JOURNAL OF BACTERIOLOGY, Jan. 1977, p. 407-414

Vol. 129, No. 1
Printed in U.S.A.

Inhibition of TnA Translocation by TnA

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Received for publication 11 August 1976

Plasmids already containing TnA showed decreased susceptibility to the translocation of a further TnA unit when compared with related plasmids that did not contain TnA. The translocation immunity imposed by TnA is exerted only on the plasmid of which it is part. It is suggested that this desensitization by a translocation unit is a general phenomenon that reduces the mutational effects of translocation.

The information available at present gives a rather unclear view of the specificity involved in the translocation of the ampicillin translocation unit TnA (10). On the one hand, this unit may be inserted at a substantial number of different sites in some small nonconjugative plasmids (e.g., RSF1010, molecular weight 5.5 $\times 10^6$ [11]; pSC101, molecular weight 6.0 $\times 10^6$ [13]) by a mechanism that apparently does not involve recombination functions of the host cell (3, 11, 13). These results imply that the specificity of the insertion process is low with respect to the base sequence of the recipient replicon. On the other hand, compelling evidence has also been presented that the ampicillin resistance determinant cannot be translocated to all replicons in *Escherichia coli* with equal efficiency (3). Furthermore, if the frequency with which the *amp* gene was translocated from the chromosome of *E. coli* UB1731 or UB1780 to R388 (3) matched by the frequency of translocation to other sites on the chromosome of these strains, TnA would appear as an element with strong mutator properties. However, cultures of *E. coli* UB1731 or UB1780 do not appear to contain abnormal numbers of mutated bacteria.

This paper seeks to clarify some of the factors that influence the ease with which a translocation unit interacts with a plasmid. In particular, we studied the effect that the acquisition of one translocation unit has on the insertion of a second into the same plasmid. The insertion of the first element greatly reduces the frequency with which a second is taken up. However, the effect is manifested only in the *cis* configuration since the presence of TnA on one plasmid does not greatly influence the translocation of TnA into a second compatible plasmid in the same host cell.

MATERIALS AND METHODS

Origin of bacterial strains and plasmids. The bacterial strains and plasmids used in these experiments are listed with their sources in Tables 1 and 2. The R-plasmid pUB501, a derivative of pUB310 that does not confer resistance to penicillins, was isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). *E. coli* UB1139 carrying the plasmid pUB310 was grown in 10 ml of nutrient broth. The cells were harvested in midlog phase, suspended in 0.1 M sodium citrate solution (pH 5.5) containing 150 μg of NTG per ml, and incubated in a shaking water bath at 37°C for 20 min. The NTG-treated cells were pelleted by centrifugation and suspended in a 10-ml nutrient broth culture of *E. coli* JC3272. The mixed-cell suspension was incubated with gentle shaking at 37°C for 30 min after which 0.1-ml volumes of suitable dilutions of the mixture were spread on minimal agar supplemented with tryptophan, histidine, and lysine (all at 20 μg/ml) and with trimethoprim (at 25 μg/ml). Colonies that arose were presumed to be JC3272 lines that had acquired plasmid pUB310 from the NTG-treated *E. coli* UB1139(pUB310) cells. These exconjugant clones were then examined to determine if they had acquired resistance to penicillins. Several clones that had acquired resistance to trimethoprim but not to penicillins were isolated. The plasmid harbored by one of these penicillin-sensitive lines was designated pUB501.

E. coli strains harboring pUB501 gave rise to penicillin-resistant derivatives at a frequency of 5.7 $\times 10^9$. Prior treatment with NTG substantially increased this frequency. The ampicillin resistance determinant carried by the revertants was shown to be plasmid borne and linked to the trimethoprim resistance determinant. Therefore, we concluded, that pUB501 is a point mutant of pUB310 that no longer expresses its *amp* resistance gene (see below).

Translocation frequency and gene transfer by mating. The procedures for determining translocation frequency and gene transfer were as described previously (3). Translocation frequencies for strains UB1731 and UB1780 are expressed as the ratio amp^r exconjugants/R^+ exconjugant.

