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Isolation, by Tetracycline Selection, of Small Plasmids Derived from R-Factor R12 in *Escherichia coli* K-12

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The examination, by agarose gel electrophoresis, of tetracycline-resistant colonies of *Escherichia coli* K-12 carrying R-factor R12 reveals the presence of smaller plasmid deoxyribonucleic acids (DNAs), incompatible with R12, in many of the clones. These plasmids are demonstrated to be homologous with R12 DNA by electron microscope heteroduplex experiments and by the production of consistent fragment patterns upon digestion with various restriction endonucleases. These autonomously replicating plasmids form a related series of covalently closed circular DNA molecules ranging in size from 3.6×10^6 to 61×10^6 daltons. Plasmids of molecular weight between 3.6×10^6 and 37×10^6 confer no antibiotic resistances, but when jointly present with R12 they nonetheless enhance the expression of the tetracycline resistance associated with this latter molecule.

Drug resistance factors are plasmid or episomal deoxyribonucleic acid (DNA) molecules that are maintained in a wide variety of bacterial types and contain one or more genes specifying products that confer resistance to antimicrobial agents. Antibiotic factor (R-factor) R12 was directly derived from R-factor NR1 by a mating between *Proteus mirabilis* Pm15/NR1 and *Escherichia coli* W677 with selection on streptomycin (SM) (11). These two plasmid DNA molecules are of the same molecular weight and confer the same resistances but otherwise differ in several respects (11). Specifically, R12 has three to four times as many copies per bacterial chromosome as NR1; R12 is more readily isolated in the form of a covalently closed circular (CCC) DNA; and the levels of drug resistance of R12 are relatively higher in the case of SM and of chloramphenicol (CM) and are lower for tetracycline (TC) (10).

We show in the present study that it is possible to select and isolate in an *E. coli* K-12 host a number of distinct, autonomously replicating plasmids. Most of these, although derived from R12, appear to lack resistance to all antibiotics. These plasmids are all CCC DNA molecules and range in size from 3.6×10^6 to 61×10^6 daltons. The DNAs from this series of related plasmids are relatively easier to isolate than the CCC DNAs that are currrently most generally used in physical studies and, in addition, they make possible the acquisition of closely related closed DNAs of essentially any desired molecular weight within the indicated range. We have found that most cells that can grow on TC-containing medium contain both R12 and one or more smaller plasmids which, because of incompatibility with R12, can then segregate as resistance-free clones.

Other small plasmids derived from R-factors have been reported previously in the literature. These include an ampicillin-resistant plasmid of about 5×10^6 daltons derived from R-factor R201 (6) and a series of small plasmids derived from a mutant of R1 (5). These latter small plasmids lie in the molecular weight range 3×10^6 to 8.4×10^6 and can be obtained simultaneously in a *dnaA* mutant host. In contrast to this latter report, the CCC plasmids that we describe here can be segregated readily from the original R-factor. In addition, their formation is unrelated to the presence of a DNA replication mutation in the host strain.

MATERIALS AND METHODS

Chemicals. BDH sodium dodecyl sulfate (SDS), specially pure grade, was purchased from Gallard Schlesinger, Carle Place, N.Y. Electrophoresis-grade agarose, Trizma base, and disodium ethylenediaminetetraacetate (EDTA) were purchased from the Sigma Chemical Co., St. Louis, Mo. Electrophoresis-grade acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from the Eastman Kodak Co., Rochester, N.Y. Optical-grade CsCl was obtained from the Harshaw Chemical Co., Solon, Ohio; ethidium bromide (EtBr) was supplied by Calbiochem, Los Angeles, Calif.; and isopropanol was purchased from the Mallinckrodt Chemical Co., St. Louis, Mo. All other chemicals were reagent grade.

Drugs. CM was purchased from Parke, Davis and Co., Detroit, Mich. SM (streptomycin sulfate), TC (tetracycline-HCl), and sulfathiazole (SA) were purchased from the Sigma Chemical Co., St. Louis, Mo.

Bacterial strains. The bacterial strains used are listed in Table 1.

Growth of strains. Penassay broth (antibiotic medium 3, Difco) was used for the growth of cells for DNA isolation or for cell lysis followed by electrophoresis on agarose gels. This was supplemented by the addition of 20 μg of thymine per ml for the growth of Thy⁻ Pm15/R12. M9 selection plates contain, in addition to M9 buffer: agar (15 g/liter); $MgSO_4$ (1 mM); glucose (0.2%); tetrazolium chloride (Sigma) (10 mg/liter); the trace metals $CaCl \cdot 2H_2O$ (5.3×10^{-5} g/liter), $ZnSO_4$ (7×10^{-4} g/liter), $FeSO_4 \cdot 7H_2O$ (2.5×10^{-4} g/liter), $CuSO_4$ (8×10^{-5} g/liter), and $MnSO_4 \cdot H_2O$ (1.9×10^{-4} g/liter); required amino acids at 20 μg/ml or thiamine at 1 μg/ml; and either CM (25 μg/ml), SA (50 μg/ml), TC (25 μg/ml), or SM (50 or 150 μg/ml). Galactose MacConkey plates, consisting of 40 g of Difco MacConkey agar base per liter with 10 g of galactose per liter, were supplemented with 20 μg of thymine per ml (GMT plates).

Agarose-gel electrophoresis. Electrophoresis buffer (E buffer) consists of 0.04 M tris(hydroxymethyl)-aminomethane (Tris)-acetate, 0.02 M sodium acetate, and 0.002 M EDTA and was prepared from stock solutions of 1 M Tris-acetate (pH 8.3), 1 M sodium acetate, and 0.5 M EDTA (pH 8.14). Either 1 or 0.7% (wt/vol) gels were poured into glass tubing (6-mm ID) and cut to 10 cm with a razor blade or, alternatively, were poured into a slab apparatus to form a slab 10 by 14 by 0.35 cm. The slab gel apparatus was sealed with silicone lubricant. Between 10 and 50% (vol/vol) of a stock solution containing 50% sucrose (wt/vol) and 0.02% bromophenol blue as the marker dye was added to samples before layering. The cylindrical gels were run at 100 V for 2 h or until the marker dye had reached the bottom of the gel. Current was applied at 80 to 100 V for 1.5 to 2.5 h or until the marker dye had moved approximately 8.5 cm.

