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Measurement of gene expression by translational coupling: effect of copy mutations on pT181 initiator synthesis

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We have prepared and analyzed two types of gene fusion between the replication initiator gene, *repC*, and the reporter gene, *blaZ*, in order to investigate the relationship between pT181 plasmid copy number and RepC initiator protein production. A series of pT181 copy mutant plasmids, with copy numbers ranging from 70 to 800 copies per cell, were analyzed. In one type of gene fusion used in this study, *blaZ* was translationally coupled to the C-terminal end of the *repC* coding sequence such that native forms of both proteins were produced. This gene fusion arrangement, which permitted monitoring of RepC production (as BlaZ activity) by plasmids using the protein for their own replication, demonstrated a linear relationship, with one exception, between RepC production and plasmid copy number over a 20-fold range. In the second type of fusion, *blaZ* was translationally fused to the C-terminal end of *repC*. As the translational fusion did not produce active RepC protein, the fusion-containing pT181 derivatives were maintained in a strain which provided RepC *in trans*, and were thus analyzed at constant copy number. In contrast to previous analyses of this type, our translational fusion constructs expressed *repC* at levels proportional to the copy numbers of the plasmids from which the fusions were prepared. Using these data, we have calculated a minimum figure for the number of RepC molecules synthesized per replication event.

Key words: copy number/plasmids/RepC/translational coupling

Introduction

As first recognized by Pritchard *et al.* (1969), the critical determinant of the plasmid way of life in bacteria is autogenous negative regulation of replication initiation. From this it follows that the rate-limiting step in plasmid replication is plasmid determined and plasmid specific, and necessarily represents the target of negative regulation. As first shown by Nordstrom and coworkers (1972), it is generally possible to obtain mutations that affect this control point and result in elevated plasmid copy numbers (copy mutations).

The key element in the life cycle of most plasmids consists of a plasmid-coded initiator (Rep) protein that binds to the plasmid replication origin in a sequence-specific manner and is synthesized in rate-limiting quantities. The synthesis of this Rep protein constitutes the above-mentioned control point. It follows that in such cases, increasing the amount of Rep synthesis will cause a proportional increase in copy number. This has been demonstrated in a limited way by experiments in which the *rep* coding sequence is cloned to an inducible promoter (Light and Molin, 1983; Manch-Citron *et al.*, 1986; Sogaard-Andersen *et al.*, 1984; Swack *et al.*, 1987). Oddly, however, induction of Rep synthesis causes only a limited increase in plasmid copy number rather than the autocatalytic runaway replication that one might expect. In fact, in the case of P1, increasing Rep concentration beyond a critical level causes a reduction in plasmid copy number (Swack *et al.*, 1987). And for R6K, copy number is unaffected by varying Rep concentration over a very wide range (Shafferman *et al.*, 1982). This type of result is explained by the fact that the Rep proteins often have inhibitory as well as stimulatory activities and that the plasmids often possess inhibitory Rep binding sites in addition to an origin of replication.

Three cases have been identified thus far in which the Rep protein seems to act only in a positive manner: plasmids of the IncFII class, typified by NR1 and R1 (Uhlin and Nordstrom, 1978), plasmids of the pT181 family, typified by pT181 and pC221 (Projan and Novick, 1988) and RSF1010 which is complex in that it encodes three different Rep proteins, one of which causes a concentration-dependent increase in copy number (Haring *et al.*, 1985). For such plasmids, one may make several predictions: (i) replication is regulated primarily at the level of Rep synthesis; (ii) the protein acts stoichiometrically and is inactivated during replication; (iii) Rep units are synthesized at a rate that approximates the number of replications occurring per cell generation; (iv) mutations causing an increase in plasmid copy number do so by increasing the rate of Rep synthesis.

The first prediction has been convincingly verified for the IncFII and pT181 families (Manch-Citron *et al.*, 1986; Stougaard *et al.*, 1982) and suggestively for RSF1010 (Haring and Scherzinger, 1989). The second prediction has recently been verified for pT181 (A.Rasooly and R.Novick, submitted) and it has been shown that in cells sustaining pT181 replication >95% of the Rep protein is present as the inactive form (A.Rasooly and R.Novick, submitted). Testing of the third and fourth predictions is the subject of this paper; it is noted that previous tests of the fourth have been unsuccessful, as discussed below.

Considering prediction (iii) for pT181 and R1, which are typical of plasmids that regulate Rep synthesis by means of inhibitory antisense RNAs (countertranscripts), most copy mutations affect either the function or the production of the countertranscripts and the molecular consequences of these mutations can be readily predicted on the basis of an understanding of the inhibitory mechanism. For pT181, this mechanism involves initial pairing between complementary loops (tg) in the mRNA leader and countertranscript (see Figure 1), which results in the formation of a transcription-