Isolation of plasmid deoxyribonucleic acid (DNA). All plasmids were isolated from overnight nutrient broth cultures (200 ml) as described by Petrocheilou et al. (15).

Estimation of the contour length of plasmids.

TABLE 1. *Strains used in these experiments*

Strain	Genotype	Source	Reference
UB 1731	*pro⁻ met⁻ nal^r amp^r*	Derived from UB281	3
UB1780	*pro⁻ met⁻ nal^r amp^r recA56*	Derived from UB1731	3
JC6310	*trp⁻ his⁻ lys⁻ str^r recA56*	A.J. Clark	1, 3
UB1139	*leu⁻ thy⁻ met⁻ nal^r*	Derived from UB1005	9

TABLE 2. *Bacterial plasmids used in these studies*

Plasmid	Markers	Alternative designation	Reference	Source
R388 derivatives				
R388	Tp Su		7	N. Datta[a]
pUB310	Tp Su Ap	R388*amp-2*	3	This laboratory
pUB501	Tp Su		This paper	See Materials and Methods
pUB508	Tp Su Ap		This paper	See Results
pUB511	Tp Su Ap		This paper	See Results
pUB514	Tp Su Ap		This paper	See Results
pUB515	Tp Su Ap		This paper	See Results
pUB518	Tp Su Ap		This paper	See Results
pUB529	Tp Su Ap		This paper	See Results
RP1 derivatives				
pUB306	Kn Tc	RP1*amp-1 irp-1*	5, 6	This laboratory
R18-18	Kn Tc		4	P. M. Chandler[b]
pUB307	KnTc		This paper	See Results
R1 derivatives				
R1*drd-19*K1	Kn		2	This laboratory
R1*drd-19 ampC45*	[Ap]^c Cm Kn Sm Su		I. Crowlesmith (personal communication)	This laboratory

[a] Royal Postgraduate Medical School, London.

[b] Department of Genetics, Monash University, Clayton, Victoria, Australia.

[c] [Ap] indicates a phenotype in which β-lactamase activity is detectable but insufficient to protect the bacteria against selection with 500 μg of carbenicillin per ml.

The procedure used to estimate plasmid size was basically that described previously with minor modification (3). DNA-cytochrome *c* films, formed by the method of Lang and Mitani (14), were transferred to electron microscope grids. The DNA was stained by immersing the grid in 5×10^{-5} M uranyl acetate in 90% (vol/vol) ethanol for 30 s followed by immersion in isopentane for 10 s, as described by Davis et al. (8). The grids carrying the stained DNA were then rotary shadowed (3).

Restriction endonuclease cleavage profiles of plasmid DNA. Cleavage of plasmid DNA by the restriction endonuclease *Bam*HI, prepared from *Bacillus amyloliquefaciens*, was performed essentially as described by Wilson and Young (16). The DNA fragments that resulted from *Bam*HI cleavage were separated electrophoretically in 0.7% (wt/vol) agarose slab gels (15).

RESULTS

Isolation and characterization of a revertible *amp^s* derivative of pUB310. A previous publication described the translocation of the *amp* gene from its chromosomal site near *his* in *E. coli* UB1731 and UB1780 to the W incompatibility group plasmid R388. The process involved the insertion of a TnA element, and this step occurred at approximately equal frequencies at a minimum of two sites on the plasmid. Moreover, similar results were obtained in both Rec⁺ (UB1731) and RecA (UB1780) strains (3).