Acrylamide-agarose gels. The same slab gel apparatus was used as described for the agarose gels. The mixed gels were made up to contain 4% acrylamide and 1% agarose in E buffer. To prepare 80 ml of agarose-acrylamide solution, 0.8 g of agarose was completely dissolved in 55 ml of water in a boiling-water bath. A second solution composed of 8 ml of 10× E buffer, 16 ml of 20% acrylamide (19% acrylamide, 1% bisacrylamide), and 23 μl of TEMED was then mixed in a 250-ml side-arm flask and heated to 40 C. The agarose-water solution was cooled to 55 C and added to the contents of the side-arm flask with swirling to mix. A 1-ml volume of 4% (wt/vol) ammonium persulfate was added, and after thorough mixing the solution was degassed under water aspiration. The spacers of the slab assembly were sealed in place with 2 to 4% agarose.

Visualization of the DNA on gels. After the completion of electrophoresis, the bands were visualized and photographed by the technique of Sharp et al. (19). The gels were soaked for 1 h in a 5 μg/ml solution of EtBr in E buffer before photographing. Ribonucleic acid fragments were occasionally found to obscure a portion of the gel. These fragments are subject to degradation by fortuitously present ribonucleases, and the digestion products diffuse out of the gel in approximately 2 days, thus enabling the visualization of any obscured DNA bands.

TABLE 1. *Bacterial strains used*

Strain	Genotype	Source and reference
E. coli K-12		
CSH-2	F⁻ *met*⁻	R. Rownd (23)
W677[a]	F⁻ *thr*⁻ *leu*⁻ B_1 *lac*⁻ *gal*⁻ *mal*⁻ *xyl*⁻	R. Rownd (23)
P. mirabilis		
Pm15/R12	*lac*⁻ *nic*⁻ TC^r *gal*⁻ *trp*⁻ *thy*⁻ *leu*⁻/TC^r CM^r SM^r SA^r Hg^r	R. Rownd (11)

[a] The requirement for B_1 appears to have been lost in the W677 strain used in the studies.

Storage of cultures. Cells were grown in Penassay broth to a turbidity of about 0.1 at a wavelength of 650 nm. Samples were stored by freezing in 10% sterile glycerol at −70 C.

Bacterial matings. Strains were grown overnight at 37 C with shaking in Penassay broth. They were then diluted 10-fold, and incubation at 37 C with shaking continued for 1.5 h. Equal volumes of donor and recipient were then vortexed and incubated for 23 h at 37 C in a new Brunswick gyratory water bath set at 42 rpm. The culture was appropriately diluted ($\sim 10^{-6}$ to 10^{-7} for R12 matings) and plated on M9 antibiotic selection plates.

Modified Hirt (8) procedure. Approximately 20 ml of cells grown in Penassay broth and maintained for 10 to 12 h in the stationary phase was pelleted at 12,000 × *g* for 10 min at 0 C. The pellet was suspended in 10 ml of room-temperature SV (0.15 M NaCl, 0.1 M sodium EDTA, pH 10.2) and pelleted again. The pellet was suspended in 6 ml of SV/10, and 0.12 ml of 25% SDS was added to a final concentration of 0.49%. After gentle mixing and incubation at 65 C for 15 to 30 min, the solution usually clarified. The sample was then placed on ice for no longer than 5 min, and 0.5 ml of water-saturated CsCl at room temperature was allowed to stream down the side of the iced tube without mixing. The tube was then slowly rotated for 30 min at 4 C, and the CsCl-SDS-DNA-protein precipitate was spun down at 17,000 × *g* for 20 min at 0 C. The supernatant, containing most of the episomal DNA, was then placed in a 12.5-ml centrifuge tube containing 6 g of CsCl. A 0.5-ml volume of stock EtBr at 5 mg/ml was added to a final concentration of approximately 300 μg/ml. Any necessary volume adjustments were made with 1 M CsCl in SV/10. The final CsCl density was about 1.6 g/cm^3. The solutions were then overlaid with Marcol, balanced, and spun at 45,000 rpm for at least 24 h at 20 C in a Ti75 rotor. The tubes were fractionated by dripping, the lower band of DNA was extracted six times with isopropanol (1), and the sample was dialyzed against 0.01 M Tris, pH 8.3.

Restriction enzymes. The *Eco*RI restriction enzyme was supplied by Geoffrey Childs and was prepared by the method of R. N. Yoshimuri (Ph.D. thesis, Univ. of California Medical Center, San Francisco, 1971). Incubation mixtures contained sufficient *Eco*RI to effect cutting, 0.1 M Tris-hydrochloride, 0.01 M $MgCl_2$ (pH 7.7), and approximately 0.1 to 0.2 μg of DNA. The usual volume was 10 to 20 μl. The mixtures were incubated at 37 C for 1 h. *Alu*I (15a) digestions were performed under the conditions stated by R. Roberts, whom we thank for generously providing that restriction enzyme.

Electron Microscopy. Heteroduplex formation and subsequent electron microscopy were carried out by published procedures (9, 17).

RESULTS

Origin of the small plasmids pSM1 and pSM2. A mating of Pm15/R12 to CSH-2 was performed with selection on 150 μg of SM per ml followed by a mating of the purified CSH-2/R12 to W677, selected on 50 μg of SM per ml. One of the recombinant colonies was found to be very unstable with respect to drug resistance. For cells plated directly from a freezer culture, 20 of 40 colonies were resistant to both SM and TC. For another sample which was removed from the freezer, diluted 10^{-6} followed by a 10^{-8} dilution (ca. 33 generations), and allowed to remain in stationary phase for 10 to 12 h before plating, only 2 of 33 colonies tested were resistant to both SM and TC. An examination of the analytical buoyant density profile in alkaline CsCl of DNA from a resistant culture indicated the presence of at least three resolvable CCC DNA bands (data not shown). To identify these species, they were examined further by SDS lysis of the corresponding cell culture (S. Mickel and W. Bauer, in preparation) and analyzed by gel electrophoresis. This latter technique, which does not require selection on drug-containing medium, permits the screening of resistance-free (R^-) colonies for plasmids which themselves carry no resistances.