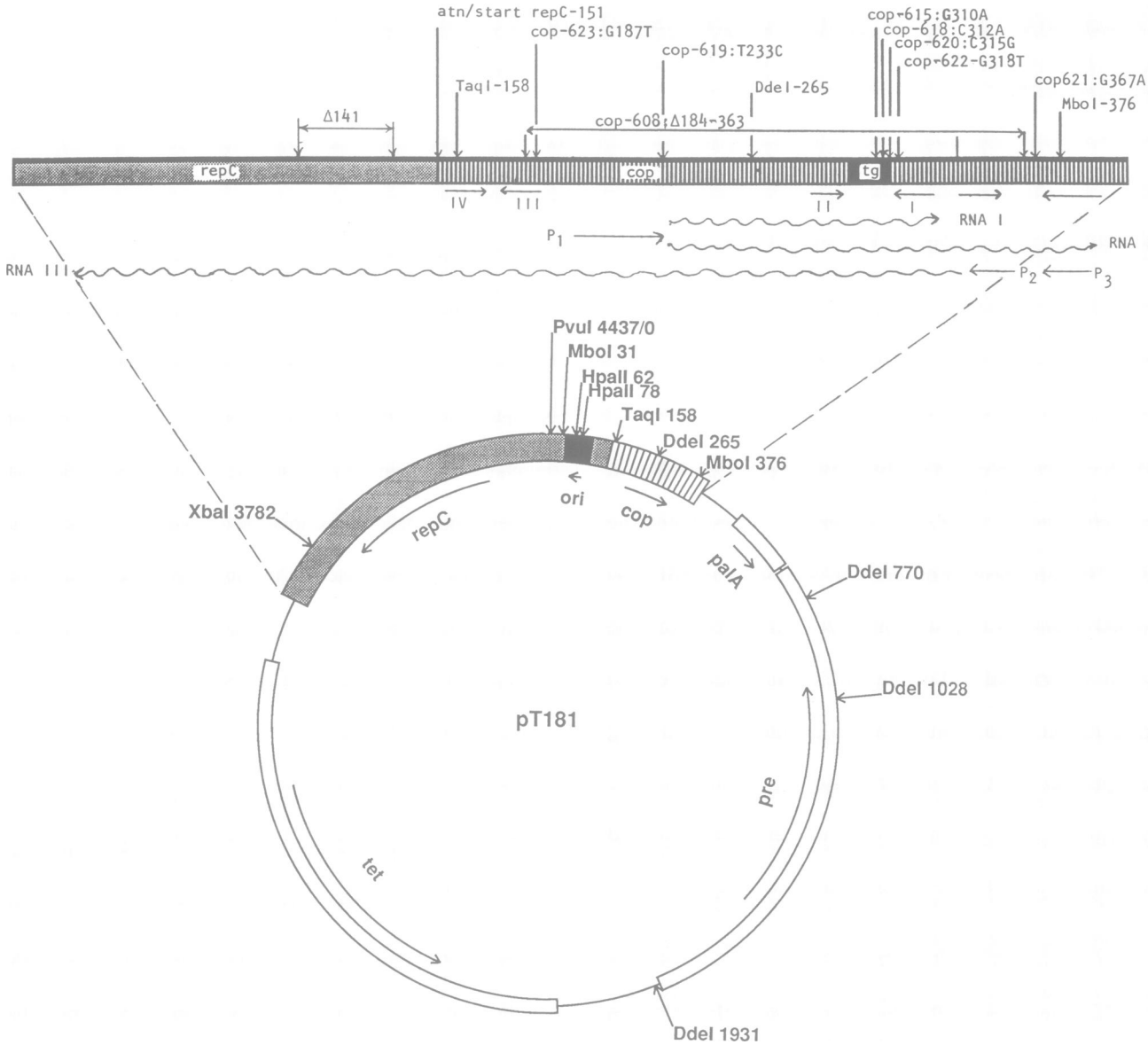


Fig. 1. pT181 genome and control region. The circular map shows major features of the pT181 genome with restriction sites important in this work, indicating nucleotide coordinates. *cop*, replication control region; *palA*, lagging strand conversion signal; *pre*, site-specific recombination function; *tet*, tetracycline resistance; *repC*, initiator; *ori*, leading strand replication origin. The enlargement displays important features of the *cop* region, including key restriction sites with nucleotide coordinates. The nucleotide substitution for each copy number mutant allele is also presented. 'tg' refers to the region of the primary countertranscript–target interaction, the 'target loop'. Wavy lines represent the known transcripts with promoters as indicated. Arrows I and II represent the target stem, which also serves as the RNA I terminator and contains the preemptor sequence (I); arrows III and IV mark the attenuator stem; and the two unlabeled arrows represent the RNA II terminator stem.

terminating hairpin (the III–IV stem–loop), just 5′ to the Rep start codon (Novick *et al.*, 1989). This attenuation mechanism is very efficient, aborting >90% of the Rep transcripts under normal conditions. Mutations have been isolated that affect the countertranscript promoter (e.g. *cop-619*, see Figure 1) or terminator (e.g. *cop-622*). These are recessive (sensitive to the wild-type countertranscripts *in trans*). Other mutations are located in the target loop and affect the rep mRNA leader as well as the countertranscript and are therefore dominant (insensitive to the wild-type countertranscripts *in trans*). One mutation, *cop-623*, is located in the proximal arm of the attenuator stem and effectively eliminates attenuation independently of the presence of the countertranscripts. It is predicted that the immediate consequence of any of these mutations would be an increase in the frequency of transcription through the attenuator, resulting in an increase in the amount of Rep synthesis per plasmid copy. This would lead to an increase in replication frequency and therefore in copy number. The increased *rep* gene dosage would cause a further increase in Rep synthesis and would also cause an increase in countertranscript concentration. Regulatory equilibrium would be re-established when the countertranscript concentration reaches the level that would ensure the synthesis of precisely the same number of Rep molecules per plasmid copy as with the wild-type plasmid (Novick *et al.*, 1985). In other words, the overall rate of Rep synthesis would be proportional to the plasmid copy number but the rate per plasmid copy would always remain constant.

A corollary of this prediction is that the countertranscript

genotype will determine a specific rate of Rep synthesis per cell irrespective of the actual copy number of the *rep-cop* element. Therefore activity measurements using gene fusions between wild-type or mutant control elements and a reporter gene, on a carrier plasmid maintained at constant copy number, should provide a valid test of the relationship between Rep protein production and the copy numbers of the mutants from which each control region is derived. Such fusions, however, have not provided a convincing test, with either R1 (Givskov and Molin, 1984) or with pT181 (Wang *et al.*, 1991). Either the above prediction is incorrect or the existing data are for some reason unsatisfactory. In our view, the regulatory formulation described above is a critical feature of any hyperbolic regulatory system and so we considered it worthwhile to perform a further test. In addition to the use of gene fusions maintained at constant copy number, which may embody an inherent artifact, we have used a technique in which the true plasmid copy number and the rate of Rep synthesis are measured simultaneously. This technique utilizes a gene fusion in which a reporter gene is C-terminally coupled to the *rep* gene by means of an overlapping start−stop configuration for the two reading frames. As re-initiation of translation at the downstream start codon, in the absence of a consensus Shine−Dalgarno site, typically occurs at a frequency of 10−20%, translational coupling is an effective natural mechanism for reducing the expression of distal genes in an operon and has been used widely in nature (Das and Yanofsky, 1984; Schumperli *et al.*, 1982; Sprengel *et al.*, 1985).

