor plasmid. Experimental conditions suitable for indirect selection were determined using the Tc resistance plasmid pSC201 and the Ap resistance plasmid RSF1030. Mixtures of these two DNA species in various ratios were used to transform $CaCl_2$-treated *E. coli* C600 cells, but only Tc resistance was used in the selection of transformants at 32 C. Tc-resistant transformants were examined for the frequency of concurrent acquisition of resistance to Ap and for the temperature sensitivity of each resistance by inoculating the clones onto plates containing either Ap or Tc and incubating both types of plates at 32 and 45 C. As shown in Table 1, when molar ratios of pSC201 to RSF1030 were 10^{-3} or less, more than half of the Tc-resistant transformants also acquired the Ap resistance of the RSF1030 plasmid. The doubly transformed cells showed resistance to Ap at both 32 and 45 C, but the resistance to Tc was expressed at only 32 C. Additional indirect selection experiments carried out with the plasmids pSC102 (17×10^6 daltons) (4) and R6K (26×10^6 daltons) (11) (Table 2) demonstrated the effectiveness of the procedure for replicons other than the RSF1030 plasmid.

Indirect selection of the P15A cryptic plasmid. *E. coli* strain 15 carries at least three distinct plasmids. The P15A cryptic plasmid present in this strain is the smallest extrachromosomal replicon yet identified, having a molecular weight of 1.5×10^6 (6, 13). A larger cryptic plasmid, P15C (13), and a third plasmid which specifies restriction and modification functions (11) are also present. A major purpose of the present experiments was the isolation of the small cryptic plasmid of *E. coli* strain 15 for further study. Because this bacterial strain also expresses a chromosomally directed type A restriction and modification system (1), an r^- mutant of *E. coli* strain C600 (3) was used as a recipient in our transformation experiments with the P15A plasmid.

Indirect selection for the P15A plasmid was carried out in a manner similar to that described above for the RSF1030, pSC102, and R6K plasmids. The molar ratio of pSC201 DNA to P15A DNA was 4×10^{-4}. Nine randomly chosen Tc-resistant transformant clones were purified on Tc plates (two streakings) and the plasmid DNA was isolated and examined by agarose gel electrophoresis. Six of the nine clones contained the small cryptic plasmid, as well as the pSC201 plasmid, while the remaining three clones carried only the indicator pSC201 replicon. An electrophoresis pattern for a clone of each type is shown in Fig. 3.

A purified colony of a clone carrying both plasmids was grown to stationary phase at 32 or 45 C in L broth and the contents of each flask were assayed on nutrient and Tc agar plates at 32 C. As expected, equal numbers of colonies from the 32 C flask were observed on both kinds of plates, but only 0.1% of the total colonies from the 45 C flask were Tc resistant. Two colonies from the nutrient agar plates, one originating from the 32 C flask and one from the 45 C flask, were randomly chosen and their CCC DNA was examined by centrifugation in neutral sucrose gradients (Fig. 4). As can be seen in this

TABLE 1. *Use of pSC201 plasmid for indirect selection of bacteria transformed by RSF1030 plasmid DNA*[a]

DNA (μg) per 5 × 10⁸ cells		Approx molar ratio[b] (pSC201/RSF1030)	% Indirect selection[c]		
RSF1030	pSC201		Expt 1	Expt 2	Expt 3
5×10^{-1}	5×10^{-1}	1	2.7 (74)[d]	10 (88)	
5×10^{-1}	5×10^{-2}	10^{-1}	3.3 (61)	6.3 (94)	
5×10^{-1}	5×10^{-3}	10^{-2}	20 (60)	32 (63)	
5×10^{-1}	5×10^{-4}	10^{-3}	53 (32)	39 (61)	85 (64)
5×10^{-1}	5×10^{-5}	10^{-4}	56 (34)	39 (53)	68 (117)
5×10^{-1}	5×10^{-6}	10^{-5}			50 (22)
1.0	1×10^{-5}	10^{-5}	71 (7)	66 (24)	

[a] Transformation frequencies observed were: pSC201 (5×10^5/μg of DNA), RSF1030 (5×10^5/μg of DNA).
[b] Molecular weights: pSC201, 5.8×10^6 (shown in text to be equivalent to pSC101 [2]); RSF1030, 5.5×10^6 (11).
[c] Percentage of indirect selection indicates the percentage of Tc^R transformants (at 32 C) that had also acquired the Ap resistance of the RSF1030 plasmid.
[d] Parentheses indicate number of transformant colonies tested for each percentage shown.