Further experiments have now generated a total of seven *amp^r* derivatives of R388. All have a molecular weight characteristic of R388 carrying TnA (approximately 25.5×10^6 [3]), and all have acquired an additional site sensitive to *Bam*HI restriction endonuclease, a situation to be expected since TnA carries a single site sensitive to this enzyme (F. Heffron, personal communication). However, an analysis in agarose gels of the polynucleotide fragments obtained from these plasmids by digestion by *Bam*HI endonuclease showed that the site of insertion of TnA was different in each case (Fig. 1). Therefore, it followed that TnA translocated to at least seven distinct sites in R388. Furthermore, the translocation events had a

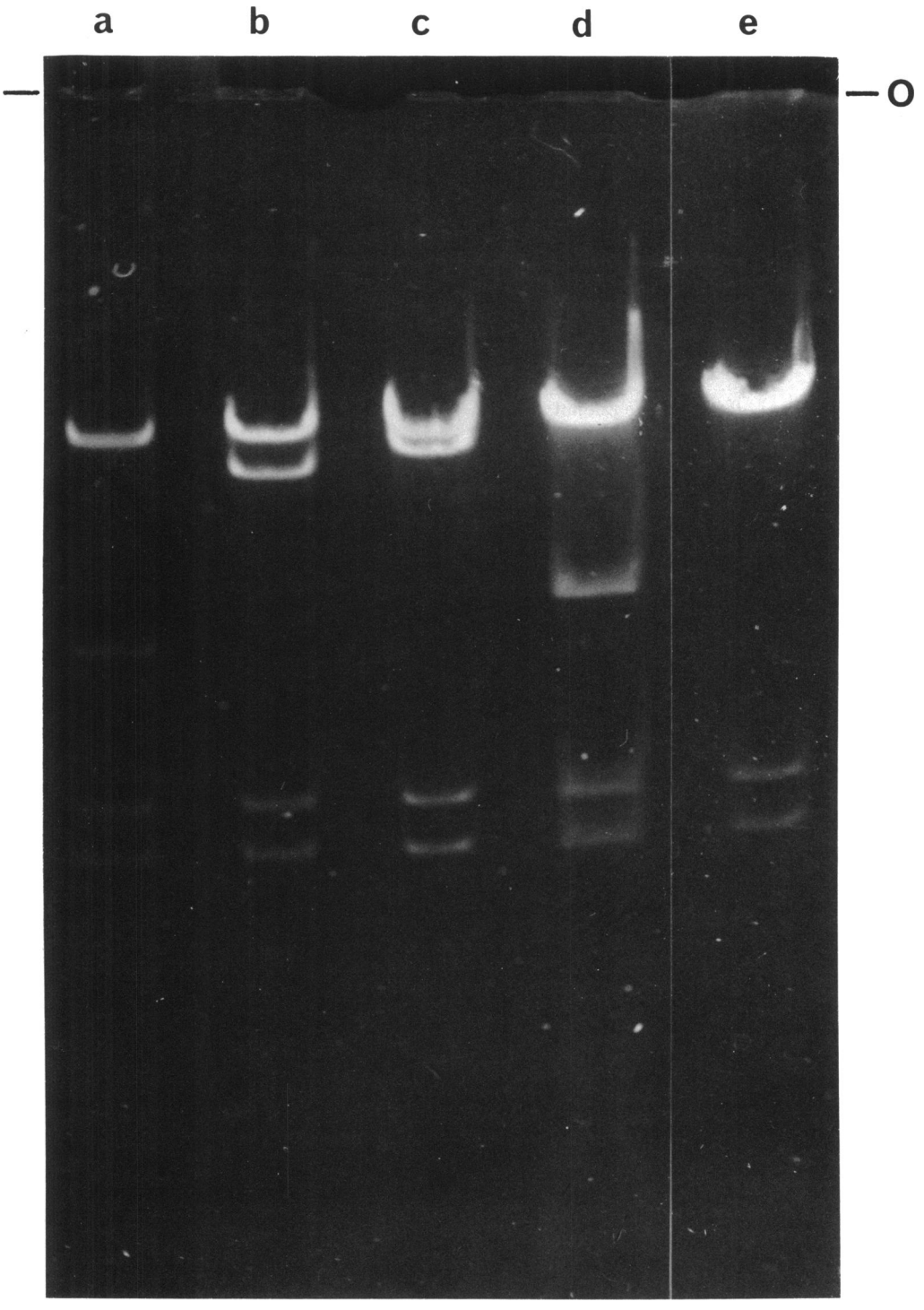

FIG. 1. *BamHI restriction endonuclease digests of R388 (e), pUB310 (a), pUB514 (b), pUB511 (c), and pUB508 (d) separated by electrophoresis in a 0.7% (wt/vol) agarose gel. O, Origin.*