Figure 1 presents the results of the electrophoresis on cylinder gels of several such whole-cell lysates. The colonies whose DNA profiles after electrophoresis on agarose gels are shown in Fig. 1, gels c through g, are all segregants obtained from the original strain (not shown). As can be seen by the pattern in Fig. 1, gels c through g, three DNA bands are present in samples derived from the unstable W677/R12 culture that are not present in W677 cultures alone (results not shown but similar to gel h). It is evident from the gels a and b in Fig. 1 that the trailing DNA band corresponds in size to unmodified R12 DNA. In Table 2 it is documented that the resistances to SM, SA, and CM are associated with the presence of the R12 band. The band designated C is present in all cultures, both those that do and that do not contain plasmids. It seems likely that this species corresponds to linear *E. coli* DNA, since the change in its mobility when the agarose content of the gel is varied differs from that seen for the other (CCC) bands and is characteristic of a large linear DNA. The faint bands migrating just ahead of the C band in gels a and c through g may be tentatively identified as linear R12 DNA or nicked circular small plasmid DNA. The faint band that migrates slightly slower than the R12 band in gel e might correspond to catenated dimers of R12 DNA. The small CCC plasmid DNA bands designated pSM1 and pSM2 carry no known drug resistances. The simultaneous presence of all of the six possible combinations of the R12-size DNA and of the smaller plasmids pSM1 and pSM2 has been observed on similar gels. No strains completely devoid of plasmids were found. It was also observed that colonies of bacteria containing both small plasmid DNA and DNA of the size of R12 grew confluently when streaked on TC test plates, whereas only a few individual colonies were observed for bacteria containing solely the R12-size DNA.

The resistance characteristics as revealed on test plates and documented in Table 2 were used to choose the strains analyzed in Fig. 1. As shown by the data presented in Table 2, segregation due to plasmid incompatibility occurs rapidly in strains containing R12 and pSM1 or pSM2. Cells were inoculated (ca. 10^{-5} dilution) from the nonselective test plate into 5 ml of Penassay broth, grown to stationary phase, and plated, and the resulting colonies were tested on drug test plates.

Stability of the plasmids. Strains W677/pSM1 and W677/pSM2 were removed from the freezer, diluted 10^{-7} in Penassay broth, and grown at 37 C to stationary phase. After 10 to

FIG. 1. *Analysis, by electrophoresis on 1% agarose gels, of DNA obtained by whole-cell lysis of cells containing, or not containing, various episomal DNAs: gels a and b, CSH-2/R12; gel c, W677/pSM1; gel d, W677/SM2; gel e, W677/R12; gel f, W677/R12 and pSM1; gel g, W677/R12 and pSM2; gel h, CSH-2 only. The clones examined in gels c through g were segregants obtained from a single clone that contained the R12, pSM1, and pSM2 bands. The band arising from the E. coli chromosomal DNA and termed the C band is present in all cultures. The profile of CSH-2 alone is indistinguishable from a profile of W677 alone.*

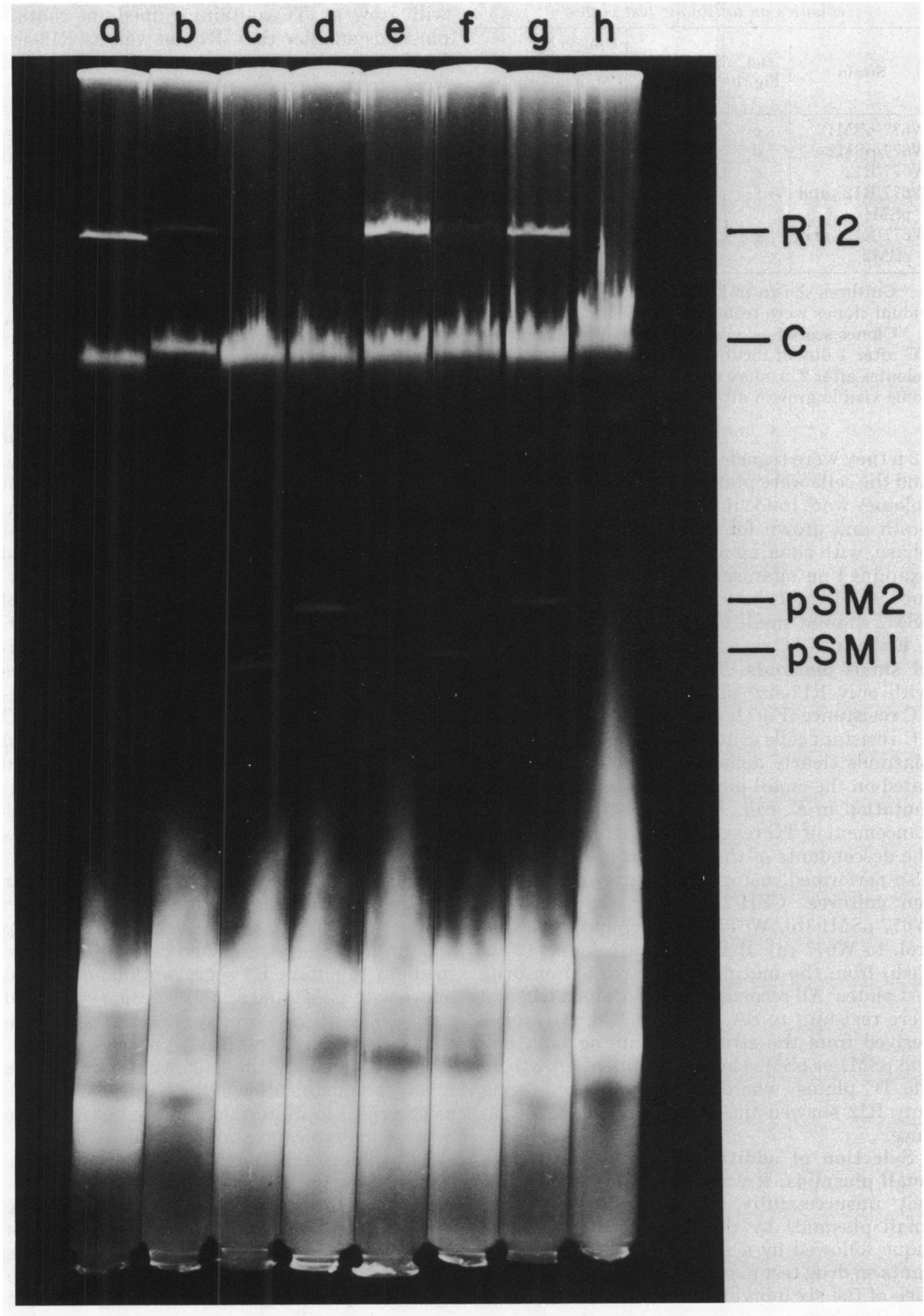
a
b
c
d
e
f
g
h
R12
C
pSM2
pSM1

TABLE 2. *Resistance characteristics of single colonies on antibiotic test plates*[a]

Strain	Gel, Fig. 1	Growth[b] on TC	Growth on SM, CM, and SA	Growth, drug-free
W677/pSM1	c	0	0	22
W677/pSM2	d	0	0	22
W677/R12	e	3+, 22±	25	25
W677/R12 and pSM1	f	1+	1	23
W677/R12 and pSM2	g	13+	13	20

[a] Cultures shown in Fig. 1 were plated, and individual clones were tested for antibiotic resistances.