TABLE 2. *Indirect selection for plasmids pSC102 and R6K*

Plasmid	Mol wt data	Approx molar ratio[a]	% Indirect selection[b]
pSC102	17	3×10^{-2}	24 (63)
		3×10^{-3}	37 (26)
		10^{-3}	32 (151)
R6K	26	2×10^{-3}	28 (35)

[a] Molecular weights used to calculate molar ratios (with respect to pSC201) were: pSC201, 5.8×10^6 (see Table 1); pSC102, 17×10^6 (4); R6K, 26×10^6 (18).
[b] Percentage of indirect selection indicates the proportion of Tc-resistant transformants that were also Km resistant for pSC102 transformation, or Ap and streptomycin resistant for R6K. The parentheses indicate the number of colonies tested for calculation of percentages. Transformants per microgram of DNA were: pSC201, (5×10^5); pSC102, (3×10^5); R6K, (2×10^4).

figure, the colony from the 32 C nutrient plate contained two separate plasmid DNA species, sedimenting at 26 *S* (pSC201, Fig. 2) and 17*S* (P15A) (19), whereas the 45 C colony contained only the 17*S* cryptic plasmid.

DISCUSSION

A major problem associated with the isolation of mutants of multicopy plasmids has been that any mutated DNA molecules must be segregated from nonmutant copies present in the same cell in order for phenotypic effects of the mutation to be detected. In the case of plasmids such as F, this may be achieved after a few rounds of replication (14). However, in the case of multicopy plasmids such as Col E1, complex experimental procedures may be required to select mutant plasmids such as those having temperature-sensitive replication properties (17). Since the plasmid DNA transformation procedure permits the cloning of individual DNA molecules, it enables propagation of a line of bacterial cells expressing a recessive plasmid mutation initially present on a single plasmid DNA molecule. Transformation of bacteria by plasmid DNA isolated from mutagenized bacteria also provides a method of ensuring that the phenotypic property being assessed is a consequence of a mutation in the plasmid, and not in the chromosome.

The observations reported here indicate that particular cells in a $CaCl_2$-treated bacterial population are preferentially competent for transformation. These bacteria are likely to acquire multiple plasmids, even when no selection is carried out for the phenotypic properties of some of the species. In addition, these experiments define a simple procedure that now permits selection of any plasmid replicon capable of being transformed into relevent bacterial cells. We have shown that the indirect selection procedure is effective for both oligocopy (e.g., pSC102) and multicopy (e.g., R6K) plasmids, and that it can be used for plasmids having sizes that extend over at least a 5-fold range. By using a low ratio of indicator plasmid DNA to DNA of the plasmid being selected indirectly in the transformation procedure, it is possible to ensure that a very high percentage of bacterial cells transformed by the indicator plasmid will also acquire the second replicon. In various experiments, DNA ratios of 10^{-3} to 10^{-5} were successful in yielding a frequency of indirect selection sufficiently high to permit convenient detection of the indirectly selected plasmids by examination of DNA from randomly chosen bacterial clones that expressed the properties of the indicator plasmid.