TABLE 3. *Comparison of the properties of R388 and its derivatives*

Plasmid	Marker pattern	β-Lactamase activity (enzyme U per mg [dry wt])	Plasmid size		No. of *Bam*HI endonuclease fragments
			Contour length (μm)	Mol wt (×10⁶)	
R388	Tp Su Tra⁺	0	10.2 ± 0.1	21.1	3
pUB310	Tp Su Ap Tra⁺	75.4	12.2 ± 0.2	25.2	4
pUB501	Tp Su Tra⁺	<1.0	11.9 ± 0.2	24.7	4
pUB529	Tp Su Ap Tra⁺	45.4	NT[a]	NT	NT
pUB508	Tp Su Ap Tra⁺	26.5	12.5 ± 0.2	25.9	4
pUB511	Tp Su AP Tra⁺	43.9	12.5 ± 0.1	25.8	4
pUB514	TP Su AP Tra⁻	NT	12.3 ± 0.1	25.4	4
pUB515	Tp Su Ap Tra⁺	21.8	11.9 ± 0.6	24.7	4
pUB518	Tp Su AP Tra⁺	41.7	12.1 ± 0.5	25.1	4

[a] NT, Not tested.

range of different effects on the phenotype specified by the plasmids. The properties of four R388 derivatives carrying TnA (namely, pUB310, pUB508, pUB511, and pUB514) are summarized in Table 3.

One R388.*amp*ʳ recombinant plasmid (pUB310, formerly R388*amp-2*; reference 3), which arose in UB1731, was chosen at random for further study. It was first treated with NTG to generate derivatives unable to express their *amp* gene (see above). One such derivative, pUB501, was unable to confer resistance to penicillins because of an inability to produce an active β-lactamase. This plasmid gave revertants that had recovered the ability to produce an active β-lactamase after treatment with NTG.

Plasmid DNA was isolated from *E. coli* strains carrying either pUB310 or pUB501, and the sizes of the circular DNA molecules were measured by electron microscopy. The plasmid pUB501 had a contour length of 11.9 μm, a value in agreement with the size determined for pUB310 (alias R388*amp-2*; reference 3; Table 3).

To confirm that pUB501 carried no extensive DNA deletions, pUB501 DNA was digested with *Bam*HI restriction endonuclease, and the resulting pattern of fragments obtained on electrophoresis in agarose gels was compared with that obtained from the parental plasmid R388 and that from pUB310 DNA (Fig. 2). TnA is known to contain one site susceptible to *Bam*HI restriction endonuclease (F. Heffron, personal communication), and both pUB310 and pUB501 showed a single extra band when their digests were compared with that of R388, indicating the presence of an extra *Bam*HI restriction endonuclease site. Moreover, the digestion patterns of pUB310 and pUB501 were indistinguishable, which confirms that the mutational change in these two plasmids is likely to be a point mutation. An additional point worth noting is that the site of TnA insertion in both pUB310 and pUB501 is in the region of R388 that gives rise to the large *Bam*HI digestion fragment running near the origin of the agarose gel (Fig. 2). Table 3 summarizes the properties of R388, pUB310 and pUB501.