Considering prediction (iv), the fact that Rep is rate-limiting implies that there will never be a significant pool of active Rep protein in the cell at any time and therefore that the number of active units of the protein will remain constant throughout the cell cycle whereas the total number of units of the protein will increase in parallel with the increase in plasmid copies that occurs by replication throughout the cell cycle. Since >90% of free RepC antigen is in the inactive, post-replicative form (A.Rasooly and R.Novick, submitted), and since an indeterminate amount may be bound to DNA or degraded, we consider the translationally coupled constructs as the only available means of directly measuring the amount of active RepC synthesized in each cell generation.

Results

Preparation and properties of repC−blaZ fusions

To study the relationship between RepC synthesis and pT181 replication, we elected to use gene fusions with β-lactamase as the reporter. Since the pT181 replication control system is likely to affect RepC translation as well as transcription (Novick *et al.*, 1989), a translational gene fusion was needed, and two different types were prepared, one in which the reporter, *blaZ*, was translationally fused, in-frame, to the C-terminus of RepC, and a second in which the genes were translationally coupled at the same point, by means of the overlapping start−stop sequence, ATGA, which has been shown to provide relatively efficient re-initiation in other systems (Schumperli *et al.*, 1982). Although an in-frame fusion has the virtue of providing 100% readthrough, its replication activity could not be predicted and so we used the coupling strategy to ensure that both (native) proteins would be produced.

Both gene fusions were prepared by inserting the 1 kb *Hin*dIII−*Taq*I fragment from pWN1818 (Wang *et al.*, 1987), containing the *blaZ* coding sequence, at the unique *Cla*I site in pT181 near the 3′ end of *repC*. Synthetic oligonucleotides were used to restore the 3′ end of *repC* distal to the *Cla*I site and to provide the required fusion junction plus a *Hin*dIII site to match that at the 5′ end of *blaZ*. These oligonucleotides are diagrammed in Figure 2 and the general structure of the resulting plasmid constructs in Figure 3.

Both fusions were prepared by ligating the *blaZ* fragment plus the adaptor to a *Cla*I-digested sample of pT181-*cop*-623 and using the ligation mixture to transform protoplasts of RN4220 (Kreiswirth *et al.*, 1983). In both cases, we also transformed a derivative of RN4220 containing a second plasmid, compatible with pT181, that contained a cloned copy of the *repC* gene and could supply RepC *in trans*, on the chance that either of the fusion plasmids did not produce active RepC. As expected, the translationally coupled *repC−blaZ* fusion produced an equal number of transformants with both recipient strains, confirming that the coupling junction gave rise to active RepC. Moreover, the plasmid, pJEB123, had essentially the same copy number in RN4220 as the native pT181-*cop-623* (not shown), suggesting that the two produced active RepC at the same rate. The in-frame fusion however, produced transformants only with the recipient containing a RepC-producing plasmid, suggesting that the fusion protein did not have RepC activity.

This was confirmed genetically by a second transfer, in which the fusion plasmid, pJEB623, was transduced to recipients with and without the RepC-producing plasmid, pSA7541. In this case, a few transductants appeared with the $RepC^-$ recipient (~1% of the number with the $RepC^+$). Ten of these were analyzed for plasmid content and all 10 were found to have received pSA7541, the RepC-providing plasmid (which was present in the donor) as well as pJEB623, the fusion plasmid. Moreover, since pSA7541 is thermosensitive for replication (Tsr) it can readily be cured by growth at 43°C. When this was done, it was found that all derivatives losing the Tsr plasmid pSA7541 had also lost pJEB623, the *repC−blaZ* in-frame translational fusion plasmid. Since translational fusions using this *blaZ* gene contain the intact *blaZ* signal peptide, the *blaZ* moiety is processed and secreted normally (Wang *et al.*, 1987). The signal sequence would remain fused to RepC and is presumably responsible for the observed lack of RepC activity. Perhaps the fused RepC is degraded or is held at the cell membrane.

Having prepared and characterized these constructions, we excised from each one the *Cla*I−*Taq*I fragment, containing the *repC* C-terminus, the *repC−blaZ* junction, and the *blaZ* gene, and used these fragments to prepare similar clones with wild-type pT181 and with several different pT181 copy mutants, as listed in Table I. All of these plasmids were prefixed pJEB; those with the coupling junction have numbers in the 100s, those with the fusion in the 600s. Copy number determinations on the pJEB100 series showed that these all had copy numbers indistinguishable from those of the plasmids from which they were derived (see Figure 4) indicating that the coupled *blaZ* gene had essentially no effect on plasmid replication. All of the translational fusion plasmids had the same copy number since all were maintained by pSA7541 which provides a constant level of RepC *in trans*.

