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Control of pT181 replication II. Mutational analysis

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We describe the isolation and analysis of mutations affecting the regulation of *Staphylococcus aureus* plasmid pT181 replication. Previous results suggested that regulation is achieved by control of the synthesis of RepC, a plasmid-coded replication protein and that the primary negative control element is CopA RNA, which consists of two transcripts that are complementary to the 5′ region of the *repC* mRNA leader. CopA inhibition probably involves a base pairing interaction with the complementary region of the RepC mRNA leader which would facilitate the formation of a downstream stem-loop in the leader that occludes the *repC* ribosome binding site. RepC is freely diffusible so that regulation of pT181 replication is indirect. Both CopA RNA-sensitive (recessive) and -insensitive (dominant) mutants were isolated. The recessives have defects in CopA RNA structure or activity, the dominants have defects in the site of action (target) of the inhibitor. Some dominants were located within the *copA* coding sequence. These therefore affect the structure of CopA RNA as well as that of its target. Other dominant mutations mapped outside of the *copA* gene and therefore produced wild-type CopA RNA. In contrast to directly regulated plasmids, pT181 copy mutants producing wild-type inhibitor could be co-maintained with the wild-type plasmid and mutational changes in inhibitor-target specificity did not change incompatibility specificity.

Key words: plasmid pT181/replication/regulation/mutations

Introduction

Bacterial plasmids maintain stable copy numbers over a wide range of growth conditions by explicit regulatory mechanisms which ensure rapid self-correction in response to fluctuations – much as is the case with biosynthetic enzymes (cf. for example, Gorini and Maas, 1957). Because plasmids control their own replication rates, the controlling element(s) are necessarily inhibitory; therefore, the regulatory circuit involves, at a minimum, an inhibitor and its site of action (target). These can be defined genetically by the isolation and characterization of control (copy) mutants affecting either the inhibitor (recessive) or target (dominant).

Replication of the ColE1 and IncFII plasmids of *Escherichia coli*, is directly regulated by an inhibitory RNA molecule transcribed from the 5′ end of the opposite strand of the coding region of a longer transcript that is required for replication. Copy control mutations commonly map in the region of overlap between the inhibitory counter-transcript and the replication transcript and define the site of primary interaction between the two RNA species. Because they affect the sequence specificity of the inhibitor-target interaction, they cause some degree of independence of mutant and wild-type copy control systems. In extreme cases, this results in new incompatibility groups (Tomizawa and Itoh, 1981; Easton and Rownd, 1982).

This report is the second of three in which it is established that pT181 replication is indirectly regulated by two counter-transcripts that inhibit the synthesis of RepC, a replication protein, in a gene dosage-dependent manner. In the first of the three papers (Novick *et al.* , accompanying paper), the genetics and physiology of the inhibitor are described and it is established that the regulatory system controls RepC synthesis. In the present paper, we report the isolation, properties and sequence analysis of copy mutants. This study has revealed that the genetic determinants of inhibitor and target overlap; many mutations are located in the *repC* mRNA leader region in a sharply defined cluster that presumably defines the primary site of the inhibitor-target interaction. In the third paper (Kumar and Novick, 1984), the counter-transcripts are identified and mapped to the 5′ end of the *repC* mRNA coding region, and shown to include the cluster of copy mutations.

It is proposed that RepC synthesis is regulated by a complementary RNA-RNA interaction which takes place at the 5′ end of the RepC mRNA leader, facilitating the formation downstream of a hairpin just 5′ to the protein start. This hairpin sequesters the ribosome binding site for the protein and would therefore be expected to interfere with translation and/or promote termination (attenuation) at that site.

Results

Isolation of copy mutants

pT181 specifies an inducible Tc^r determinant that confers basal and induced resistance levels of 5 and 40 μg/ml, respectively. Copy mutants were obtained by selecting for resistance to 75–150 μg/ml and screening by gel electrophoresis of sheared whole-cell lysates for visually detectable copy effects. Spontaneous mutants with plasmid-linked copy effects were obtained at a frequency of 10^{-7}–10^{-6}/colony-forming unit. Twenty of these were chosen for further study, and in Figure 1 is shown the stained agarose gel pattern obtained with whole-cell lysates of strains carrying mutant plasmids. One additional plasmid, pSA0331, a pT181 derivative with a thermosensitive replication defect (Iordanescu, 1976a) was studied. This plasmid has a high copy number at 32°C and was predicted to be a double mutant (see below).

pT181 specifies a *trans*-active replication inhibitor, CopA, which, when cloned at an elevated copy number, excludes autonomous pT181 (Novick *et al.*, accompanying paper). Thus, an alternative means of obtaining control mutants was by selection for CopA resistance. Such mutants were obtained by transducing pT181 into a strain containing pRN6116, a