[b] Clones scored as ± were negative for growth on TC after 1 day of incubation and showed scattered colonies after 2 or more days. Those scored as + had some visible growth after 1 day.

12 h they were transferred to room temperature and the cells were plated on GMT plates. Single colonies were innoculated into 5 ml of Penassay broth and grown for 7 h, just into stationary phase, with shaking at 37 C, and the DNA was examined on agarose gels. All colonies examined (10/10) for both W677/pSM1 and W677/pSM2 showed small plasmid DNA bands.

Enhanced TC resistances of R12 in presence of small plasmids. The fact that segregants with only R12-size plasmid DNA and limited TC resistance (Fig. 1, gel e) can be isolated from TC-resistant cells containing R12 and two small plasmids clearly demonstrates that a gene located on the small plasmid, rather than a host mutation in *E. coli*, is responsible for the enhancement of TC resistance by R12. Matings of the descendants of those shown in Fig. 1 were also performed, using strains grown from frozen cultures. CSH-2/R12 (a) was mated to W677/pSM1 (b), W677/pSM2 (c) and, as a control, to W677 (h). Recombinant colonies (25 of each) from the mating were streaked on drug test plates. All recombinants contained R12 and were resistant to SA, SM, and CM. All clones derived from the strains containing both R12 and pSM1 or pSM2 showed confluent growth on the TC plates, whereas the strain containing only R12 showed the usual limited TC resistance.

Selection of additional strains containing small plasmids. It was attempted initially, albeit unsuccessfully, to generate additional small plasmids by the original mating technique followed by a screening of the recombinants on drug test plates. It was discovered that each of the six individual colonies that grew at the site where the clones had been streaked on TC test plates contained plasmids in addition to R12 DNA. We found that most colonies that will grow on TC-containing medium contain plasmids smaller than R12 as well as R12-size DNA molecules. When colonies containing R12 were streaked on TC plates after being previously selected on CM, SM, or SA or even when no antibiotic selection has been present, plasmids could often be seen as additional bands when cultures were lysed and subjected to electrophoresis on a 1% agarose gel. (13/18 clones from a mating and 6/16 clones from CSH-2/R12 cells treated similarly to the mating culture showed visible smaller plasmid bands.) Even if no plasmid band was visible under these electrophoresis conditions, bands could in some cases be seen if the sample was run on a 0.7% agarose gel. A prior mating therefore appears to be unnecessary for plasmid generation. These clones from the TC plates were grown in drug-free medium to allow segregation to occur and then streaked with a sterile toothpick onto antibiotic test plates. Four types of segregant colonies were found: those showing no resistance; those resistant only to TC; those resistant to SM, CM, and SA but sensitive to TC; and those sensitive to SM, CM, and SA but displaying the limited TC resistance characteristic of R12. In general, small plasmids were most often found after matings or incubation of a culture for 24 h at 37 C with selection on M9 plates containing SM, CM, or SA followed by TC selection (streaking colonies on minimal TC plates). In particular, 38/38 cultures examined that were selected on SM, CM, or SA failed to show plasmids, although two TC^+ SM^- CM^- SA^- clones were obtained upon testing clones from a mating that had grown on GMT plates with no selection.

Isolation and purification of the smaller plasmids. To further characterize the plasmids it was necessary to purify them. The first step was to grow the TC-selected cells in drug-free medium to obtain R^- segregants as described above with pSM1 and pSM2. We then examined the lysed cultures directly on gels to confirm that only a single plasmid type was present. The remainder of the purification procedure involves a modification of the Hirt (8) procedure as described in Materials and Methods. Figure 2 presents typical results obtained after the CsCl banding step. Much of the *E. coli* DNA is removed by the SDS-salt precipitation treatment prior to banding. We are able to isolate approximately 1 to 2 μg of DNA per ml of culture medium for the smaller plasmids. This method does not work well for NR1 DNA grown in Penassay broth but is remarkably effective for round-of-replication mutants (11), resulting in relatively pure DNA after one banding with