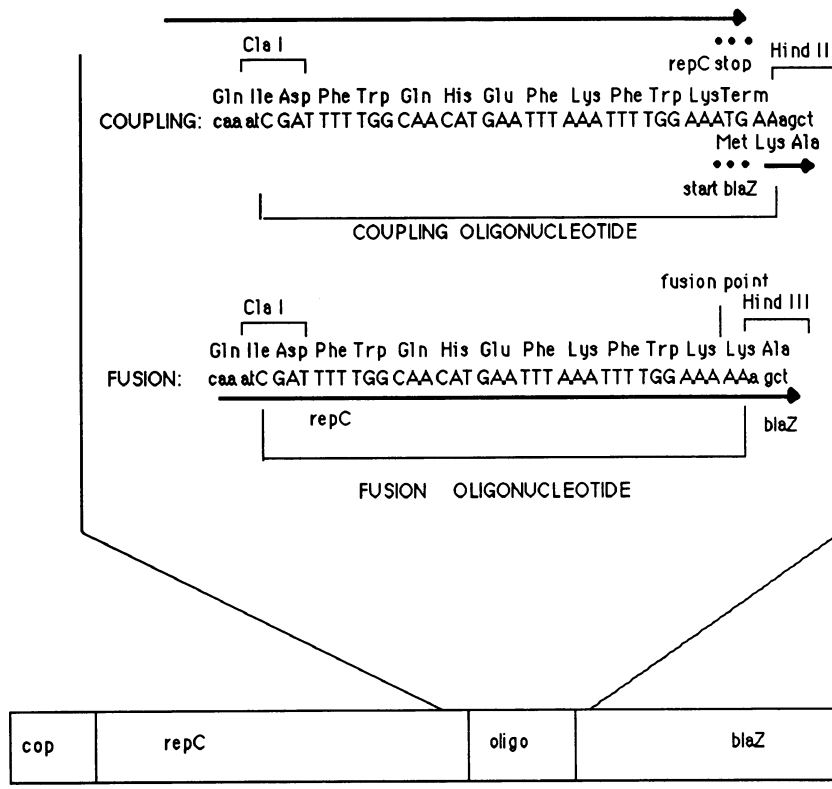


Fig. 2. Construction of *repC–blaZ* fusions. Nucleotide sequences of the synthetic oligonucleotide linkers used to construct the coupling and fusion are shown. The linkers contain sequences that re-establish the C-terminal end of the *repC* gene distal to the *ClaI* site. The coupling linker contains overlapping initiation/termination codons (ATGA) while the fusion linker contains the sequence AA at the corresponding position. *ClaI* linearizes pT181, cutting uniquely at nucleotide position 3677 and the linker plus the β-lactamase gene were inserted into pT181 at this site.

We note that the coupled plasmids showed higher copy numbers than the fusion plasmids when coresident with the RepC-producing plasmid, pSA7541 (Figure 5, lanes 1–4). This effect was not seen with parallel constructs prepared from a derivative of pT181 with a deletion in *repC* (pRN6238) (Figure 5, lanes 5 and 6). The most likely interpretation of these results is that the copy numbers of the coupling constructs reflect additivity between the active RepC synthesized by the pT181 coupled plasmid and that provided *in trans* by pSA7541, whereas the copy numbers of the fusion constructs reflect only the RepC synthesized by pSA6541. Alternatively, it is possible that the defective RepC synthesized by the fusion plasmids interferes with the functional RepC encoded by pSA7541.

Relation of blaZ expression to plasmid copy number

On the basis of the above results, it was assumed that expression of β-lactamase activity by the coupled plasmids would provide an accurate estimate of the amount of RepC synthesized per cell generation relative to plasmid copy number, assuming that the efficiency of translational read-through across the coupling junction is constant. Note that the measured β-lactamase activity is a direct indication of the rate of RepC synthesis per cell per generation as it represents exactly twice the amount of enzyme synthesized during the generation preceding the measurement. These β-lactamase activity measurements (shown in Table I, column headed 'coupled'), obtained with exponentially growing bacteria at 10^9 cells/ml, demonstrated a linear proportionality between plasmid copy number and RepC synthesis over a 20-fold range of copy numbers. As can be seen in Figure 6, this linearity exists for all the plasmids studied except for one, pT181-*cop-608*, which has a deletion of the entire control region including the primary *repC* promoter. Consequently *cop-608* is not functionally comparable with the other plasmids.

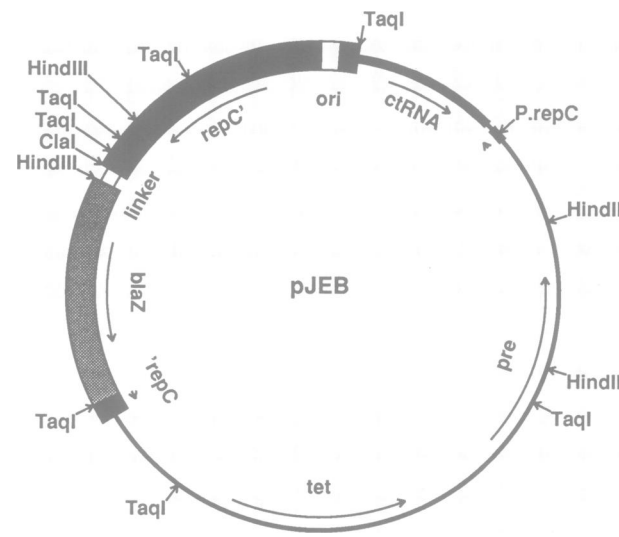


Fig. 3. Map of the pJEB plasmids with *repC–blaZ* fusions. These plasmids represent a series in which the C-terminal end of *repC* fused to *blaZ* was cloned to the unique *ClaI* site in pT181, near the C-terminal end of *repC*. Note that *blaZ* is followed by a duplication of the short C-terminal segment of *repC* distal to the *ClaI* site. P.repC, *repC* promoter; ctRNA, countertranscript; tet, tetracycline resistance determinant; pre, site-specific recombination function.

Table I. β-lactamase activities of *repC−blaZ* fusions

Plasmid mutation	Copy number	Replications per cell cycle	β-lactamase activity (U/mg)[a]			
			Coupled[b]	Fused	RepC monomers per cell[c]	RepC monomers per replication event
WT	22	16	0.13	0.055	15	0.94
cop-620	70	49	0.39		45	0.92
cop-622	120	85	0.45		53	0.61
cop-619	140	95	0.58	0.48	66	0.69
cop-621	150	110	0.79		90	0.82
cop-618	210	150	1.21		138	0.92
cop-623	400	280	2.55	2.13	291	1.04
cop-608	800	570	2.24	1.92	256	0.45

[a]U, μM/h, expressed as pencillin G activities.
[b]These figures have been corrected for the step-down ratio of 2.5 across the coupling junction, to facilitate comparsion with the figures derived from the fusion constructs.
[c]Calculated from β-lactamase activities of *repC−blaZ* couplings, using the value of 1.2×10^6 U/μM for the specific activity of *S.aureus* β-lactamase (Richmond, 1965). These figures represent the amount of RepC synthesis per cell per generation.

