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Characterization of SmSu Plasmids by Restriction Endonuclease Cleavage and Compatibility Testing

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Twelve plasmids carrying genes for streptomycin and sulfonamide resistance were studied for the number and distribution of sites on the plasmid molecules susceptible to cleavage by the restriction endonuclease *Eco*RI. Ten of the twelve were found to have a single cut site, one plasmid (R678) had three such sites, and plasmid PB165, which was isolated as three supercoiled deoxyribonucleic acid species with molecular weights 7.4×10^6, 14.7×10^6, and 21.4×10^6 was reduced to a single (linear) species of molecular weight 7.6×10^6 after cutting with *Eco*RI. We conclude that PB165 forms oligomers in *Escherichia coli* and that the number of copies of these per chromosome is more consistent with a negative than a positive control mechanism for plasmid replication. Compatibility testing of these plasmids showed they all belong to the same incompatibility group, which we designate IncQ, suggesting that they may have come from a common ancestor.

In a previous paper (4) we concluded that the majority of a selection of plasmids conferring linked streptomycin (Sm) and sulfonamide (Su) resistance were very closely related, on the basis of molecular weight comparisons and deoxyribonucleic acid (DNA)-DNA hybridizations. Only 2 of the 12 SmSu determinants studied were markedly different from the rest of the group, namely, R678, which has a molecular weight 60% greater than the others, and PB165, which, instead of one plasmid, consisted of at least three supercoiled species with molecular weights approximately in the ratio 1:2:3.

Another technique for characterizing and differentiating between closely related plasmids involves the treatment of plasmid DNA with restriction endonucleases followed by agarose gel electrophoresis of the DNA fragments generated (17). Small differences in polynucleotide sequences, not detectable by our previous methods, can alter the number or distribution of restriction endonuclease-susceptible sites on a plasmid. We therefore analyzed our SmSu plasmids by this technique. We also hoped to find out whether the plasmids of PB165 are related as oligomers.

The classification of plasmids into incompatibility groups correlates with their polynucleotide sequence relationships (9). Studies on SmSu plasmids by Smith et al. (16) have shown that 19 out of 26 tested belonged to the same incompatibility group, but the recombinant plasmid used by them to test incompatibility was not available to us. We used instead Ap201, which is a transposition (11) derivative of the SmSu plasmid RSF1010, isolated by Heffron et al. (12). Ap201 has acquired a piece of DNA (about 3×10^6 daltons [Mdal]) specifying resistance to ampicillin (Ap), the insertion resulting in the loss of Su^r and a reduction of the level of Sm^r. We suppose that our plasmids are related to RSF1010 because heteroduplex analysis by the same workers has shown that this plasmid hybridizes with R684 (Table 1), leaving only a short sequence of R684 unpaired (12).

MATERIALS AND METHODS

Bacterial strains. Strains of *Escherichia coli* K-12 used were J53 *pro met* (λ) (7), W3110T$^-$ *thy dra* (1), and ATCC 14948 *thy* (4). A *thy* mutant of *E. coli* C (*lac met*) was isolated by trimethoprim selection (4).

Bacterial plasmids. Bacterial plasmids used are listed in Table 1.

Isolation of radioactively labeled plasmid DNA. Thymine-requiring strains containing the SmSu plasmids were labeled with radioactive thymine, and their DNA was isolated by CsCl equilibrium centrifugation in the presence of ethidium bromide as previously described (4).