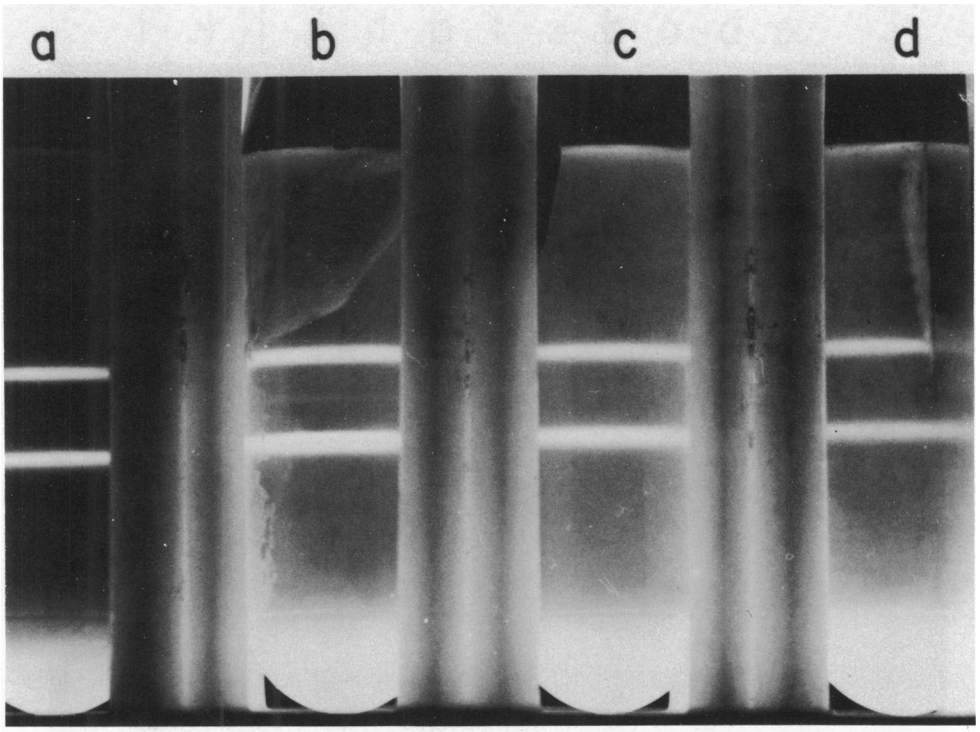

FIG. 2. *CsCl/EtBr buoyant banding of Hirt-extracted CSH-2 cells containing four different plasmids: a, pSM6; b, pSM9; c, pSM7; d, pSM5.*

EtBr in CsCl. That is, when purified plasmid DNA is examined on gels, contaminating bacterial DNA is not observed.

Ordering the fragments. The results of limit digestion of the purified plasmid DNAs with *Eco*RI restriction endonucleases are shown in Fig. 3 and the fragment sizes are presented in Table 3. Two classes of resistance-free small plasmids were examined, those that were cut once and those that were cut more than once by *Eco*RI. If the circular plasmids are cut n times, they produce n fragments upon complete digestion. These fragments represent a subset of the R12 fragments if they are derived from R12 DNA, except for one fragment. This unusual fragment consists of the site where the intramolecular recombination occurred in R12, as well as the adjacent DNA. The first identifiable fragment observed (Fig. 3) after two or more cuts is the second largest, r_2 (terminology of E. Ohtsubo). According to Ohtsubo (personal communication), who has mapped the *Eco*RI cutting sites of R100, fragment r_2, in the RTF-TC (resistance-transfer function-tetracycline resistance) region, contains an origin of replication for this R-factor. R12 was derived from the same source (NR1) as R100 (13, 16, 20) and yields the same size fragments when cut with *Eco*RI (Ohtsubo, personal communication). The order of appearance of additional R12-size *Eco*RI fragments with an increase in plasmid size and number of cuts is: r_6, r_5, and r_3. The order r_2, r_6, r_5, r_3 based upon data presented in Fig. 3 is in agreement with the order obtained by Ohtsubo for R100 and, in addition, suggests that the recombination occurred in the same region between r_2 and the next cut for all the multiply cut plasmids. The fact that the correct R12-size fragments are generated, in the proper order, implies very strongly that these plasmids are derived from intramolecular recombination in R12 DNA.

The large plasmid pSM9 shown in Fig. 3, channel b, is of a different type in that it does contain Hg, SM, CM, and SA resistances. This relatively large plasmid was originally selected from a mating on CM followed by streaking on TC. A TC-resistant colony was grown in drug-free medium, and an SM^+ CM^+ SA^+ TC^- segregant colony was obtained. It is not clear whether the augmented TC resistance originally observed arose from this plasmid or from a small plasmid that possibly passed unobserved in our 1% agarose gel screening and

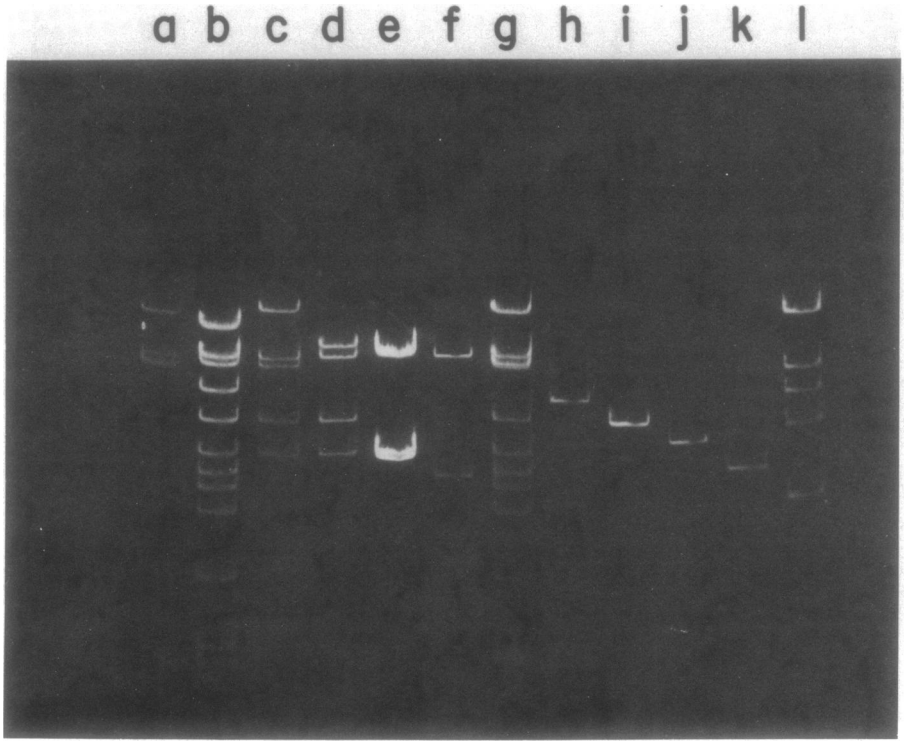

FIG. 3. *Analysis of plasmid DNAs digested with EcoRI on a 0.7% agarose gel. Channels a and g, R12; b, pSM9; c, pSM8; d, pSM7; e, pSM6; f, pSM5; h, pSM4; i, pSM3; j, pSM2; k, pSM1; and l, F8(P6) from E. Ohtsubo. Plasmids pSM1 through pSM8 carry no resistance markers; pSM9 is TC sensitive and is resistant to SA, SM, and CM. Plasmids pSM1 through pSM4 each have one cutting site. Plasmids pSM5 through pSM8 have two, three, four, and five cutting sites, respectively. The sizes of the digestion fragments arising from F8(P6) were determined by E. Ohtsubo by electron microscopy. Refer to Table 3 for a list of the fragment sizes.*

was not obtained by segregation. Two fragments differing from R12 occur in the digests of this plasmid. The mechanism of generation of this plasmid is currently under investigation.