As noted above, it is predicted that initiator expression by a passively maintained initiator gene will also be directly proportional to the copy number of the plasmid used for the construction, so long as the regulatory circuits are intact and functional. This relation would not hold for *cop-608*, which lacks countertranscript regulation; the amount of RepC produced by *cop-608* should always be a linear function of gene dosage.

As shown in Table I (column headed 'fused'), the β-lactamase activities for two of the fusion constructs, *cop-619* and *cop-623*, closely match the β-lactamase activities for the corresponding coupling constructs, whereas the fusion construct using pT181 wild-type produces less β-lactamase than the corresponding coupling construct. This difference can be explained by the presence of the *cop-620* countertranscripts encoded by the RepC-producing plasmid pSA7541. It has previously been shown that the pT181 copy mutants are indifferent to the *cop-620* countertranscripts *in trans* whereas pT181 wild-type is sensitive (Highlander and Novick, 1990).

The failure of the pT181 *cop-608* construct to show the expected gene dosage dependence of *blaZ* expression is not understood; possibly there is a second control mechanism, as yet unidentified, that functions with this plasmid. Such a control mechanism might explain why *cop-608* does not show autocatalytic runaway replication (Carleton *et al.*, 1984).

Estimation of RepC synthesis per cell

Given that β-lactamase is expressed by the coupling constructs in direct proportion to the RepC that is being used for plasmid replication, BlaZ measurements provide a direct indication of the amount of RepC synthesis for each plasmid at its native copy number. We have converted these β-lactamase measurements to RepC molecules per cell by utilizing the known specific activity of pure staphylococcal β-lactamase on penicillin G (1.2×10^6 U/μmol; Richmond, 1965), and two additional correction factors: because nitrocefin was used to assay the β-lactamase activity, the measurements had to be corrected for the substrate activity of nitrocefin relative to penicillin G (see Materials and methods). The nitrocefin−penicillin G conversion factor was found to be 8.9 (data not shown). Since the frequency of re-initiation across the translational coupling junction is considerably less than 100%, the β-lactamase measurements had also to be corrected for the re-initiation frequency (the step-down ratio). The step-down ratio was determined to be 2.5 ± 0.25 by comparing the β-lactamase activities of *repC−blaZ* translationally fused and translationally coupled constructs (see Figure 2) held at the same copy number. The *repC−blaZ* fusion and coupling junctions were inserted at the *Cla*I site of pRN6238, a derivative of pT181 wild-type with an inactivating deletion in the *repC* gene (Δ141) (Novick *et al.*, 1984). These homologous constructs were maintained at the same copy number by a constant source of RepC provided by a compatible co-resident plasmid, pSA7541, containing the cloned *repC* gene (see Figure 5, lanes 5 and 6). The β-lactamase activity of the strain containing the fusion plasmid, pJEB641, was 0.051 ± 0.010 U/mg

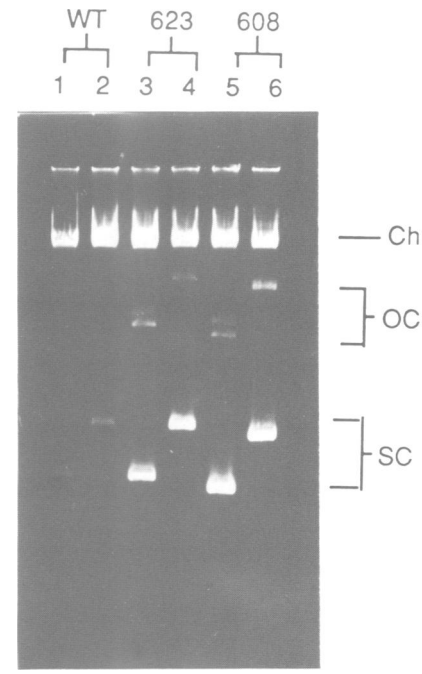


Fig. 4. Copy number measurements. Whole-cell sheared mini-lysates prepared with measured cell samples were separated on 1% agarose. Chr, chromosomal DNA; OC, nicked plasmid DNA; SC, supercoiled plasmid DNA. Lanes 1, 3 and 5 contain pT181, *cop-623* and *cop-608*, respectively. Lanes 2, 4 and 6 contain the coupling derivatives of these plasmids, namely, pJEB100, pJEB123 and pJEB108, respectively.

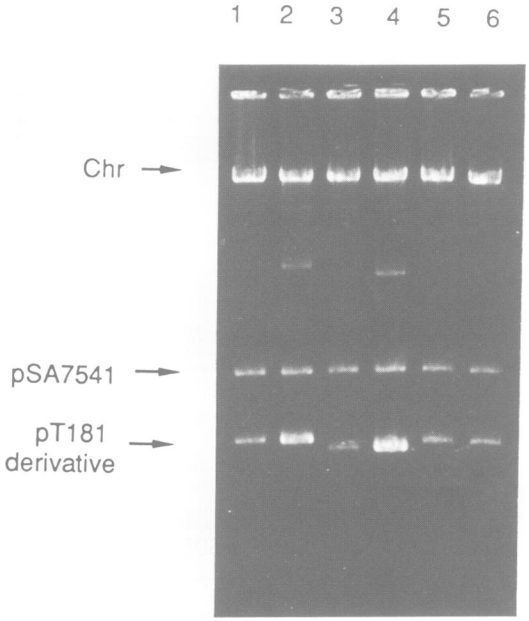
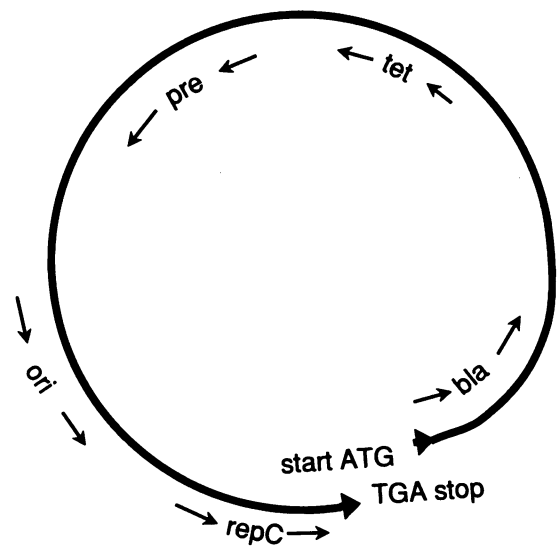