Sucrose gradient sedimentation analysis. Sucrose gradient sedimentation analysis was by centrifugation through freeze-thaw-generated, 5 to 20% sucrose gradients as described previously (4). We calculated molecular weights (M) of DNA molecular species from their relative sedimentation distances (d) from the meniscus, using the relationship (5) $M_1^a = M_2^a \times (d_1/d_2)$, where a, the sedimentation exponent, has been empirically derived by us (manuscript in preparation) as 0.43 for covalently closed circular (CCC) forms, 0.36 for open circular (OC)

TABLE 1. *Bacterial plasmids used*

Plasmid	Molecular mass (Mdal)	Resistance markers[a]	Reference
R300B	5.7	Sm^rSu^r	4
R305C	5.7	Sm^rSu^r	4
R310	5.7	Sm^rSu^r	4
R450B	5.7	Sm^rSu^r	4
R464C	5.7	Sm^rSu^r	4
R676	5.7	Sm^rSu^r	4
R678	9.2	Sm^rSu^r	4
R682	5.7	Sm^rSu^r	4
R684	6.3	Sm^rSu^r	4
R750	5.9	Sm^rSu^r	4
R1162	5.5	Sm^rSu^r	4
PB165	7.4, 14.7, 21.4	Sm^rSu^r	4
R7K-TnC1	33.5	$Ap^rTp^rSm^r$	3
Ap201	9.1	Ap^rSm^r	12
R144*drd*3	65	Tc^rKm^r	14

[a] Resistance marker symbols are: Sm, streptomycin; Su, sulfonamide; Ap, ampicillin; Tp, trimethoprim; Tc, tetracycline; and Km, kanamycin.

forms, and 0.35 for linear (LIN) DNA. For comparison between unlike tertiary forms, we used: $d_{CCC}/d_{OC} = 1.21 \times M^{0.07}$; $d_{OC}/d_{LIN} = 1.11 \times M^{0.01}$.

Transformation. Transformation of *E. coli* C with plasmid DNA was by the method developed by A. E. Jacob and S. J. Hobbs, described previously (4).

Cleavage of plasmid DNA with restriction endonuclease *Eco*RI and agarose gel electrophoresis. The *Eco*RI reaction was carried out at 37°C for 30 min in a reaction mixture containing: about 50 ng of DNA in 10 to 30 μl of TES buffer [50 mM tris(hydroxymethyl)aminomethane, 5 mM ethylenediaminetetraacetic acid, 50 mM NaCl, pH 8]; 10 μl of 0.4 M tris(hydroxymethyl)aminomethane, 0.15 M $MgSO_4$, pH 7.4, and 5 μl of *Eco*RI (a generous gift from S. Cohen); TES buffer to 50 μl. The same mixture without enzyme was used as a control. After incubation, the reaction mixture was placed on ice and 25 μl of 0.05 M ethylenediaminetetraacetic acid-30% sucrose-0.01% bromophenol blue was added. After mixing, 50 μl was layered carefully onto the top of a 1% agarose gel (Seakem Limited) held in glass tubing (5-mm ID) by means of a constricted lower end. Electrophoresis at 3 mA/gel was continued for about 4.5 h in EB buffer [0.5 M tris(hydroxymethyl)aminomethane, 0.2 M sodium acetate, 1.5% (vol/vol) glacial acetic acid, 0.01 M ethylenediaminetetraacetic acid, 0.5 μg of ethidium bromide per ml, pH 7.9], which had also been used to make the gels. Bromophenol blue served as a tracking dye.

After electrophoresis, the gels were removed from their holders, illuminated with ultraviolet light, and photographed through a red filter. Exposure times at f4.5 on Polaroid type 107 film were chosen to give maximum contrast. These were 1 min for gels 15 and 16 and 4 min for the rest.

Incompatibility testing. We used the *E. coli thy* strains in which we have previously isolated each of the 12 SmSu plasmids (4); i.e., the majority of plasmids were in W3110T$^-$, R676 and R682 were in *E. coli* C *thy*, and R1162 was in ATCC 14948 *thy*. We used J53(R144*drd*3)(Ap201) to mobilize Ap201 into the SmSu plasmid-containing strains, selecting for ampicillin resistance and counterselecting the donor J53. Recipient clones were purified on nutrient medium with ampicillin (100 μg/ml) and then tested for the unselected *thy* marker by their failure to grow on DST medium containing 4% lysed horse blood (2). Testing for the presence of a Su^r plasmid in a *thy* strain requires a high (1 mg/ml) concentration of sulfonamide because the thymine (20 μM) necessary for growth acts as a sulfonamide antagonist.