Homology between the small plasmids and R12. To demonstrate unequivocally that the plasmids were derived from R12, heteroduplex formation was performed between R6-5 and two of the plasmids. R6-5 contains three small insertion regions but is otherwise entirely homologous with R100-1 (17). R12 and R100-1 are indistinguishable by restriction enzyme mapping experiments (E. Ohtsubo, personal communication). The occurrence of the characteristic inversion loops in R6-5 DNA, after denaturation and renaturation, allows the region of plasmid homology to be mapped. Plasmid pSM5, which was cut twice, and pSM1, the smallest of the original plasmids, were chosen for this purpose (see Fig. 3). The results are shown in Fig. 4. Plasmid pSM5 is entirely homologous with R6-5, as is pSM1. The deletion of a short region occurred in pSM1 in addition to the recombination event generating the plasmid, since the length of the duplex region of the heteroduplex molecules is indistinguishable from that of pSM1 on the same grid. It seems likely that one of the recombination sites involved in formation of these plasmids is the IS1 sequence, which is known to be a "hot spot" for recombination (4, 7, 3, 15). Heteroduplex mapping experiments are currently being conducted to examine the possible role of the IS1 DNA in plasmid formation (Mickel, Ohtsubo, and Bauer, in preparation).

The data shown in Fig. 5 indirectly demonstrate the sequence relatedness of the other small plasmids with pSM1. In this experiment several of the small plasmids were digested with restriction endonuclease *Alu*I (15a), and the resulting fragments were compared. Although the digestion did not proceed to completion, resulting in the presence of some partially

digested fragments, the degree of digestion is similar among the various small plasmids compared. The remarkable similarity in the band patterns, down to the presence of the same partially digested fragments, is evident. If only 1 or 2 major bands differ in position out of the 12 to 20 or more bands per plasmid, the nearly complete correspondence would seem unlikely to represent an artifact of partial digestion. If this premise is granted, then two significant conclusions can be drawn. The first is that, since all of the plasmids have approximately 12 of the 13 major bands of pSM1 (a) in common, they too are derived from R12. Similar results for bands not present in pSM1 digests can barely be discerned for the larger plasmid pSM5 (channels i and j). The second conclusion concerns the four plasmids (channels d, g, k, and l) of the same overall size as determined with *Eco*RI-digested DNA on 1% agarose gels. Three of these appear to differ from each other by a few hundred base pairs (d = l, l ≠ k, l ≠ g, and k ≠ g) when a band-by-band comparison is made. Such a comparison requires the disregarding of partial digestion products that are present in lesser amounts than the major bands and therefore clearly lie outside the expected limit digest pattern. A similar but less obvious difference exists for the smaller plasmids of similar size, pSM3 and pSM13, as shown in channels c and h. The second largest of the major bands migrated slightly farther in the case of pSM13 (channel h), a difference that was clearer for an aliquot of the same sample run on a 2.5% agarose gel (data not shown).

DISCUSSION

Mechanism of generation of plasmids. The plasmids described in this publication all appear to be derived from R12, based upon the heteroduplex data for pSM1 and pSM2, the restriction enzyme data in Fig. 3 and 5, and the incompatibility of the plasmids with R12 in an *E. coli* K-12 host. The generation of small plasmids almost certainly occurs during normal growth of the bacteria containing R12 rather than as a result of induction specifically related to the presence of TC. This latter conclusion is based upon the observation that individual sporadic colonies are obtained when single clones containing R12 are streaked on TC test plates. Further conclusions from this observation are that all cells containing R12 are capable of generating plasmids and that the frequency of such generation may vary between hosts, since an estimated 10-fold more colonies were seen on TC plates for CSH-2/R12 than for W677/R12. Also from the number of colonies that grow on a streak on TC test plates it can be estimated that the frequency of cells containing a small

TABLE 3. *Fragment sizes of EcoRI-digested plasmids*[a]

Channel in Fig. 3	Plasmid	Size (kb)	Fragment size (kb) common with R12	Size (kb) of odd fragment
k	pSM1	5.51 ± 0.14		5.51 ± 0.14
j	pSM2	6.49 ± 0.14		6.49 ± 0.14
i	pSM3	7.16 ± 0.21		7.16 ± 0.21
h	pSM4	8.29 ± 0.21		8.29 ± 0.21
f	pSM5	17.3 ± 1.3[b]	12.3 ± 1.2	5.0 ± 0.2
e	pSM6	23.6 ± 1.7[b]	12.6 ± 1.0, 5.8 ± 0.3	5.2 ± 0.4
d	pSM7	38.5 ± 3.1[b]	12.1 ± 1.2, 5.8 ± 0.2, 7.3 ± 0.2	13.3 ± 1.5[b]
c	pSM8	56.6 ± 3.2[b]	12.4 ± 1.0, 5.6 ± 0.1, 7.1 ± 0.1, 11.2 ± 1.1[b]	20.3 ± 1.0[b]
g and a	R12	88.2[b] + smaller bands	r_1 = 20.2 ± 0.9,[b] r_2 = 12.5 ± 1.1,[b] r_3 = 11.5 ± 1.0,[b] r_4 = 11.2 ± 0.6,[b] r_5 = 7.28 ± 0.37, r_6 = 5.78 ± 0.27, r_7 = 5.09 ± 0.20, r_8 = 4.56 ± 0.21, r_9 = 3.85 ± 0.18, r_{10} = 2.64 ± 0.49, r_{11} = 1.45 ± 0.15, r_{12} = 1.19 ± 0.17, r_{13} = 0.97 ± 0.15 (other smaller bands are not visible)	
b	pSM9	83.0[b] + smaller bands	Same as R12 except that r_1 and r_4 are replaced by 16.9 ± 1.8[b] and 9.29 ± 0.81	

[a] Size calculations were based on measurements from at least three gels in which *Eco*RI-digested F8(P6) or λ_{CI857} were included for calibration purposes.

[b] Fragments larger than 9.5 kb lie outside the linear range of the gel; hence some systematic error may have been introduced by the curve-fitting program used to obtain these data.