Effect of one TnA unit on the acquisition of a second. The availability of pUB501, in which the TnA unit was present but carried an inactive *amp* gene, allowed the frequency of translocation to this plasmid of a further TnA unit carrying an unmutated *amp* gene to be compared with the rate of translocation of TnA to R388, which does not carry any part of the TnA sequence. To this end, pUB501 was transferred to *E. coli* UB1731 and to UB1780 by conjugation, and the exconjugants that carried pUB501 were selected with trimethoprim. *E. coli* UB1731(pUB501) and *E. coli* UB1780(pUB501) were then outcrossed with *E. coli* JC6310 to assess the extent of *amp* translocation that had taken place (see above and reference 3). For this purpose, the exconjugant colonies were selected on minimal agar containing carbenicillin, and the colonies obtained in this way were then scored for co-inheritance of trimethoprim resistance. A similar experiment in which *E. coli* UB1731(R388) and UB1780(R388) were outcrossed into JC6310, exactly as described previously (3), acted as a control. Table 4 summarizes the results of this experiment. In the case of R388, about 1% of all the trimethoprim-resistant exconjugants were also carbenicillin resistant, and this frequency was similar whether the host cell was Rec⁺ or RecA. Thus, the frequency of *amp* translocation in both UB1731-(R388) and UB1780(R388) was about 10^{-2}.

The frequency with which pUB501 *amp*⁺ recombinants emerged from *E. coli* UB1731-(pUB501) was about 6×10^{-3}, which was only about 10-fold lower than that at which