RESULTS

Analysis of DNA fragments produced by *Eco*RI. The banding patterns produced by agarose gel electrophoresis of plasmid DNA before and after cutting by the restriction endonuclease *Eco*RI are shown in Fig. 1. With the exception of R678 and PB165, the SmSu plasmids all gave two bands before enzyme treatment (a faster-running dense band [a] and a fainter band [b] running about half as far as band a). *Eco*RI treatment reduced these two bands to a single one (c).

We correlated the gel bands with the tertiary DNA forms by sucrose gradient analysis of ^{3}H-labeled R300B before and after *Eco*RI treatment, using ^{14}C-labeled R300B DNA as a marker. The results show that the native DNA existed mostly in the CCC form, with a minority in the OC form (Fig. 2a). We therefore conclude that bands a and b in the gels are CCC and OC molecules, respectively. After *Eco*RI cleavage, the DNA sedimented as a single peak (Fig. 2b). Calculation, using the equations given in Materials and Methods, shows that the position of this peak is consistent with its being linear DNA of the same molecular weight as the untreated plasmid (5.7×10^6). Thus, band c is the complete plasmid DNA molecule in linear form, and we conclude that each of the SmSu plasmids apart from R678 and PB165 contains a single site susceptible to *Eco*RI cleavage.

Analysis of R678. R678 has a molecular weight of 9.2×10^6 which is significantly higher than the majority of the SmSu plasmids (5.7×10^6) (4). Gel electrophoresis of untreated R678 DNA gave three bands, presumably representing CCC, OC, and linear DNA forms, the faintest band being linear (Fig. 1, gel 13). After *Eco*RI cleavage, two bands were discernible (gel 14). Sucrose gradient analysis of this cloven R678 DNA, however, gave three peaks (Fig. 3), representing three linear DNA fragments of 7.0, 2.3, and 0.8 Mdal. These three peaks were seen in each of three sucrose gradient analyses of two separate isolations of R678 DNA. Also, the ratios of the radioactive