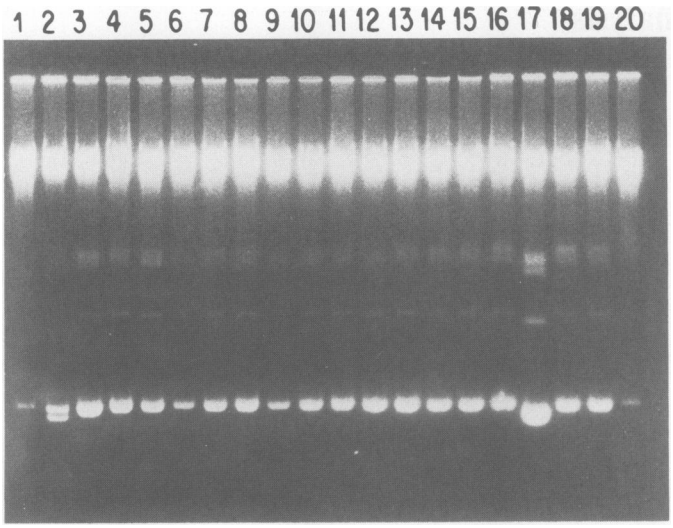

Fig. 1. Agarose gel electrophoretic analysis of copy mutants. Whole-cell sheared mini-lysates were separated on 1% agarose in Tris borate buffer for 18 h at 2.5 V/cm, stained with ethidium bromide and photographed. Samples corresponding to equivalent numbers of starting cells were loaded ($\sim 10^8$ cells/sample) in order to permit comparison. Gels of this type were used for copy number determination. **Tracks 1,20**: pT181; **2**: *cop-626;* **3**: *623*; **4**: *622*; **5**: *621*; **6**: *620*; **7**: *619*; **8**: *618*; **9**: *617*; **10**: *615*; **11**: *614*; **12**: *613*; **13**: *612*; **14**: *611*; **15**: *610*; **16**: *609*; **17**: *608*; **18**: *607*; **19**: *601*. The heavy upper and lower bands respresent sheared chromosomal and supercoiled plasmid monomeric DNA, respectively. The uppermost band represents a small amount of material that remains in the sample well. The intermediate bands represent plasmid isomers including oligomers and relaxed monomers.

CopA$^+$ test plasmid, with selection for Tc^r. Of >300 transductants screened, three contained pT181-linked mutations of the desired type, most of the others containing recombinant plasmids. All three had elevated copy numbers and were designated *cop-640, 641* and *642*. As shown in Table I, the mutants had copy numbers ranging from 70 to ~900, as compared with ~22 for the wild-type, with most falling between 100 and 200.

Classification of copy mutants

Mutants insensitive to CopA inhibition are referred to as dominant, those sensitive to CopA as recessive. Dominance and recessivity were scored by reciprocal transductions with the CopA$^+$ test plasmid, pRN6116. In one direction ability of the mutant to become established in the presence of pRN6116 was measured; in the other direction, displacement of the mutant by the incoming CopA$^+$ test plasmid was scored With one exception, mutants that were unable to establish in the presence of the co-integrate (recessives) were quantitatively displaced by it and those that were able to establish (dominants) were not displaced. The exception, *cop-623*, was not displaced but was able to establish only at a greatly reduced frequency. Electrophoretic analysis revealed that the copy numbers of the dominant mutants were generally unaffected by the presence of pRN6116. A summary of these results is presented in Table I, in which the inability of a mutant to coexist with pRN6116 is indicated by a (−) sign. For the dominant mutants, copy numbers in the presence as well as in the absence of pRN6116 are listed.

In addition to *copA*, which is located between nucleotides 158 and 570 (Novick *et al.*, accompanying paper), pT181 has a second, weaker incompatibility determinant, *inc3B*, which is located to the left (Novick *et al.*, accompanying paper; see Figure 2). The copy mutants were also tested for sensitivity to Inc3B. Here, the test plasmid was pRN6264, a derivative of a pT181::pE194 co-integrate with a deletion of *copA* (Novick *et al.*, accompanying paper). Most of the mutants were indifferent to Inc3B in transduction tests; however, the copy numbers were reduced, to a greater or lesser extent as shown in Table II. It is noted that the *inc3B* region contains the origin of pT181 replication and it is possible that the inhibitory activity of *inc3B* involves titration of the RepC protein by binding to the origin.

Mapping of copy mutations

Nucleotide sequence analysis (Khan *et al.*, 1982) has shown that pT181 *cop-608*, which lacks both *copA* and the target of the inhibition, *copT*, has a 180-bp deletion extending from nucleotides 183 to 362 (see Figure 2). Two other dominant copy mutants, *cop-626* and *cop-633*, were also found to have deletions within the 345-bp *Mbo*I-D fragment (nucleotides 31−376). This suggested that the point mutations might also be located in this region. Accordingly, the *Mbo*I-D fragment was isolated and sequenced for each of the spontaneous dominant and recessive mutants under consideration. These results are shown in Figure 3 and confirm that the copy mutations are accompanied by single base changes in the *Mbo*I-D fragment.

Verification of copy number mutant sequence positions

Removal of *Dde*I fragments B (1028−1931) and D (770−1028) generated a viable, 3.3-kb plasmid expressing tetracycline resistance and consisting of two *Dde*I fragments A (1931−265) and C (265−770). This plasmid (pRN8099) was found to have the same copy number, incompatibility, and stability properties as the parent, wild-type plasmid. The *Dde*I site at 265 is located within the pT181 control region, some of the copy mutations falling to its left, others to its right. A series of reconstructions was then performed in which specific segments of the reduced wild-type plasmid were replaced by the corresponding segment of a copy mutant plasmid and *vice versa*. If the nucleotide changed was to the right of *Dde*I (265), the mutant segment from 265 (*Dde*I) to 377 (*Mbo*I) was ligated to a 377 (*Mbo*I)-770 (*Dde*I) segment from the wild-type and this hybrid segment then ligated to a preparation of *Dde*I-A (1931−265) DNA from the wild-type plasmid. In all cases, Tc^r transformants obtained with such constructions displayed the copy number of the mutant plasmid. As a control, in each case, the *Dde*I-A fragment from the mutant plasmid was ligated to *Dde*I-C from the wild-type. Here, the transformants uniformly displayed the wild-type copy number. A similar strategy was employed for the mutants that had base changes to the left of *Dde*I (265). Here, a *Hinf*I-*Dde*I co-fragment (191−265) was ligated to a partial *Hinf*I digest of *Dde*I-A from the wild-type (note that there is also a *Hinf*I site at 160) and the resulting reconstructed *Dde*I-A fragment ligated to *Dde*I-C DNA from the wild-type. Again, the reconstructed plasmids recovered by transformation always displayed the mutant copy number and the control plasmids, constructed by combining intact wild-type *Dde*I-A with mutant *Dde*I-C, uniformly gave transformants with the wild-type copy number. Note also that for each mutant, the phenotype was recovered upon the insertion of a fully sequenced segment, confirming definitively the assignment of copy mutations to the base sequence changes as shown in Figure 3. In this manner the sequence positions of *cop-607, 609, 613, 614, 615, 617, 618, 619, 620,*