Fig. 5. Comparison of copy numbers of coupled versus fused plasmids. Whole-cell sheared mini-lysates of heteroplasmid strains were run on a 1% agarose gel. The two plasmids in each heteroplasmid strain were the RepC donor plasmid (pSA7541) and one of the pT181 derivative plasmids. Lanes 1, 3 and 5 show the *repC−blaZ* translational fusion in *cop-623* (pJEBN623), *cop-608* (pJEB608) and pRN6238 (pJEB641) respectively. Lanes 2, 4 and 6 show the corresponding couplings, pJEB123, pJEB1908 and pJEB141.

(average of five determinations) while that of the corresponding coupling, pJEB141, was 0.020 ± 0.004 U/mg (average of five determinations). From these measurements it was concluded that β-lactamase was expressed by the *repC−blaZ* couplings at 40 ± 10% the level expressed by the fusion plasmids. Using the correction factors described above, we calculated values for RepC monomers synthesized per cell per generation (shown in Table I) from the β-lactamase activities measured with the constructs described in Figure 6. Specifically, all of the β-lactamase activities in Table II have been corrected by the nitrocefin−penicillin G factor of 8.9. To facilitate comparison with the translational fusion constructs, the β-lactamase activities for the coupling plasmids have been multiplied by 2.5, to correct for the step-down ratio.

Discussion

In this paper we have studied the relationship between the initiator protein RepC and the replication of pT181 wild-type and copy mutant plasmids. A β-lactamase (*blaZ*) reporter gene was translationally coupled to the pT181 *repC* gene via an overlapping start−stop codon in order to permit measurement of RepC production by the plasmids utilizing the protein for replication. Using this system we have observed a linear relationship between plasmid copy number and Rep production for pT181 and its copy mutants. We have also observed this same relationship for C-terminal



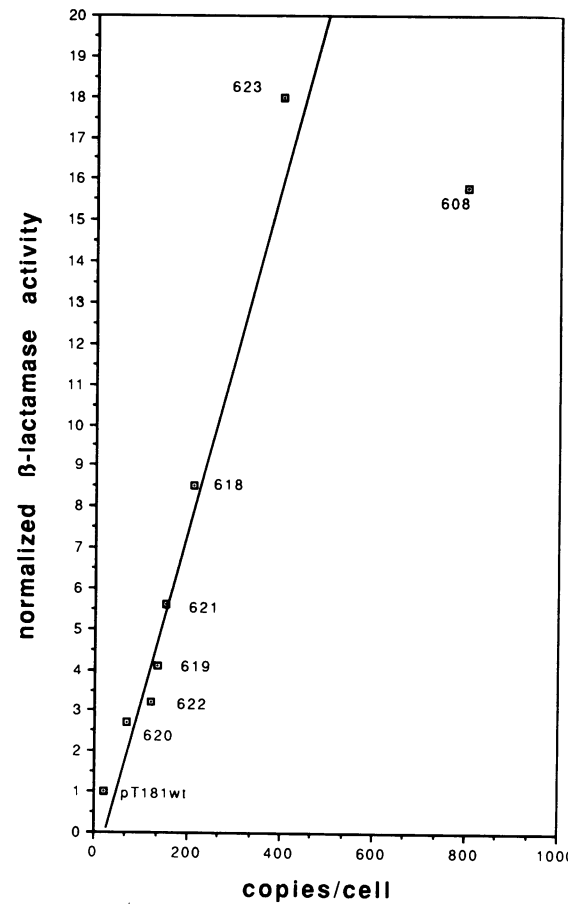


Fig. 6. RepC production in relation to plasmid copy number. RepC production is expressed as normalized β-lactamase activity, which refers to the β-lactamase activity relative to the wild-type coupled plasmid in RN451 (RN6962). Each copy mutant number is written beside its corresponding point on the graph.

repC−blaZ translational fusions constructed with intact pT181 plasmids. However, we have observed a disparity between the relative β-lactamase activities produced by gene fusion constructs with intact pT181 plasmids and N-terminal *repC−blaZ* translational fusions constructed in a carrier plasmid (Wang *et al.*, 1991). Moreover, the N-terminal fusions showed only a poor correlation between β-lactamase activity and the corresponding pT181 plasmid copy number. Similarly, in the IncFII plasmids, it has been shown that plasmid copy number increases in response to increasing Rep concentration (Light and Molin, 1983), but no correlation could be detected between Rep synthesis and copy number for several copy mutants when analyzed by translational *repA−blaZ* N-terminal fusions at constant copy number (Givskov and Molin, 1984). We suggest that the difference between the fusion results presented here and the results of Wang *et al.* (1991) and of Givskov and Molin (1984) is that our fusions preserved the native sequence context of the *rep* gene whereas the others did not.