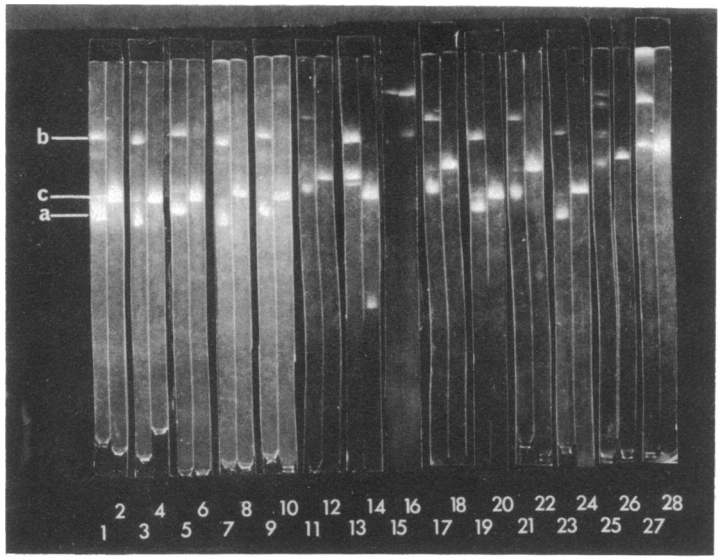

FIG. 1. *Plasmid DNA after electrophoresis on agarose gels in the presence of ethidium bromide. Apart from gels 15 and 16, electrophoresis was continued for 4.5 h. The numbered gels are in pairs containing the same plasmid DNA, the first being native and the second EcoRI-treated DNA as follows: 1 and 2, R300B; 3 and 4, R305C; 5 and 6, R310; 7 and 8, R450B; 9 and 10, R464C; 11 and 12, R676; 13 and 14, R678; 15 and 16, also R678 but subjected to electrophoresis for only 90 min (see text); 17 and 18, R682; 19 and 20, R684; 21 and 22, R750; 23 and 24, R1162; 25 and 26, PB165; 27 and 28, R7K-TnC1. The DNA bands were visualized by illumination with ultraviolet light, which fluoresces with the DNA-ethidium bromide complex. The bands in gels 1 and 2 are the CCC (a), OC (b), and linear (c) tertiary forms of R300B DNA (see text).*

counts in the peaks were proportional to our estimates of their molecular weights. Thus, we are confident that R678 is cut into three DNA fragments by *Eco*RI. We repeated the gel electrophoresis with a fresh preparation of R678 DNA using more DNA than previously (ca. 100 ng) and running it for a shorter time (90 min). Under these conditions, three bands were seen in the gel containing the *Eco*RI-treated DNA (Fig. 1, gel 16). The faint, fast-running band had presumably run off the end in the longer run (4.5 h, gel 14).

Clearly, both sucrose gradient analysis and gel electrophoresis can detect DNA fragments only above a limiting molecular weight. We feel that it is unlikely that we have failed to detect fragments above this limit from the other plasmid DNAs, because the distances travelled by the visible band in each correlate very well with the molecular weights of each entire plasmid (Table 1). There is no evidence of any small DNA fragments in the sucrose gradient analyses of *Eco*RI cloven R300B (Fig. 2b, 3, and 4).

Analysis of PB165. PB165 has been shown to consist of three supercoiled molecules of molecular masses 7.4, 14.7, and 21.4 Mdal (4), and we have suggested that these might be oligomers of a 7.4-Mdal plasmid.

We have also examined a clone of *E. coli* C transformed with DNA from PB165 and found the plasmid DNA to consist of the above three molecular species (data not shown). If this transformation involved the uptake of only a single DNA molecule, then the observed species must be oligomerically related.

Gel electrophoresis of untreated PB165 DNA gave six bands (presumably three CCC and three OC forms; gel 25, Fig. 1). After *Eco*RI treatment only a single band is visible (gel 26). This was confirmed by sucrose gradient analysis, which showed a single linear DNA species of molecular weight 7.6×10^6 (Fig. 4). We conclude that PB165 exists in the cell as oligomers, the monomer having a single *Eco*RI cutting site.

Compatibility testing. As we had established by DNA-DNA hybridization (4) that 10 of this group of SmSu plasmids were very closely related, we expected them to belong to the same incompatibility group. But as R678 and PB165 were less closely related to this group, and especially as PB165 exists intracellularly as oligomers, we considered that they might belong to different groups.

We therefore tested the compatibility of each of the SmSu plasmids with the ampicillin resistance transposition derivative (Ap201) of the