Table I. Copy mutants

Plasmid	Allele	Copy no. ± S.D.	Copy no. in the presence of: pRN6116	pRN6264	Base change[a]
pT181	Wild-type	22 ± 5 (10)	–	<5	None
pRN8044	cop-404	160 ± 20 (4)	150	100	308 C-A
pRN8007	cop-607	170 ± 20 (3)	190	110	244 T-C
pRN8008 Δ144	cop-608	800 ± 190 (7)	900	650	Δ183–362
pRN8009	cop-609	74 (1)	–	82	207 A-G
pRN8010	cop-610	280 ± 80 (3)	140	ND	–
pRN8011 pRN8013	cop-611 cop-613	195 ± 35 (6)	190	110	310 G-T
pRN8019 pRN8020	cop-614 cop-615	150 ± 25 (7)	130	85	310 G-A
pRN8022	cop-617	75 ± 14 (3)	89	ND	312 C-T
pRN8023	cop-618	210 ± 50 (3)	165	92	312 C-A
pRN8024	cop-619	135 ± 22 (5)	–	ND	233 A-G
pRN8025 pRN8130	cop-620 cop-642	70 ± 15 (3)	56	<5	315 G-C
pRN8026	cop-621	150 ± 25 (3)	–	27	367 C-T
pRN8027	cop-622	120 ± 10 (4)	–	ND	318 C-A
pRN8061	cop-623	400 ± 75 (3)	360	300	187 C-A
pRN8062 Δ255	cop-626	800 (1)	+	ND	Δ157-314
pRN8109	cop-632	145 ± 20 (3)	120	30	314 C-A
pRN8110 Δ257	cop-633	300 (1)	+	ND	Δ208–280
pSA0331	cop-634	145 ± 25 (3)	140	ND	314 C-A β 275 A-G
pRN8133	cop-640	220 ± 40 (4)	200	ND	310 G-A
pRN8135	cop-641	130 ± 15 (4)	110	ND	316 C-T

[a]Base changes are given for the 3′ strand as shown in Figure 4.
[b]'–' signifies inability of the mutant to co-exist with the test plasmid; '+' signifies stable co-existence but copy numbers were not measured.
ND = not done.

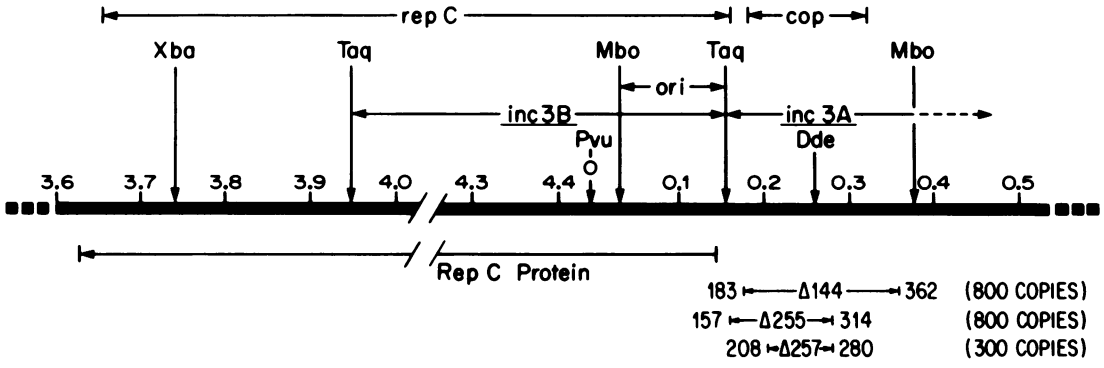

Fig. 2. Linear map of the pT181 copy control region. Heavy line represents the pT181 genome with figures in kb and critical restriction sites indicated. Approximate extents of the various known genetic determinants are indicated above; extents of the three deletions affecting copy control are indicated below, including copy numbers and nucleotide coordinates.

621, 622, 623, 632 and *641*, were verified and shown to correlate with the phenotype of the original mutant plasmids. Fragment exchange experiments with pSA0331 revealed that there were two separate mutations, one responsible for the thermosensitivity and a second responsible for the elevated copy number. The former was identified as an A to C change at position 275, the latter as a G to A change at position 314 – the same change as that found for *cop-632*. The location of the thermosensitive mutation in the 5′-untranslated region of the *repC* gene (Kumar and Novick, 1984) suggests that the thermosensitivity must be due to a temperature-dependent deficiency in production of RepC rather than to a defect in