Since the coupling method permitted a direct measurement of the Rep protein in cells in which it is being used for replication, an estimate of the molecular ratio of Rep protein production to replication events in the cell could be made. Plasmid copy numbers determined on random cultures represent the overall average of plasmid copies per cell. Assuming that plasmid replication events proceed throughout the cell cycle at a constant overall frequency, the plasmid copy number as measured is a close approximation of the number of plasmid copies in a cell of median age, namely age 0.415. [Median age is calculated from the standard equation for population distribution in a random exponential bacterial culture, $Y = 2^{(1-x)}$, where Y is the frequency of cells of age x ($0 < x < 1$).] For pT181 wild-type, with a copy number of 22, a newborn cell (age 0) would have $22/1.415 = 16$ copies and a cell about to divide (age 1) would therefore have 32, so that the number of replication events per cell cycle would be 16. If replication frequency is regulated by the amount of Rep synthesis, it is necessary that the protein act stoichiometrically rather than catalytically; in other words, it must be inactivated as a consequence of or immediately following replication. This would predict that the number of Rep molecules produced per cell cycle would closely approximate the number of replication events. In Table I calculations of RepC concentration in molecules per cell on the basis of β-lactamase activities of translational couplings are presented. These figures represent the cellular average in the same way that the measured copy number does—so that the number of new molecules synthesized per cell would be given by the cellular average divided by 1.415. The simplest prediction would be that, on average, one molecule of RepC is synthesized per replication event. The figures given in Table I are consistent with this prediction but are too low by at least a factor of 2 (since only half of the measured β-lactamase will have been synthesized during the preceding cell generation). RepC acts as a dimer (Thomas *et al.*, 1990) and it is likely that one new dimer is required per replication event, which would make the estimates too low by an additional factor of 2. However, it is conceivable that only one Rep subunit is inactivated and then replaced by a new active one. In any case, further refinements in these measurements are expected to provide a more accurate test of the above prediction. One important possibility is that our calculated frequency of translational re-initiation across the coupling junction is artifactually too high: the β-lactamase produced by the couplings is normal wild-type β-lactamase, whereas that produced by the fusions has a long N-terminal extension. Although β-lactamase is processed and secreted despite this N-terminal extension (Wang *et al.*, 1987), its processing and secretion may be less efficient than that of the native protein. We note that the re-initiation frequency

Table II. Plasmids

Plasmid	Description	Reference
pT181	Tc^r, wild-type, 22 copies/cell	Iordanescu (1976a)
pRN8008	Tc^r, pT181 *cop-608*, 800 copies per cell	Carleton *et al.* (1984)
pRN8023	Tc^r, pT181 *cop-618*, 210 copies per cell	Carleton *et al.* (1984)
pRN8024	Tc^r, pT181 *cop-619*, 135 copies per cell	Carleton *et al.* (1984)
pRN8025	Tc^r, pT181 *cop-620*, 70 copies per cell	Carleton *et al.* (1984)
pRN8026	Tc^r, pT181 *cop-621*, 150 copies per cell	Carleton *et al.* (1984)
pRN8027	Tc^r, pT181 *cop-622*, 120 copies per cell	Carleton *et al.* (1984)
pRB8061	Tc^r, pT181 *cop-623*, 400 copies per cell	Carleton *et al.* (1984)
pRN6238	Tc^r, pT181Δ141, $RepC^-$	Novick *et al.* (1982)
pSA7541	Em^r, pE5::pT181Δori, Δtet, *cop-620*	Iordanescu (1989)
pWN1850	pC194-based expression vector for using blaZ as the reporter gene	Wang *et al.* (1987)
pJEB100	Tc^r, *blaZ* translationally coupled to *repC* in pT181 wild-type	This work
pJEB108	Tc^r, *blaZ* translationally coupled to *repC* in *cop-608*	This work
pJEB118	Tc^r, *blaZ* translationally coupled to *repC* in *cop-618*	This work
pJEB119	Tc^r, *blaZ* translationally coupled to *repC* in *cop-619*	This work
pJEB120	Tc^r, *blaZ* translationally coupled to *repC* in *cop-620*	This work
pJEB121	Tc^r, *blaZ* translationally coupled to *repC* in *cop-621*	This work
pJEB122	Tc^r, *blaZ* translationally coupled to *repC* in *cop-622*	This work
pJEB123	Tc^r, *blaZ* translationally coupled to *repC* in *cop-623*	This work
pJEB141	Tc^r, *blaZ* translationally coupled to *repC* in pRN6238	This work
pJEB600	Tc^r, *blaZ* translationally fused to *repC* in pT181 wild-type	This work
pJEB608	Tc^r, *blaZ* translationally fused to *repC* in *cop-608*	This work
pJEB619	Tc^r, *blaZ* translationally fused to *repC* in *cop-619*	This work
pJEB623	Tc^r, *blaZ* translationally fused to *repC* in *cop-623*	This work
pJEB641	Tc^r, *blaZ* translationally fused to *repC* in pRN6238	This work

calculated for the *repC−blaZ* couplings is 2- to 3-fold higher than that seen with other translationally coupled genes (Schumperli *et al.*, 1982; Das and Yanofsky, 1985; Sprengel *et al.*, 1985); an artifactually low β-lactamase activity for the fusions could account for this. Consequently, we regard the figures presented in Table II for molecules per replication event as minimum estimates. Experiments are in progress to refine these measurements.

Materials and methods

Bacterial strains and plasmids

The bacterial plasmids used are listed in Table II. In Figure 1 a map of pT181 is presented showing the locations and nucleotide changes for the high copy number mutants listed in Table II. These mutants were isolated and characterized by Carleton *et al.* (1984). The *Staphylococcus aureus* strains used in this work were derivatives of NTCC 8325 (Novick, 1967). RN451 is a derivative lacking two prophages (φ12 and φ15) (Novick, 1967).

RN4220 (Kreiswirth *et al.*, 1983) is a restriction-defective mutant which is an efficient recipient for *Escherichia coli* DNA. pT181 is a naturally occurring 4.4 kb multicopy Tc^r plasmid from *S.aureus* (Iordanescu, 1976b). pWN1850 is a shuttle vector with the ColE1 and pC194 replication origins, the pC194 chloramphenicol resistance (*cat*) marker, and the β-lactamase gene from pI258 (Novick *et al.*, 1979) deleted for the *blaZ* promoter and Shine−Dalgarno site (Wang *et al.*, 1987). pSA7541 is cointegrate between a pT181 derivative, defective for both pT181 origin function (synonymous replacement of four nucleotides at nick site) and for tetracycline resistance, and Em^r plasmid pE5; pSA7541 is present in *S.aureus* strain SA1906 (Iordanescu, 1989). pRN6238 (Δ141) is a pT181 plasmid defective for RepC function (owing to the deletion of nucleotides 4381−15) and containing an active pT181 origin of replication (Novick *et al.*, 1982).