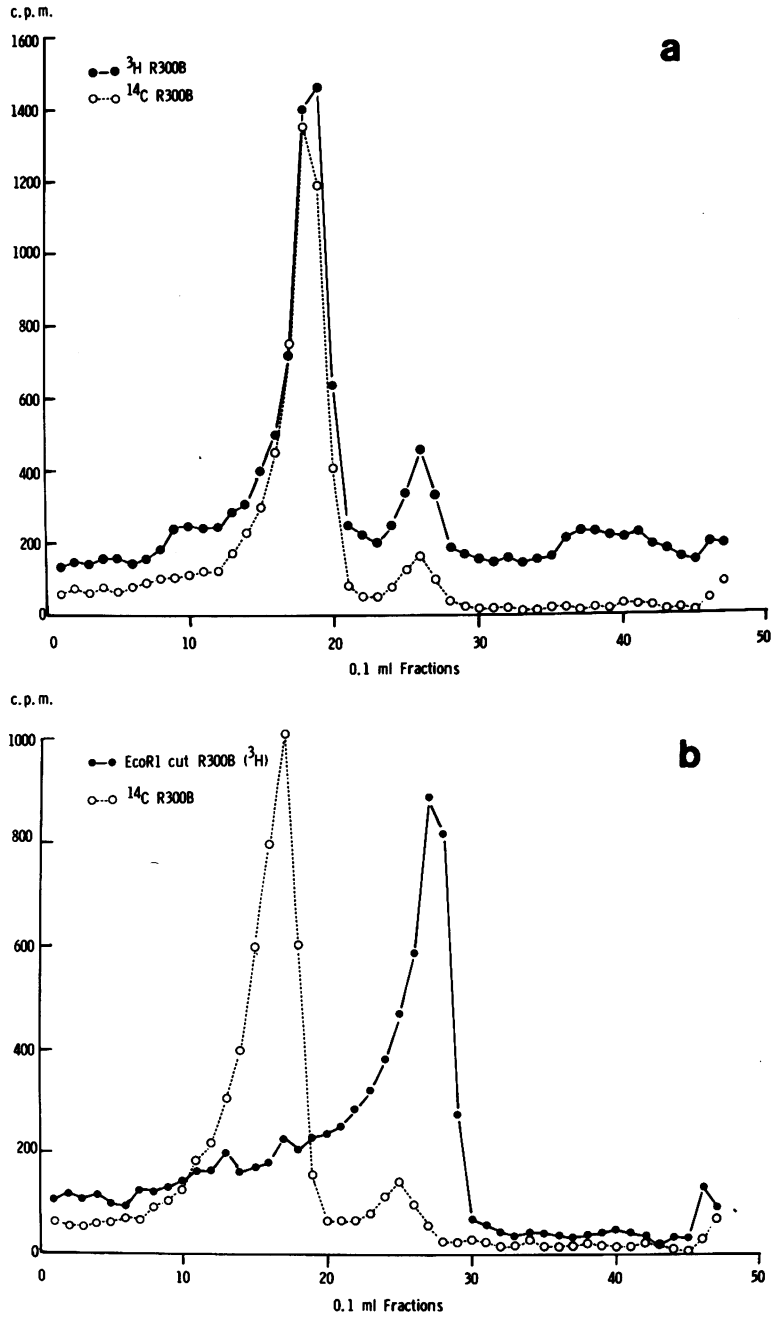

FIG. 2. *Sucrose gradient sedimentation analyses of R300B DNA. (a) Control of untreated ^{3}H-labeled R300B with ^{14}C-labeled R300B. The two peaks for each sample are, left to right, CCC and OC forms, respectively. (b) EcoRI-treated ^{3}H-labeled R300B with untreated ^{14}C-labeled R300B. The single ^{3}H peak represents linear DNA (see text). Sedimentation was from right to left at 100,000 × g for 4 h.*

SmSu plasmid RSF1010. This derivative no longer confers sulfonamide resistance (12). R144*drd*3 (14) was used to mobilize Ap201 into recipient strains carrying the SmSu plasmids, selecting for Ap^r recipient clones. After purification, 20 transconjugants from each cross were tested on plates containing ampicillin, kanamycin (Km), or sulfonamide ditches. All 20 were Ap^r and Su^s, from which we conclude that the introduction of Ap201 has eliminated the