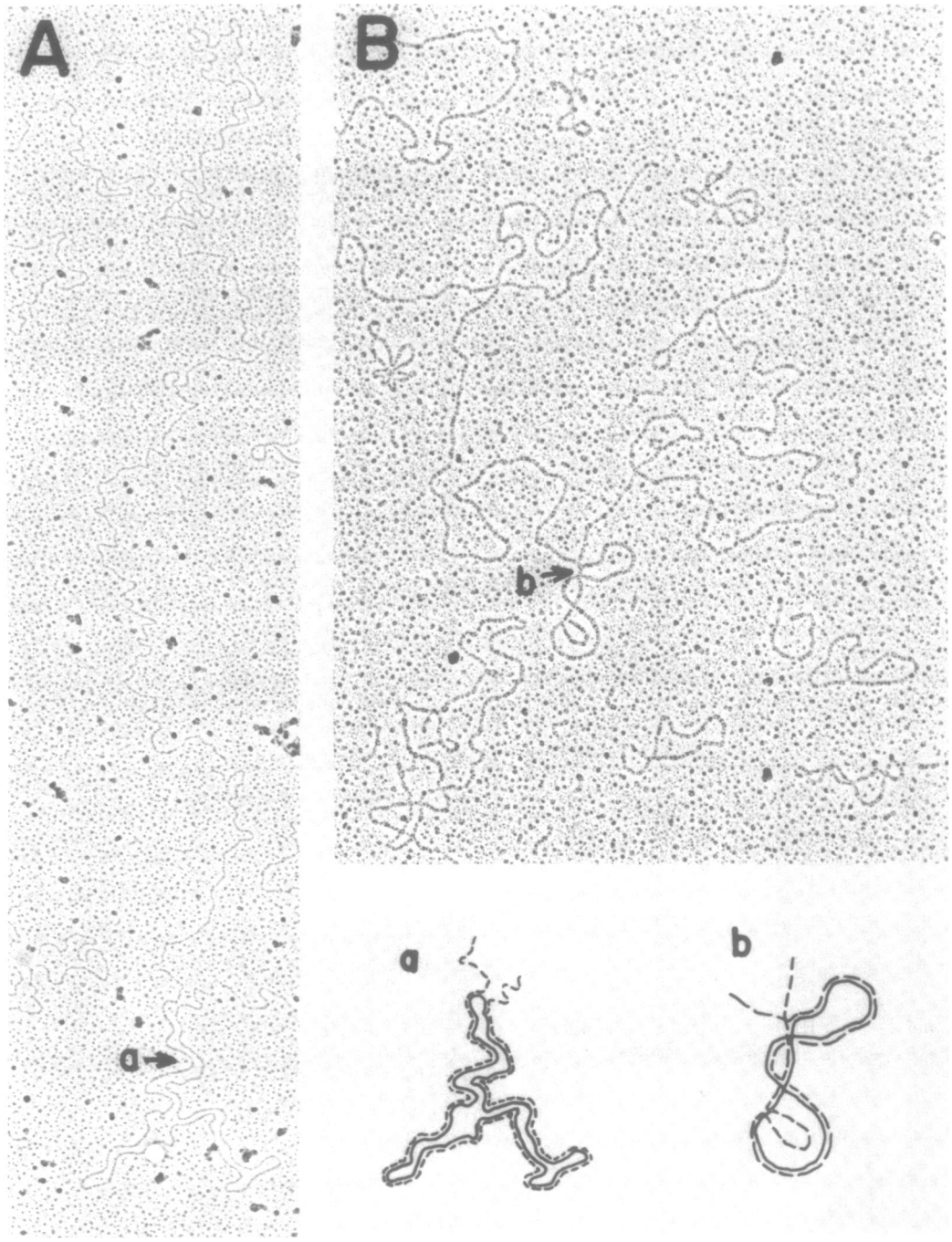

FIG. 4. *Heteroduplexes R6-5/pSM5 (A) and R6-5/pSM1 (B). As can be seen by the drawings of the heteroduplex region of the molecules (a) and (b), all of the plasmid DNA in each case forms a heteroduplex with R6-5. The single-stranded loop (dashed line in b), due to a deletion that occurred in pSM1, is R6-5 DNA. (A) and (B) are at the same magnification and, for the purpose of approximate size calibration, the small molecule at lower center in (B) is a single-stranded φX174 molecule.*

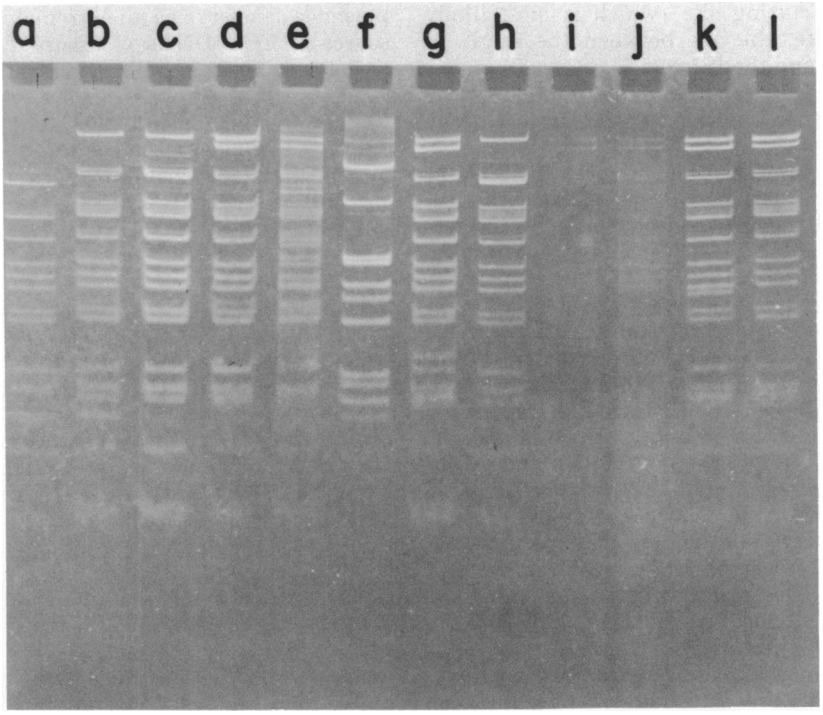

FIG. 5. *Acrylamide-agarose gel patterns obtained with small plasmid DNAs digested with AluI (15a). Simian virus 40 DNA, which had been digested with HindII plus HindIII (2) and was a generous gift of Walter Keller, is shown in channel c for approximating fragment sizes even though the distance migrated in such gels is a function of structure as well as of molecular weight (21). The following are the estimated sizes in base pairs of the fragments produced by the HindII plus HindIII digestion of simian virus 40 DNA based upon a molecular weight of 3.2 × 10^6 and the data of Danna et al. (2): 1,108, 738, 517, 492, 418, 369, 345, 271, 246, 221, and 196. The samples listed in order of increasing plasmid size to within 10% are: a, pSM1 (5.5 kb); b, pSM2 (6.5 kb); c and h, pSM3 and pSM13* (7.2 kb); d, g, k, and l, pSM4, pSM11, pSM12, and pSM14* (8.3 kb); e, pSM10* (14.5 kb); i and j, both pSM5* (17.3 kb). (An asterisk [*] indicates a CSH-2 host; others had a W677 host.) (It should be noted that the plasmids in d, g, and k did not necessarily arise independently since they came from separate clones of the same culture.)*