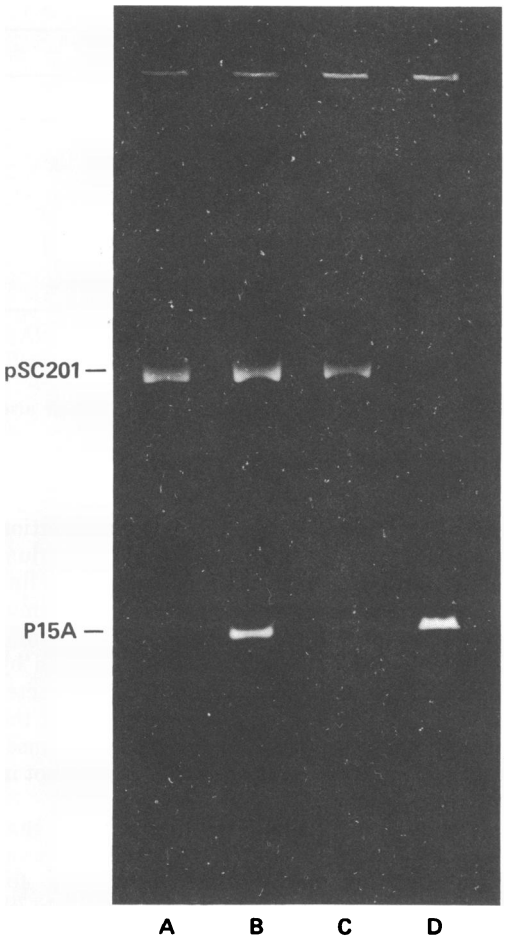

FIG. 3. *Agarose gel analysis of CCC DNA of Tc-resistant transformants. Indirect selection for cells containing the P15A plasmid was carried out as described in the text. Nine Tc-resistant clones were purified and used to inoculate 250 ml of L broth. After overnight incubation at 32 C (stationary-phase cells), the CCC DNA was isolated after lysis with 1% Triton X (16) and dye-bouyant density centrifugation. The CCC DNA was analyzed by electrophoresis in 0.8% agarose gels; three clones contained only pSC201 plasmid DNA while six clones contained P15A plasmid DNA in addition to the pSC201 plasmid species. (A) Agarose gel electrophoresis of CCC DNA from a clone containing only pSC201 DNA; (B) electrophoresis of CCC DNA from a clone containing pSC201 and P15A plasmid species; (C) and (D) represent gel electrophoresis of control DNA species, pSC201 (C) and P15A (D).*

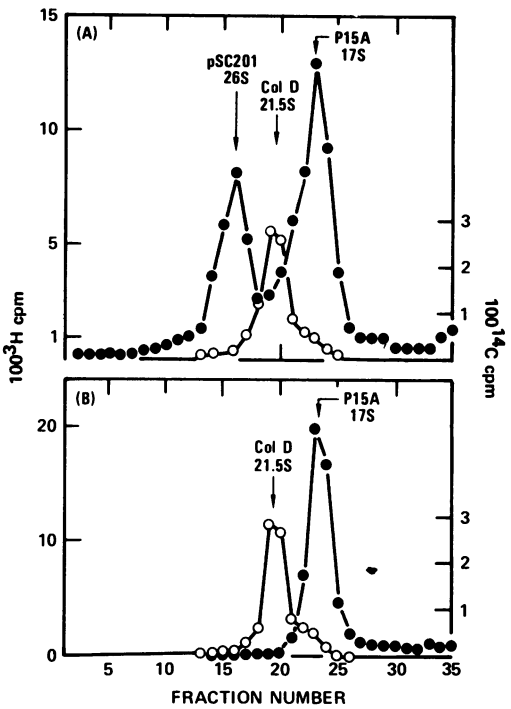

FIG. 4. *Sucrose gradient analysis of CCC DNA of colonies resulting from growth of a strain containing pSC201 and P15A at 32 and 45 C. A doubly transformed clone containing pSC201 and P15A was grown for 16 h in L broth at 32 and 45 C and assayed on nutrient agar plates (see text). Two colonies, one from the 32 C and one from the 45 C nutrient plate, were each inoculated into 250 ml of L broth containing 3 μCi of [^{3}H]thymidine per ml. After 16 h of growth at 32 C, the stationary-phase cells were harvested and lysed with 1% Triton X (16), and CCC DNA was isolated by dye-bouyant density centrifugation. The CCC DNA was analyzed by centrifugation in a 5 to 20% sucrose gradient in an SW50.1 rotor for 150 min at 39,000 rpm. A 10-μl volume of ^{14}C-labeled ColD DNA (21.5S, D. J. Kopecko, personal communication) was used as reference marker. Top curve (A) is CCC DNA originating from a colony incubated at 32 C. Bottom curve (B) is CCC DNA originating from a colony incubated at 45 C.*

Although we initially determined the appropriate experimental conditions for indirect selection by use of a second plasmid that expressed phenotypic traits that could be identified in the transformed cells, the procedure is suitable for the selection and biological isolation of cryptic plasmids. The P15A cryptic plasmid has been of special interest because it is the smallest segment of DNA known to be capable of autonomous replication in *E. coli* (6). Because this extrachromosomal element coexists with two other plasmids in its natural host bacterial strain (13), it could not be determined previously whether the plasmid, which can contain enough genetic information to code for only two to four proteins, requires genes residing on the coexisting replicons for it to exist in the plasmid state.

Purification of the P15A plasmid by indirect

selection, and propagation of a line of cells carrying only this replicon, demonstrated that this 2,300-nucleotide-long DNA segment is competent for autonomous replication. The ability to introduce the P15A plasmid into particular mutant strains of *E. coli* by indirect selection now permits elucidation of functional interactions between this plasmid and the chromosomal genes involved in bacterial DNA replication. It should also facilitate identification of specific plasmid gene products involved in the replication of extrachromosomal elements.

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