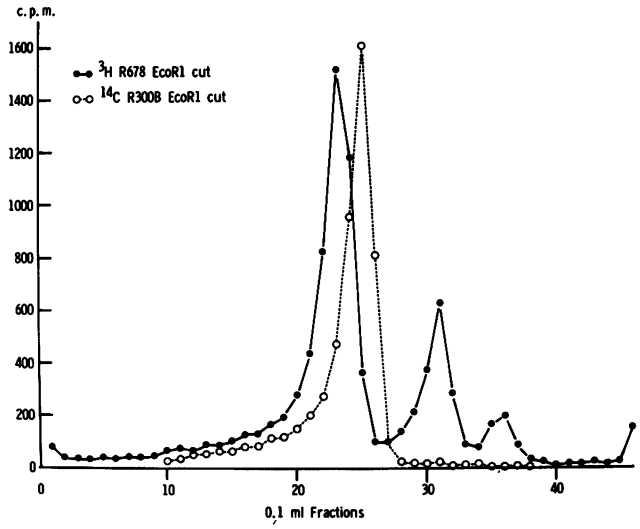

FIG. 3. *Sucrose gradient sedimentation analysis of an EcoRI-treated mixture of ^{3}H-labeled R678 and ^{14}C-labeled R300B DNA samples. R678 is cut into three DNA fragments. Sedimentation was at 150,000 × g for 3 h.*

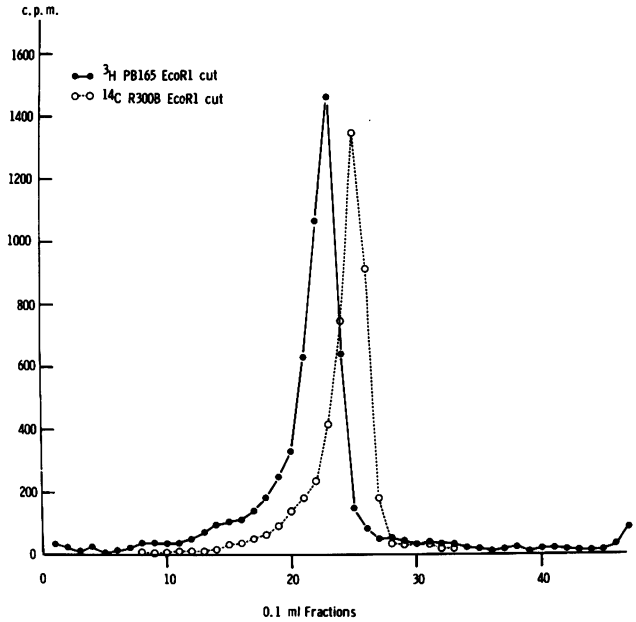

FIG. 4. *Sucrose gradient sedimentation analysis of an EcoRI-treated mixture of ^{3}H-labeled PB165 and ^{14}C-labeled R300B DNA samples. The three DNA species seen in analyses of untreated PB165 (4) have been reduced to a single linear DNA peak of 7.6 Mdal. Sedimentation was at 150,000 × g for 3 h.*

SmSu plasmid. This was true for each of the 12 plasmids tested (Table 1). The majority of the recipients also received R144*drd*3 (as shown by Km^{r}), which belongs to incompatibility group (Inc) Iα (10) and which does not itself eliminate SmSu plasmids (8). We conclude that these SmSu plasmids all belong to the same incompatibility group, which we propose to designate IncQ.

Relationship between electrophoretic mo-

bility and DNA molecular weight. We noted above that the tertiary forms of DNA from the 5.7-Mdal plasmids were separated by gel electrophoresis in the following order from the origin: OC, linear, and CCC. In contrast, Thompson et al. (17) found that under their experimental conditions, the order of DNA tertiary forms was: CCC and then OC and linear together. They studied plasmid DNA of molecular weights 25×10^6 to 65×10^6. To see whether this discrepancy is due to the different molecular weights of the DNAs used, we ran agarose gels with a larger plasmid, R7K-TnC1, which has a molecular weight of 33.5×10^6 and a single *Eco*RI cleavage site (3). A preparation of DNA of this plasmid (shown by sucrose gradient analysis to consist of predominantly CCC molecules, data not presented) gave two bands after electrophoresis, in order from the origin: a dense (CCC) band and a fainter (OC) band that had moved about twice as far through the gel (gel 27, Fig. 1). *Eco*RI-cut DNA gave a single dense band coincident with the OC band of the control (gel 28, Fig. 1). These observations agree with those of Thompson et al. (17), and thus we conclude that the relative electrophoretic mobilities of the three tertiary forms of a plasmid DNA molecule are critically dependent on the molecular weight of the DNA being analyzed. For the 5.7- to 6.3-Mdal DNA, the relative mobilities in increasing order are OC, linear, and CCC. By 9.2 Mdal (R678, gel 13) the linear form has become the fastest, and by 33.5 Mdal (R7K-TnC1, gels 27 and 28) the order is CCC and then coincident OC and linear.