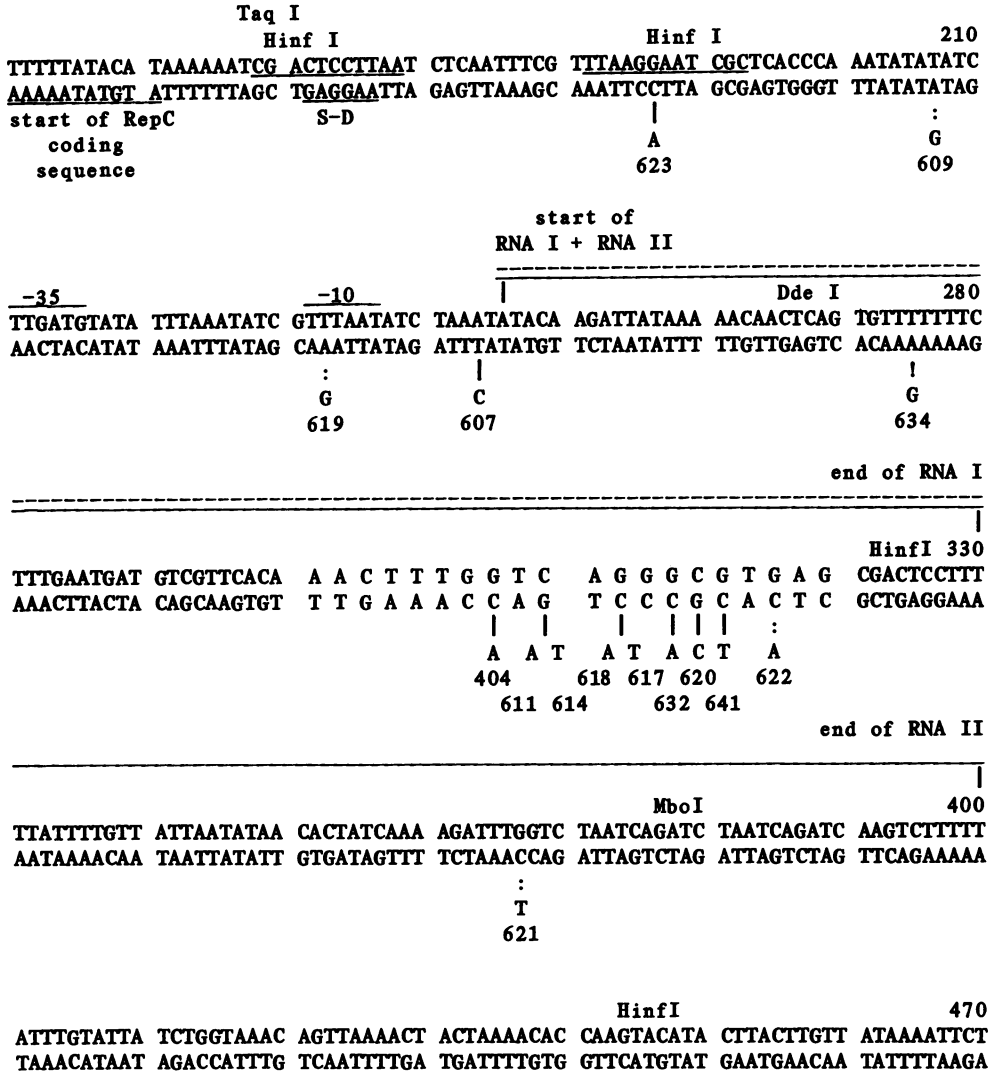

Fig. 3. Sequence of the pT181 copy control region between bases 141 and 470. Restriction enzyme recognition sites are indicated as well as a putative ribosome binding site and start site for the RepC coding sequence – read from right to left. Base sequence changes in several independently isolated copy mutants of pT181 are as follows: | = dominant mutation; : = recessive; ! = temperature-sensitive for replication.

the protein itself.

Complementation analysis

CopA inhibition can be overcome in *trans* by any dominant pT181 copy mutant (Novick *et al.*, accompanying paper). This result establishes that pT181 is regulated indirectly (Grindley *et al.*, 1978) and predicts that wild-type pT181 or recessive copy mutants will be able to co-exist with any dominant copy mutant.

As illustrated in Figure 4, it was possible to establish heteroplasmids with pSA5000 and pT181 or any of its copy mutants. It was also possible to establish heteroplasmids containing either of two pSA5000 copy mutants and pT181 or its copy mutants. Most of these heteroplasmids were unstable, segregating monoplasmid variants, and required doubly selective media for maintenance. Heteroplasmid instability was not observed for pairs of mutants with very high copy numbers. To determine whether this type of stability was due to genotypic changes in incompatibilty type, as has been observed for other plasmids, or was due simply to the high copy numbers *per se*, we treated the heteroplasmids for reciprocal intrapool amplification by propagating them under different selective conditions. With genotypically incompatible plasmids, the plasmid favored by selective conditions is amplified at the expense of the other, the total pool size remaining constant (Projan and Novick, 1984). With compatible plasmids, the pools are independent and indifferent to selective conditions.

Heteroplasmids containing either pRN8008, pRN8061, pRN8023, pRN8110, or pRN8133 and pSA5000 were constructed and found to show reciprocal intrapool amplification as were heteroplasmids containing pRN8044, or pRN8045 and pT181. No pair of single replicons was found that did not show the effect.

Table II lists the copy numbers of heteroplasmids containing pT181 or one of its copy mutants plus pSA5000 grown under conditions of minimal selection to eliminate selective amplification. In most cases, the copy numbers of the two are approximately equal, consistent with the unrestricted availability in *trans* of all RepC protein in the cell. Note in particular that the wild-type plasmid and recessive mutants are able to co-exist with *cop-623*, a dominant mutation mapping

outside the *copA* coding sequence (Kumar and Novick, 1984) and expressing wild-type CopA activity (Novick *et al.*, accompanying paper). For recessive/wild-type heteroplasmids, the copy pool is approximately double that of the wild-type alone. This is expected since only one of the pair has an active inhibitor gene whereas both actively produce RepC. For dominant/wild-type pairs, the overall pool is substantially elevated because the dominant mutant produces RepC independently of the presence of the wild-type inhibitory activity. The overall pool in this case is lower than that of the mutant alone because both plasmids utilize the available RepC activity, all of which is produced by the mutant – RepC production by the wild-type would be shut off by its own elevated inhibitory activity plus that of the mutant (Novick *et al.*, accompanying paper). The basis of the asymmetric pools observed in a few cases is unknown.