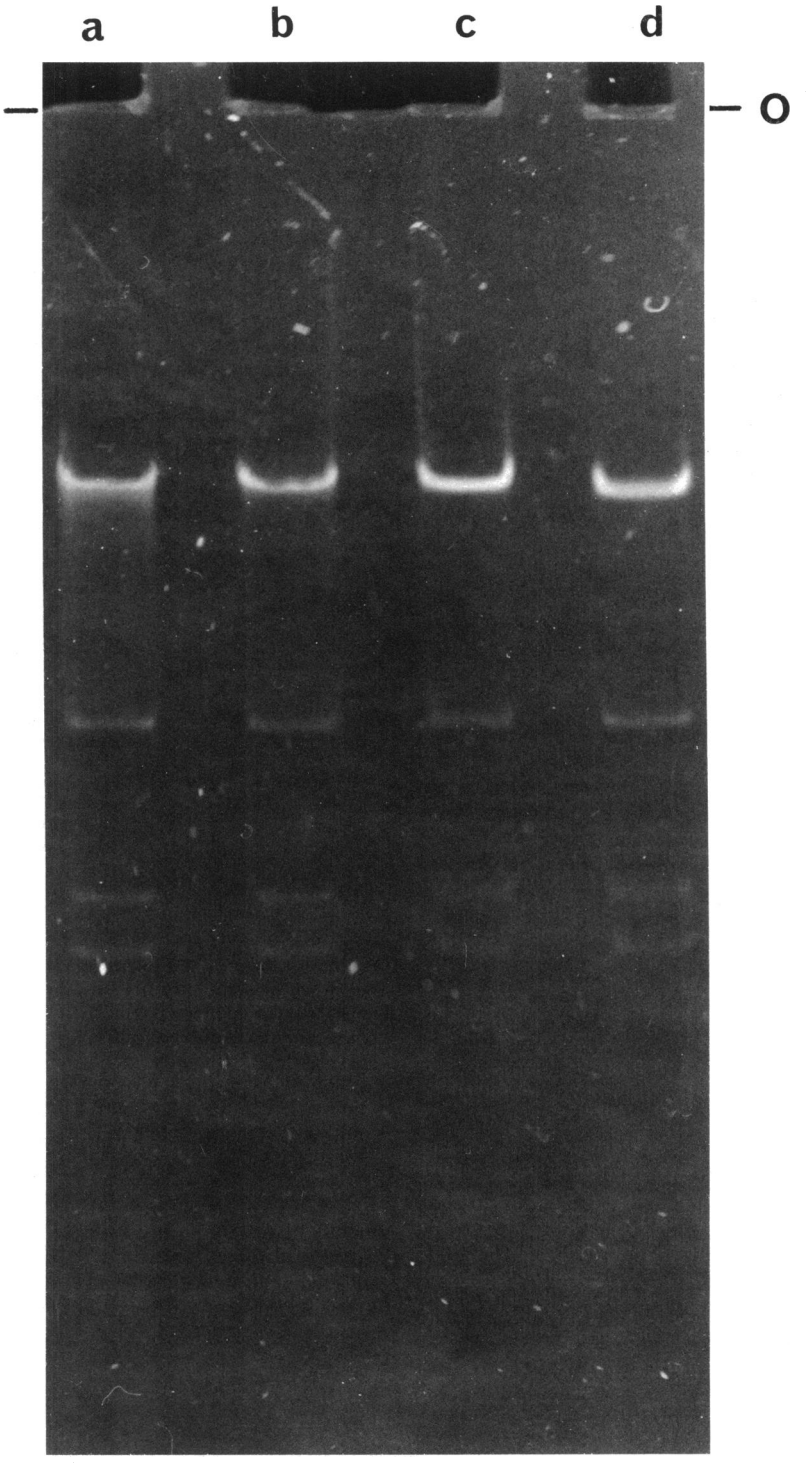

FIG. 2. *BamHI restriction endonuclease digests of pUB515 (a), pUB518 (b), pUB501 (c) and pUB310 (d) separated by electrophoresis in a 0.7% (wt/vol) agarose gel. O, Origin.*

TABLE 4. *Frequency of translocation of TnA from its chromosomal site in UB1731 and UB1780 to R388 and to pUB501*

Acceptor plasmid	Translocation frequency	
	UB1731 (Rec$^+$)	UB1780 (RecA)
R388	1.1×10^{-2}	2.4×10^{-2}
pUB501	6.1×10^{-3}	2.8×10^{-7}

exconjugants with a similar phenotype (but not necessarily with a similar genotype; see below) were derived from UB1731 (R388) (Table 4). In contrast, *amp*^{r} derivatives of pUB501 were derived from UB1780(pUB501) at a frequency of about 3×10^{-7}, i.e., at a frequency about 10,000-fold lower than that found when R388 was used in place of pUB501 in a *recA* host strain (Table 4).

Translocation of the *amp* gene between replicons is normally associated with the acquisition by the recipient replicon of a piece of DNA with a molecular weight of about 4.0×10^6 (3). On the other hand, normal recombination is a reciprocal event, and no increase in molecular weight of the recipient plasmid should occur. To determine whether the *amp*^{r} recombinants that emerged from UB1731(pUB501) were the result of a translocation event, plasmid DNA from *E. coli* JC6310 carrying pUB515 (an *amp*^{r} derivative of pUB501) was isolated, and its size was determined by electron microscopy (Table 3). The contour length of pUB515 was the same as that of pUB310 and pUB501; i.e., the acquisition of a functional *amp*^{r} determinant by pUB501 seems to have occurred with no increase in plasmid size. This is likely to have occurred by reciprocal recombination and not be the translocation of an additional TnA unit into the plasmid. Similarly, examination of an *amp*^{r} derivative of pUB501 (namely, pUB518) that emerged from the *recA* host, UB1780, also indicated that the repair of the *amp* mutation on pUB501 had been by reciprocal recombination and not by translocation (Table 3). The *recA56* mutation present in UB1780 is known to be slightly leaky (12), and this could account for the apparent repair of the *amp* lesion on pUB501 by recombination.

If the repair of the *amp* mutation on pUB501 was by recombination, the reciprocal nature of the event should not alter the size of the fragments obtained from these plasmids after endonuclease digestion. To test this point, pUB515 DNA was prepared and the material was digested with *Bam*HI endonuclease. The digest was then separated by ionophoresis in an agarose slab gel, and the pattern of fragments obtained was compared with those arising from pUB310 and from pUB501. Despite the range of ampicillin phenotypes specified by these plasmids, the *Bam*HI digests showed identical patterns in the agarose gels for all three plasmids (Fig. 2), providing additional evidence that pUB515 was derived from pUB501 by reciprocal recombination rather than by translocation.