Media and growth conditions

Staphylococcus aureus strains were grown with aeration at 37°C (unless otherwise indicated) in CY broth (Novick and Brodsky, 1972) or on GL agar supplemented with antibiotics as required. Tetracycline was used at 5 μg/ml and erythromycin (Em) at 10 μg/ml. Transformation of *S.aureus* was by means of protoplasts (Gotz *et al.*, 1981) and that of *E.coli* was by the standard procedure (Sambrook *et al.*, 1989).

A Klett−Summerson colorimeter with green filter was used to monitor growth. A Klett reading of 343 corresponds to 1.0 μg (dry weight) of bacteria per ml. This bacterial concentration corresponds to $\sim 4.4 \times 10^9$ cells per ml (Wang and Novick, 1987).

General methods

Phage mediated transduction, protoplast transformation and media for *S.aureus* were as previously described (Novick and Brodsky, 1972; Chang and Cohen, 1979; Carleton *et al.*, 1984). Preparation of plasmid DNA from *S.aureus* was as previously described (Novick *et al.*, 1979). Preparation of plasmid DNA, restriction endonuclease digestions, agarose and acrylamide gel electrophoresis, and molecular cloning were performed using standard procedures as described by Maniatis *et al.* (1982) except that lysostaphin was substituted for lysozyme in the preparation of *S.aureus* protoplasts, lysates, etc. DNA restriction fragments for molecular cloning were isolated from polyacrylamide gels as described by Maxam and Gilbert (1980). DNA sequencing was performed by the dideoxy nucleotide chain termination method using [γ-^{35}S]SdATP (Sanger *et al.*, 1977; Biggin *et al.*, 1983); fragments to be sequenced were cloned into M13. An M13 universal primer was used for sequencing.

Chemicals

Tetracycline, erythromycin, soluble potato starch and pancreatic RNase were purchased from Sigma. Penicillin G sodium, T4 DNA ligase and T4 polynucleotide kinase were purchased from United States Biochemical. *Taq*I, *Cla*I, *Hin*dIII and proteinase K were purchased from Boehringer Mannheim. Nitrocefin was purchased from Baltimore Biological Labs. Lysostaphin was a gift from Applied Microbiology Inc. [γ-^{35}]SdATP and [^{3}H]dThd were purchased from New England Nuclear. Oligonucleotides were purchased from New York University Medical School.

Plasmid constructions

repC−blaZ gene fusions were constructed by means of synthetic oligonucleotides. pT181-*cop-623* was digested with *Cla*I, which recognizes a single site near the C-terminal end of *repC* (see Figure 1), and ligated to a 1 kb *Hin*dIII−*Taq*I fragment from pWN1850 (which contains the *blaZ* coding sequence) plus a double-stranded synthetic oligonucleotide that contains the C-terminal end of the RepC including the *Cla*I site and the desired *repC−blaZ* junction for either translational coupling or translational fusion (see Figure 2), followed by a *Hin*dIII site. The single-stranded oligonucleotides, purchased from NYU Medical School (see Figure 2), were annealed for 30 min at 55°C in hybridization buffer (Sambrook *et al.*, 1989), phosphorylated, then cloned into M13mp19 and sequenced before use. The first translational coupling construct was prepared with pT181-*cop-623*, and designated pJEB123 (see Figure 3). This construct was introduced by transformation into *S.aureus* strain RN4220 and shown to replicate normally and to produce β-lactamase. The plasmid pJEB123 was then used for the preparation of similar constructs with different pT181 derivatives as listed in Table II. For these constructions, the *Taq*I fragment containing the end of *repC* translationally coupled to *blaZ* was excised from pJEB123 and ligated to a *Cla*I-digested preparation of each of the other plasmids and the ligation product introduced in turn into RN451. These were forced clonings since restoration of the continuity of *repC* was required for plasmid viability. C-terminal *repC−blaZ* translational fusions were constructed by the same method, using an oligonucleotide linker fusing the two reading frames and lacking the *repC−blaZ* overlapping stop−start sequence (see Figure 2). These latter constructs could not be introduced by transformation into the plasmid negative strain RN451. However, they were able to be introduced by transformation into *S.aureus* strain SA1906, which contains a RepC-producing plasmid.

Figure 3 shows the structure of the basic gene fusion used in this study. The series of gene fusion plasmids prefixed pJEB is listed in Table II.

Determination of plasmid copy numbers

Plasmid copy numbers were determined by a modification of the agarose gel method described by Weisblum *et al.* (1979).

Copy numbers were calculated from the ratio of radioactive counts incorporated into the plasmid and chromosomal DNA. The determinations were based on an *S.aureus* cellular DNA content of 3.95×10^9 daltons of chromosomal DNA/cell (Projan *et al.*, 1983).

β-lactamase assays

β-lactamase activity was detected in bacterial colonies by starch−iodide decolorization (Perret, 1954); positive colonies have a clear halo on a dark blue background. For quantitative β-lactamase determinations, bacteria were grown overnight on GL plates containing 5 μg/ml of tetracycline. The cells were then used to inoculate 15 ml of CY broth to a cell density of 30 Klett units, and then grown to a final density of 200 Klett units. β-lactamase activity was assayed spectrophotometrically at pH 5.8 employing a chromogenic substrate, nitrocefin (O'Callaghan *et al.*, 1972). For measurements of low β-lactamase activity, a microiodometric method (Novick, 1962) was used. β-lactamase activity is expressed as units/mg dry wt where one unit represents 1 μmol substrate hydrolyzed per hour at 30°C.

We have found that with a standard enzyme sample assayed with nitrocefin and pencillin G, the activity with nitrocefin was 11.2% of that with penicillin G and the activity values given in the text have therefore been corrected by this factor so as to represent penicillin G activities.

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