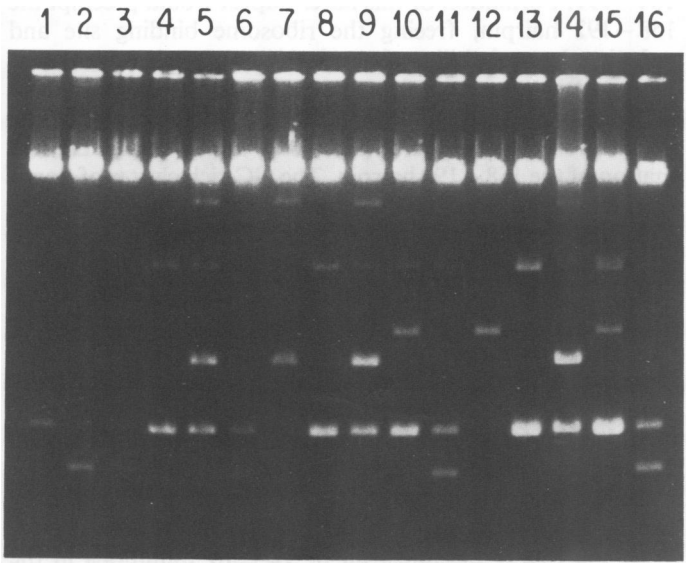

Fig. 4. Agarose gel electrophoretic analysis of copy mutant interactions. Sheared whole-cell mini-lysates were prepared and analyzed as in the experiment shown in Figure 1. **Track 1**: pT181; **2**: pSA5000; **3**: pT181; pSA5000; **4**: pRN8024 (*cop-619*); **5**: pRN8024; pRN6264 (Inc3A$^-$,Inc3B$^+$); **6**: pRN8024; pSA5000; **7**: pRN6264 (Inc3A$^-$,Inc3B$^+$); **8**: pRN8020 (*cop-615*); **9**: pRN8020; pRN6264 (Inc3A$^-$,Inc3B$^+$); **10**: pRN8020; pRN6116 (Inc3A$^+$,Inc3B$^-$); **11**: pRN8020; pSA5000; **12**: pRN6116 (Inc3A$^+$,Inc3B$^-$); **13**: pRN8061 (*cop-623*); **14**: pRN8061; pRN6264 (Inc3A$^-$,Inc3B$^+$); **15**: pRN8061; pRN6116 (Inc3A$^+$;Inc3B$^-$); **16**: pRN8061; pSA5000.

Inhibitor-target specificity and incompatibilty.

Changes in inhibitor-target specificity for directly regulated plasmids such as of the ColE1 and IncFII types result in the establishment of new incompatibility groups (Tomizawa and Itoh, 1981; Lacatena and Cesareni, 1981; Easton and Rownd, 1982). We considered it probable that this would not be the case for indirectly regulated plasmids such as pT181 because the product of the regulated step, RepC, would always be mutually available to all pT181-like plasmids regardless of the specificities of their copy control elements. To test this prediction, we cloned the *copA* determinants (as described by Novick *et al.*, accompanying paper) from several plasmids with target mutations and tested these for their ability to inhibit replication of the wild-type and mutant plasmids. The cloned *copA* gene from each of two mutants, *cop-404* and *640*, at 55 copies/cell, was found to have no detectable effect in *trans* on the copy number of either of the plasmids (not shown). Further, heteroplasmid strains containing either of the copy mutants and either of the *copA* clones showed no segregation of the copy mutant plasmid during growth on non-selective media. A heteroplasmid containing the two mutant plasmids showed reciprocal intrapool amplification with approximately the same kinetics as did other pairs of mutant plasmids with similar copy numbers, including pairs containing the same *cop* allele. These heteroplasmids also segregated during growth on non-selective media, but at very slow rates due to their high copy numbers. These results suggest that for pT181, segregational incompatibility is independent of the target specificities of the inhibitor RNAs.

Behavior of copy mutants of B. subtilis

In view of the possibility that genetic control elements may function differently in different species, we transferred several of the pT181 copy mutants, *cop-608, 614, 615, 618, 619, 621, 622* and *623* to *B. subtilis* by protoplast or competent cell transformation, selecting for tetracycline resistance.

Table II. Interactions between pT181 copy mutants and pSA5000

Plasmid	cop allele	Copy no. in heteroplasmid state		Sum of wild-type plus mutant	Homoplasmid copy number
		pT181 replicon	pSA5000		
pT181	Wild-type	–	–	–	22
pSA5000	Wild-type	–	–	–	22
pT181	Wild-type	12	10	22	22
pRN8009	cop-609(R)[a]	22	32	54	74
pRN8011	cop-611(D)[a]	25	110	135	200
pRN8020	cop-615(D)	70	63	133	150
pRN8023	cop-618(D)	46	170	216	210
pRN8024	cop-619(R)	20	21	41	140
pRN8026	cop-621(R)	85	23	108	150
pRN8027	cop-622(R)	32	22	54	120
pRN8061	cop-623(D)	110	130	240	400

[a]R = recessive, D = dominant or overlap mutant.

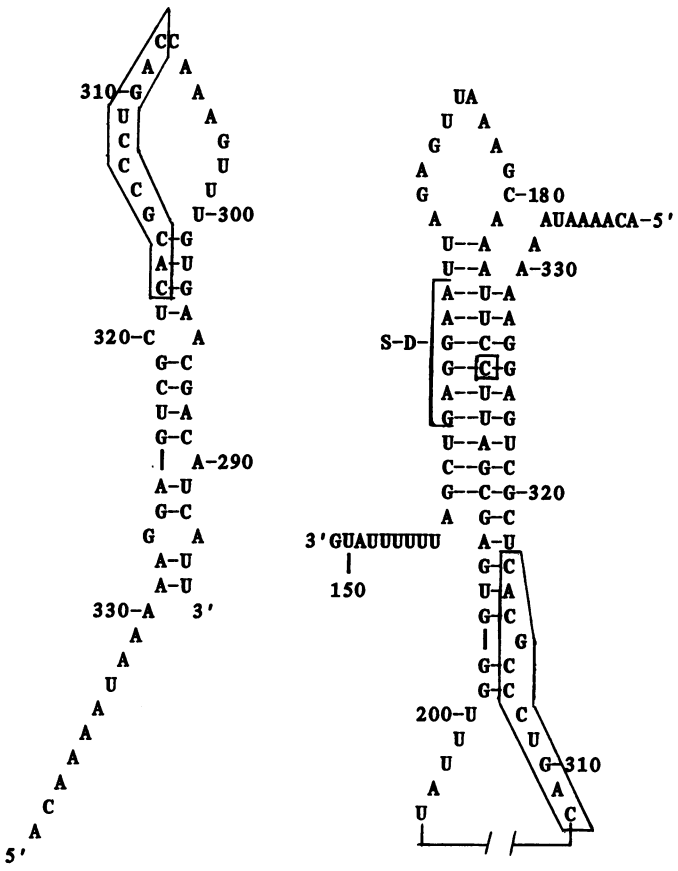

Fig. 5. Possible RNA structures involved in RepC expression (see text).