Similar studies with *Bam*HI restriction endonuclease on the plasmid pUB518 have shown that it has arisen by reciprocal recombination involving pUB501, although the plasmid arose in a RecA host in this case (data not shown). Several other pUB501*amp* recombinants have been examined, and all appear to have acquired the *amp* gene by reciprocal recombination, rather than by translocation.

Translocation of TnA to other plasmids already carrying TnA. In view of the ability of an ampicillin translocation unit already located on R388 to reduce the frequency of translocation of a second unit to the same plasmid, it was decided to see whether a similar phenomenon could be detected with other R-plasmids. Therefore, studies similar to those already reported with R388 were carried out with derivatives of RP1 and of R1.

A number of RP1 derivatives were available. RP1*amp-1 irp-1* (alias pUB306) is a mutant of RP1, which is of the same size as the parent plasmid, but which cannot express either its β-lactamase (*amp*) or its intrinsic penicillin resistance (*irp*) genes (5, 6). R18-18 does not specify β-lactamase production and is a revertible mutant of R18, a plasmid indistinguishable from RP1. Plasmid pUB307, on the other hand, is a derivative of RP1 that carries a deletion of approximately 3.3×10^6 and that cannot specify β-lactamase production. Therefore, the first two plasmids carry TnA in a mutated form, whereas pUB307 carries a deletion that has removed *amp*.

Each of these plasmids was transferred to the Rec$^+$ and Rec$^-$ *E. coli* strains UB1731 and UB1780, respectively, by standard mating procedures (see above). These two strains with their carried plasmids were then outcrossed with *E. coli* strain JC6310 to measure the translocation frequency of the *amp* gene from its site in the *E. coli* chromosome to the resident plasmid (Table 5). The frequency of translocation of TnA from the chromosome to pUB307 occurred at a frequency of about 2×10^{-2}, regardless of whether the host cell was Rec$^+$ (UB1731) or RecA (UB1780) (Table 5). In contrast, the translocation of TnA from the *E. coli* chromosome to pUB306 or to R18-18 occurred at a frequency of only 10^{-5} in an RecA background, a reduction of about 2,000-fold over the frequency obtained when no TnA was already present in the recipi-

TABLE 5. *Frequency of translocation of TnA from its chromosomal site in UB1731 and UB1780 to derivatives of RP1 and R1*

Acceptor plasmid	Translocation frequency	
	UB1731 (Rec+)	UB1780 (RecA)
pUB306	1.3×10^{-2}	1.4×10^{-5}
pUB307	2.5×10^{-2}	2.7×10^{-2}
R18-18	2.0×10^{-2}	1.0×10^{-5}
R1*drd-19 ampC45*	4.6×10^{-6}	5.8×10^{-7}[a]
R1*drd-19*K1	2.4×10^{-3}	1.4×10^{-3}

[a] Maximum value.

ent plasmid. However, as was the case with the derivatives of R388 described earlier, *amp*^{r} derivatives did emerge at a frequency of about 10^{-2} when pUB306 and R18-18 were used in a Rec$^+$ host (UB1731; Table 5).

These studies showed exactly the same pattern as was found with the derivatives of R388. If the recipient plasmid still carried a TnA unit with its *amp* gene inactivated, then the translocation of another TnA to the same plasmid could not be detected, and all the *amp*^{r} derivatives that were isolated from this plasmid arose by a reciprocal recombination, presumably because of the leakiness of UB1780 (see above). However, when the recipient plasmid did not carry TnA, translocation of the ampicillin translocation unit occurred at high frequency from its site on the chromosome of either UB1731 or UB1780.