DISCUSSION

We have studied the *Eco*RI cleavage susceptibility of a group of SmSu plasmids that we have previously shown to be related (4). Each of the 10 very closely related plasmids has a single *Eco*RI cleavage site, whereas R678, which is 60% larger and only 45% homologous with the reference plasmid (R300B, one of the ten), has three such sites. Further, as only a single DNA band is seen in the analysis of *Eco*RI-cut PB165 DNA, we have concluded that the three supercoiled molecular species of PB165 are oligomerically related (as their molecular weights suggest). We also conclude that, unless the *Eco*RI site is in the middle of the monomer subunit, these monomers must be joined "head to tail" within the oligomers. We have found that all 12 SmSu plasmids are incompatible with Ap201 and are therefore all in the same incompatibility group (IncQ).

We suggest that the sharing of a common incompatibility group reflects a fundamental common property of these plasmids, namely, their replication control mechanism. This supports our previous contention (4) that these widely spread and very common SmSu plasmids may have originated from a common ancestor.

Whereas we expected the 10 very similar plasmids to be in the same group, we supposed that PB165, with its markedly different molecular weight, DNA homology, and, most important, its existence as oligomers, would probably be in a different incompatibility group. Data from our previous paper (4) show that PB165 has 0.8, 1.3, and 4.1 copies of the trimer, dimer, and monomer, respectively, per bacterial chromosome, which add up to 9 copies of the monomer subunit per chromosome. This matches closely the average of 10 copies/chromosome found for the other SmSu plasmids (especially when one also includes the small amount of tetramer we have observed in some sucrose gradient analyses of PB165 DNA). Thus, PB165 has the same incompatibility function and control of copy numbers (considering the monomer subunit) as the other SmSu plasmids. This is consistent with the model of Pritchard et al. (15) that incompatibility between two plasmids is brought about by their producing an identical or similar "initiation repressor" that controls DNA replication negatively. Each type of initiation repressor gives a characteristic number of plasmid copies per cell, so that two plasmids producing the same repressor within the same cell could not replicate to give more than this characteristic number of copies irrespective of whether these copies exist as monomers or are joined up into oligomers. If segregation at cell division is random with respect to plasmid type, the two plasmids will be observed to segregate, i.e., be incompatible. A negative control model of DNA replication has been supported by recent data (6, 18). The positive control (attachment site) model of Jacob et al. (13) predicts, on the other hand, that the total number of PB165 replicons (as opposed to monomer subunits) would be controlled at the number characteristic of the particular attachment site used by the plasmid for replication. Since PB165 is incompatible with, and thus uses the same attachment site as, the other SmSu plasmids, this model would predict the presence of 10 PB165 replicons/chromosome. Our data of 0.8 + 1.3 + 4.1 giving 6.2 total replicons are less consistent with this model than with a negative control model.

We must also conclude from the above that the formation of oligomers by a plasmid depends upon controls (and replication or recom-

bination mechanisms?) that are different from those regulating its replication; i.e., the initiation control of a plasmid, and its subsequent type of replication, are not necessarily linked. We have observed another example of this phenomenon: RP4 and R751 both belong to incompatibility group P, but whereas R751 produces oligomers, RP4 does not (unpublished data).

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