Transformants were analyzed for plasmid copy number and, in some cases, for vegetative plasmid stability. Two of the copy mutants (*cop-615* and *cop-618*) had approximately the same plasmid-to-chromosome ratios in *B. subtilis* as in *S. aureus*. This is consistent with a similar functionality for the copy control system in the two species. One mutant, *cop-63*, had about 1/3 as much plasmid DNA in *B. subtilis* as in *S. aureus*. The other, *cop-608*, had about 1/20 as much – in fact, it had only twice the level of plasmid DNA as wild-type pT181 in this host species.

Four of the copy mutants were tested for vegetative stability and were as unstable in *B. subtilis* as the wild-type plasmid (D. Usadi and R. Novick, unpublished data), having segregation rates of ~1% per generation. This indicates that instability in *B. subtilis* is not a simple function of copy number.

Discussion

All of the mutations and deletions affecting copy control are located in a region of some 200 bp immediately 5′ to the start of the *repC* coding sequence. Most of the dominant mutations involve changes within a very short GC-rich segment (303–316), which probably represents the physical target of the inhibition. The similarity of this organization to that of the control regions of the ColE1 and IncFII plasmids of *E. coli* suggests that there may be a parallelism between the functional elements of pT181 and those of the other plasmids. Kumar and Novick (1984) have demonstrated two tandem leftward starts for the *repC* mRNA at positions 339 and 410, respectively and two rightward counter-transcripts, 80 and 150 nucleotides in length, initiating at position 246 (see Figure 3). Both of these counter-transcripts are involved in copy control (unpublished data) and their complementarity with the mRNA leader region suggests that the regulatory mechanism may involve RNA-RNA interactions between counter-transcript and mRNA leader. RNA-DNA interactions are also theoretically possible.

The region from 158 to 192 (see Figure 5) can form a hairpin which resembles rho-independent terminators (Rosenberg and Court, 1978) and in which the *repC* ribosome binding site is fully base paired. An upstream segment, nucleotides 313–329 can form a strong duplex with nucleotides 184–197. Formation of this latter duplex would preempt the 158–192 hairpin, freeing the ribosome binding site and reducing the probability of termination (see Figure 5). The inhibitory RNAs are complementary to the upstream portion of the leader; the formation of an RNA-RNA duplex would tie up the preemptor sequence (313–329) and so permit the formation of the 158–192 hairpin. The GC-rich cluster of dominant mutations presumably defines the site of the primary interaction between the two RNAs. This region is single-stranded in the optimal computer-generated (Zuker and Stiegler, 1981) secondary structures of RNA I, RNA II and the mRNA leader (Kumar and Novick, 1984), except that for the latter, this is true only early in transcription (see Figure 5); once the region from 200 to 180 has been transcribed, the optimal folding includes the structure shown at right in Figure 5, in which the target region is partially base-paired. Nearly all of the mutations in this region involve a GC to AT change – a change that would reduce the efficiency of any primary pairing interaction between the two polynucleotide chains. With the IncFII plasmids, all of 12 copy mutations in the overlap region also involve GC to AT changes in the target loop. With pT181, only copy mutants with GC to AT changes in this region were obtained even if selection was for resistance to inhibition by wild-type CopA.

This result is different from the results with ColE1, in which selection for resistance to WT RNA I inhibition resulted in GC to AT and AT to GC changes in the target loop with about equal frequency (Lacatena and Cesareni 1981). Moreover, most of these mutations had very little effect on copy number, regardless of the type of base change involved, unless the mutation had a primary effect on the secondary structure of the interacting species. It appears, therefore, that the pT181 and the IncFII copy control mechanisms are much more sensitive to complementary GC to AT target loop changes than is ColE1.

The postulated role of the 158–192 hairpin is supported by the location of *cop-623* (position 187, see Figure 5). This mutation lowers the calculated stability of the hairpin from −13.9 kcal to −6.0 kcal, and effectively bypasses the pT181 copy control circuit.

Other mutants mapping outside of the leader-counter-transcript overlap region are *cop-607, 609* and *619*. The latter two are recessive and are located in the counter-transcript promoter; both produce decreased amounts of the two counter-transcripts (Kumar and Novick, 1984). The third mutant, *cop-607*, is dominant and the basis of its cop$^-$ phenotype is not presently understood.

It is noted that the IncFII plasmids are controlled by a counter-transcript that inhibits translation of the mRNA for RepA, a replication protein (Light and Molin, 1982). CopA RNA may also act by complexing a preemptor sequence in