plasmid in addition to R12 is approximately 10^{-2} to 10^{-5}. This frequency range is much higher than mutation frequencies, but cells containing small plasmids are not likely to be randomly selected. The TC furnishes selection and, for reasons not yet understood, confers an advantage on the cells containing both small plasmids and R12. One result is that most colonies from such plates yield small plasmids, implying that the regulation of TC resistance can also involve a genetic locus distant from the site at which the gene for TC maps (17). With SA, SM, or CM selection no small plasmids were seen either in or derived from R12-containing cells. We have also recently found that selection for cells containing plasmids in addition to R12 occurs when stationary cultures are stored at 37 C for several days. The mechanism of this latter process is currently under investigation. The selection of the initial plasmids pSM1 and pSM2 was probably by this mechanism, since laboratory temperatures were in excess of 30 C for several days immediately preceding the time of their discovery. All of the other plasmids discussed in this paper were selected on TC-containing medium. The *Eco*RI restriction enzyme digestion data shown in Fig. 3 indicate the presence of fragments from only one side of fragment r_2 (containing the origin). The order of appearance of the digestion fragments with increasing plasmid size is r_2, r_6, r_5, and r_3. According to the data of Ohtsubo (personal communication), the *Eco*RI digestion product on the r-determinant side of the origin is fragment r_8 and is of a length equal to 4.8 kilobases (kb). This fragment contains the IS1 sequence. All of the plasmids examined contain the origin of replication and at least

one *Eco*RI cutting site (which is most likely the r_2-r_R site, the one between the origin of replication and the IS1 region). Since fragment r_R itself was never observed, a limit of 4.8 kb on the distance from the r_2-r_R cutting site to the region where recombination must have occurred is obtained for all the plasmids. In light of the data of other investigators that the termini of IS1 regions may be "hot spots" for recombination (4, 7, 3, 15) and of our heteroduplex data for pSM1 and pSM5, it appears likely that IS1 is involved in plasmid formation also. Heteroduplex experiments with several of our plasmids are in progress to experimentally verify this. Recombination occurring between the IS1 sequences in NR1 have been suggested (9, 15) to be instrumental in the formation of RTF-TC (resistance-transfer function and tetracycline resistance DNA) molecules or of molecules with tandem r-determinants. From the data in Fig. 5, it appears as though the site with which IS1 recombines can be practically anywhere; therefore, as others have suggested (4, 7), if sequence specificity is involved, it must be of a very low order in this case.

Other small plasmids derived from R-factors. The data presented here on the generation of small plasmids from an R-factor bear some similarity to the work of Goebel and Bonewald (5) involving a mutant R-factor. They were able to obtain small plasmids from R-factor R1*drd*-19B2 by growing in *E. coli* K-12 strain CRT46, a temperature-sensitive *dnaA* (initiation) mutant at the permissive temperature. In some cases, these plasmids carried the ampicillin marker. It is probable that the relationship of R12 to NR1 is similar to that of R1*drd*-19B2 to R1*drd*-19. R12 and R1*drd*-19B2 are both present in 3.5- to 4-fold excess over the amount seen for NR1 and R1*drd*-19, respectively. In both cases, the sizes of the members of the cognate pairs are virtually identical. R1 and R100-1 were shown (17) to be largely homologous in the region now known to be the replication origin (14, 22), and these strains are closely related to R1*drd*-19 and NR1, respectively. Since R12 has a 100-fold higher frequency of transmission than NR1 (R. Rownd, personal communication), it is possibly also derepressed. Although the families of plasmids obtained from R12 and R1*drd*-19B2, respectively, are in many ways similar, the means of selection were entirely different. The generation of the plasmids reported by Goebel and Bonewald (5) must occur in a large number of cells, since so many plasmids of different size classes are seen in a single culture. In our case a single plasmid, which is stable in the host, was isolated.

Potential uses for the plasmid DNAs. These plasmids can serve as an extremely convenient source of CCC DNAs of assorted sizes for a variety of physical and biological studies. They range in size from 5.5 kb (3.6×10^6 daltons) to the size of compete R-factors, 91 kb (61×10^6 daltons). The plasmids are stable and are easier to grow than most bacteriophages since stationary cultures can be used and no prior viral infection is necessary. With standard procedures (18), the DNA is easy to isolate and the quantities present in the cell are comparable to those obtained with bacteriophage PM2, which is currently the most generally used source of circular DNA for physical studies. Possibly our modification of the Hirt (8) procedure, which yields 20 to 40 μg of DNA from 20 ml of cells, could be further refined to substitute NaCl for CsCl and to include a pelleting step to concentrate the DNA before banding in CsCl, so that still larger quantities of DNA could be obtained. There is also a good possibility that with our plasmids, as with those obtained by Goebel and Bonewald (5), an increased number of copies could be obtained by transfer to an *E. coli* C host.

Nature of TC resistance. The results presented above indicate that cells which contain both R12 and one or more small plasmids exhibit enhanced TC resistance. This is of special interest in that the small plasmids apparently contain no sequences not found in R12, nor do they contain the known TC region of R12 (17). Two explanations for this phenomenon appear possible based upon our present data. First, the recombination event that resulted in the creation of a given small plasmid separated the DNA from the sequence normally regulating its transcription. This might lead to an altered or lost transcriptional control of the expression of TC resistance in the presence of the small plasmids. Second, the enhanced TC resistance might be due to a gene dosage effect involving an unknown gene product or control mechanism. Further experiments will be needed to distinguish between these two possibilities.

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