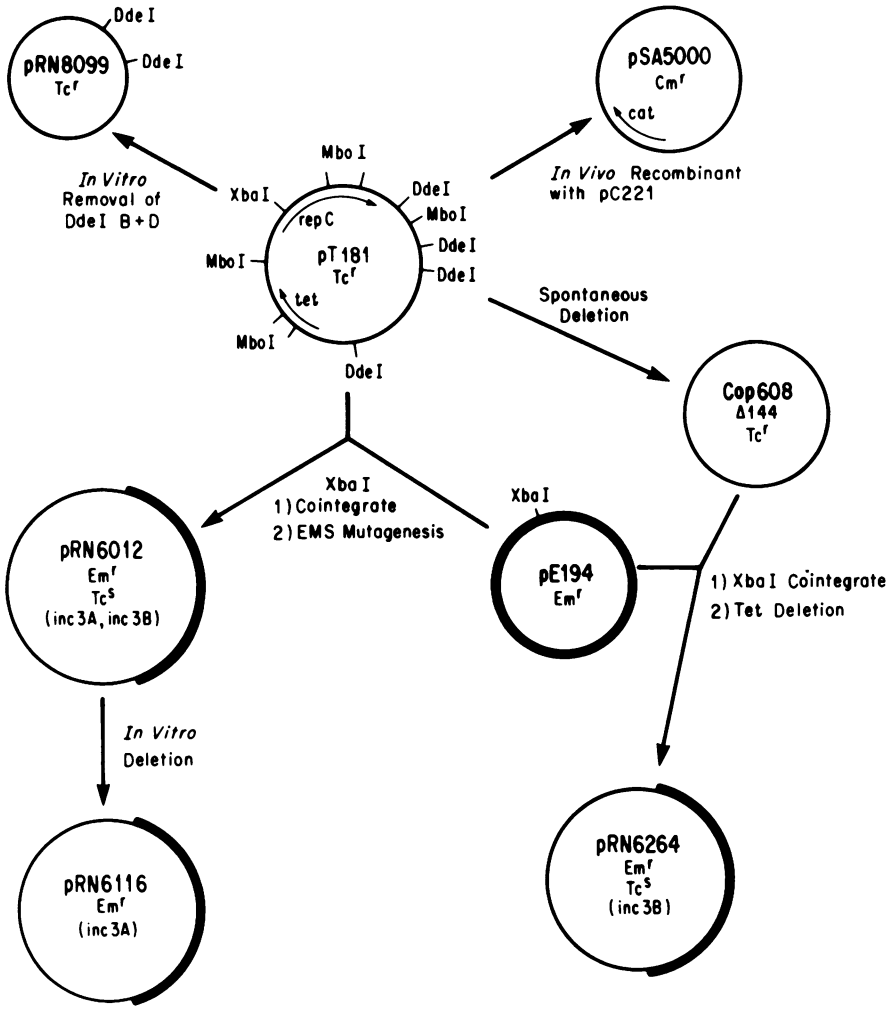

Fig. 6. Plasmid pedigrees. Contributions from pT181 are shown as thin lines, from pE194 as heavy. The plasmid pRN6012 was derived in two steps. pT181 and pE194 were joined *in vitro* at their unique *Xba*I sites and a Tc^s mutant of the resulting co-integrate was isolated following ethyl methanesulphonate mutagenesis. pRN6264 was derived similarly, except that the second step involved the isolation of a spontaneous Tc^s deletion as previously described (Novick *et al.*, accompanying paper).

the *repA* message and thus permitting the formation of a downstream hairpin that occludes the ribosome binding site (R. Rownd, personal communication).

Consequences of indirect versus direct control

For counter-transcript regulations such as ColE1, pT181 and the IncFII plasmids, the commonest class of copy mutations occur in the RNA overlap region, affecting both inhibitor and target in a complementary manner. Although we score these as dominant, their inhibitor activity is also affected and so we propose to refer to them as overlap mutants. For directly regulated plasmids, dominant mutants (I^+, T^-) unilaterally exclude either wild-type or pure recessives (I^-, T^+) and can usually be co-maintained with one another or with any of the three other types. In fact, some overlap mutants define new incompatibility groups. With indirectly regulated plasmids, such as pT181, dominant mutants do not express unilateral incompatibility and overlap mutants do not establish new incompatibility groups, even if their inhibitor-target specificities are changed completely. These observations are readily understood in terms of a stochastic model which holds that incompatible heteroplasmids segregate because the inevitable stochastic fluctuations in their copy numbers cannot be corrected (Novick and Hoppensteadt, 1978). For directly regulated plasmids, mutants with changed inhibitor-target recognition specificity are compatible because they can self-correct their copy pools independently; for indirectly regulated plasmids, such mutants remain segregationally incompatible because they cannot.

Materials and methods

Bacterial strains and plasmids

The staphylococcal strains used are derivatives of strain 8325 (Novick, 1967). RN27 is lysogenic for phage 80α and was used as recipient for transductions performed with this phage. pT181 is a naturally-occurring 4.4-kb plasmid from *S. aureus* (Iordanescu, 1976b). The plasmid, pSA5000 is an *in vivo* recombinant between pT181 and pC221, possibly having a contribution from a third related plasmid, pSA0501, as well (Iordanescu and Surdeanu, 1980). pSA5000 consists mainly of pT181 DNA with a replacement of a 2-kb stretch containing the Cm^r determinant of pC221. Its sequence in the replication control region from nucleotides 3941 to 376 is identical to that of pT181 in the corresponding region (unpublished data). pSA5000 has the same copy number and incompatiblity properties as pT181 and is therefore utilized as a differentially marked equivalent replicon (see Figure 6). pRN6012 is an *Xba*I co-integrate between pT181 and pE194 (Novick *et al.*, accompanying paper) which was used to generate *in vitro* deletions affecting the pT181 replicon. pRN6116 is a derivative of pRN6012 with a deletion removing the pT181 origin but leaving intact the *copA* determinant (Novick *et al.*, acompanying

paper). pRN6264 is an *in vivo* recombinant between pRN6012 and pT181 *cop-608* (see text), and lacks *copA* activity.

Media and culture conditions

CY (Novick and Brodsky, 1972) was used for broth cultures which were shaken at 37°C and monitored turbidimetrically. GL agar (Novick and Brodsky, 1972) was supplemented with antibiotics as required. Tetracycline, chloramphenicol and erythromycin were used at 5 μg/ml unless otherwise specified.

Transduction was with phages φ11 or 80α (Novick, 1967). Competent cell and protoplast trnsformation were as described (Pattee and Neveln, 1975; Chang and Cohen, 1979).

CopA activity was scored by displacement tests; one plasmid was transduced to a strain containing the other with selection for the donor plasmid. Transductant colonies were toothpicked to the same medium and were then replicated to score for the resident plasmid. Segregational incompatibility was scored by measuring the segregation rates of heteroplasmid strains during growth in liquid cultures in non-selective media. Samples were plated at appropriate time intervals and colonies were scored by replica plating. Alternatively, heteroplasmids were tested for reciprocal intrapool variation (Projan and Novick, 1984, see text).

Isolation and analysis of plasmid DNA

DNA was prepared and analyzed as described (Novick *et al.*, 1979; Projan *et al.*, 1983). Copy numbers were determined by fluorimetric densitometry of ethidium bromide-stained agarose gels run on sheared whole-cell mini-lysates of exponentially-growing cultures (Projan *et al.*, 1983). Determinations were done on at least three independent cultures for each of the copy mutants; replicate determinations were consistent to within 20%.

Determination of DNA content of S. aureus cells

Exponential cultures growing at 30, 37 and 43°C were harvested by centrifugation, and the cells were washed and resuspended in phage buffer (Novick, 1967). A sample was subjected to sonic oscillation to disrupt clumps (Novick and Brodsky, 1972) and the cells were counted in a model B Coulter counter and in a Petroff-Hauser counter. A second sample was fixed, sectioned, and examined in the electron microscope to determine the proportion of septated cells. A third sample was analyzed for total DNA content by the modified diphenylamine reaction (Giles and Myers, 1965). The DNA content per cell was determined by dividing the total DNA by the cell counts after correction for residual clumps and septated cells (which totalled some 20% in exponential cultures). The figures obtained for the three temperatures were 3.0, 3.5 and 5.0 x 10^9 daltons per cell, respectively. These figures may represent moderate overestimates because it is difficult to obtain cell counts of *S. aureus* that are not underestimated. Since the genome of *S. aureus* has been estimated at 1.4−1.6 x 10^9 dalton (Bak *et al.*, 1970; H. Tzagoloff and R.P. Novick, unpublished data), these figures for exponential cells represent 2−3 genome equivalents/cell.

Restriction mapping and cloning

Restriction enzymes were purchased from New England Biolabs and Bethesda Research Labs. Restriction mapping was performed with dye-cesium-purified plasmid DNA samples. For molecular cloning, specific fragments were extracted from polyacrylamide gels, phenol extracted, alcohol precipitated, and re-dissolved in DNA ligase buffer (Maxam and Gilbert, 1980). Samples were mixed in equimolar ratios and incubated with T4 DNA ligase at a DNA concentration of ~10 μg/ml and a ligase concentration of 40 units/ml for 18 h at 4°C. These samples were used directly to transform *S. aureus* protoplasts with selection for the appropriate antibiotics resistance marker. Transformants were screened for plasmid content and those having plasmids of the expected size were used to prepare plasmid DNA for confirmatory restriction analysis, and, if necessary, sequencing.

DNA sequencing

Determination of nucleotide sequences was by the method of Maxam and Gilbert (1980). In all cases, sequence determinations were performed on both strands in the region of interest.

Computer analysis

Possible secondary structures of RNA molecules were predicted by the computer program of Zuker and Stiegler (1981).

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References

Bak,A.L., Christiansen,C. and Stenderup,A. (1970) *J. Gen. Microbiol.*, **64**, 377-380.
Chang,S. and Cohen,S.N. (1979) *Mol. Gen. Genet.*, **168**, 111-115.
Easton,A.M. and Rownd,R.H. (1982) *J. Bacteriol.*, **152**, 829-839.
Giles,K.W. and Myers,A. (1965) *Nature*, **206**, 93-94.
Gorini,L. and Maas,W.K. (1957) *Biochim. Biophys. Acta*, **25**, 208-209.
Grindley,N.D.F., Grindley,J.N., Kelley,W.S. (1978) in Schlessinger,D. (ed.), *Microbiology – 1978*, American Society of Microbiology, pp. 71-73.
Iordanescu,S. (1976a) *Arch. Roum. Pathol. Exp. Microbiol.*, **35**, 111-118.
Iordanescu,S. (1976b) *Arch. Roum. Pathol. Exp. Microbiol.*, **35**, 207-215.
Iordanescu,S. and Surdeanu,M. (1980) *Plasmid*, **4**, 1-9.
Khan,S.A., Adler,G.K. and Novick,R.P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4580-4584.
Kumar,C. and Novick,R.P. (1984) *Proc. Natl. Acad. Sci. USA*, in press.
Lacatena,R.M. and Cesareni,G. (1981) *Nature*, **294**, 623-626.
Light,J. and Molin,S. (1982) *Mol. Gen. Genet.*, **187**, 486-493.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.
Novick,R.P. (1967) *Virology*, **33**, 155-166.
Novick,R.P. and Brodsky,R. (1972) *J. Mol. Biol.*, **68**, 285-302.
Novick,R.P. and Hoppensteadt,F.C. (1978) *Plasmid*, **1**, 421-434.
Novick,R.P., Murphy,E., Gryczan,T., Baron,E. and Edelman,I. (1979) *Plasmid*, **2**, 109-129.
Novick,R.P., Adler,G.K., Majumder,S., Khan,S.A., Carleton,S., Rosenblum,W.D. and Iordanescu,S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4108-4112.
Pattee,P.A. and Neveln,D.S. (1975) *J. Bacteriol.*, **124**, 201-211.
Projan,S.J. and Novick,R.P. (1984) *Plasmid*, in press.
Projan,S.J., Carleton,S. and Novick,R.P. (1983) *Plasmid*, **9**, 182-190.
Rosenberg,M. and Court,D. (1978) *Annu. Rev. Genet.*, **13**, 319-353.
Tomizawa,J., Itoh,T., Selzer,G. and Som,T. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1421-1425.
Zuker,M. and Stiegler,P. (1981) *Nucleic Acids Res.*, **9**, 133-148.

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