Similar experiments, in which the translocation of TnA to R1*drd-19*K1 and to R1*drd-19 ampC45* from UB1731 and UB1780 were measured, gave similar results (Table 5). Translocation to R1*drd-19*K1 occurred at a frequency of approximately 2×10^{-3}, irrespective of the state of the *recA* gene in the host cell, and this correlates with the fact that this plasmid carries an extensive deletion of R1 that has removed several functions, including the ability to mediate resistance to penicillins (2). R1*drd-19 ampC45*, on the other hand, is a point mutant of R1*drd-19* that still carries TnA, although the expression of the *amp* gene by this plasmid is so poor that one can select against *E. coli*, carrying it with ampicillin (I. Crowlesmith, personal communication). Translocation of TnA to this plasmid was not detected in an *recA* host (limit of detection, 6×10^{-7}; Table 5). Therefore, these results are consistent with those already described for derivatives of R388 and RP1, that carriage of a TnA unit in a plasmid greatly reduces the ability of that plasmid to accept another unit of the same type.

Does TnA block the translocation of TnA when located on a separate plasmid? Since the presence of TnA on a plasmid blocks the uptake of a second TnA unit onto the same plasmid, the question arises as to whether a TnA unit located on a separate plasmid can block insertion of TnA into a plasmid that does not carry a copy of a TnA element.

To test this point, the RP1 derivative pUB307 was transferred to both UB1731(pUB501) and UB1780(pUB501), i.e., to both *E. coli* UB1731 and UB1780, which already carried the R388 derivative pUB501. Exconjugant colonies obtained in this way were then outcrossed to *E. coli* JC6310 as a recipient, and the transfer of plasmid-borne ampicillin resistance was selected for with carbenicillin. Exconjugants that arose from this cross were tested to determine whether they had also acquired resistance to kanamycin and to trimethoprim in addition to carbenicillin. All clones tested had acquired resistance to kanamycin but not to trimethoprim regardless of the recombination proficiency of the host strain. The results of this experiment (Table 6) show that pUB307.*amp*^{r} recombinants arose at a frequency of about 1.7×10^{-2} per pUB307 in UB1731 and at a frequency of about 2.3×10^{-3} per recipient replicon in UB1780. These frequencies of translocation are not significantly lower than those observed for translocation of TnA to pUB307 in the absence of pUB501 and, consequently, the TnA unit in pUB501 does not seem to be exerting a *trans* effect. The apparent lack of any pUB501.*amp*^{r} recombinants in UB1731 carrying pUB307 was not unexpected since conjugal transfer of R388 is severely inhibited when derivatives of RP1 are carried in the same *E. coli* cell (V. Stanisich, personal communication).

DISCUSSION

The experimental data described here argue strongly that the presence of a TnA unit in a bacterial plasmid exerts a strong inhibitory effect against the uptake of a second TnA unit by the same replicon. So strong is this effect that the uptake of a second TnA into a recipient plasmid has not yet been observed even when the uptake of the first element occurs at high frequency.

This phenomenon does not seem to be site specific. Not only did it appear in a fully devel-

TABLE 6. *Translocation of TnA onto pUB307 in the presence of pUB501*

Acceptor plasmid	Second plasmid in donor	Translocation frequency	
		UB1731 (Rec+)	UB1780 (RecA)
pUB307	pUB501	1.7×10^{-2}	2.3×10^{-3}
pUB307	Nil	2.5×10^{-2}	2.7×10^{-2}

oped form in the first R388.*amp*^{r} recombinant tested in these experiments, but it was also found associated with TnA units in the P class plasmid RP1 and the FII plasmid R1. Therefore, it seems certain that this phenomenon is expressed by TnA, at least, in a wide range of plasmid replicons.

The existence of the desensitizing effect goes some way to explain why translocation units such as TnA are not powerful mutator elements. The frequency of TnA translocation to recipient replicons reported by a number of workers (3, 11, 13), if applied to the bacterial chromosome, would argue that TnA should have a marked lethal effect. However, there is no evidence that strains such as UB1731 and UB1780, in which TnA is integrated into the chromosome, contain a large population of dead bacteria, even though these strains are potent sources of TnA elements for translocation to other replicons.

In general, natural processes that have the effect of altering bacterial DNA tend to have counterbalancing forces. Thus, mutation is balanced by repair and restriction, balanced by modification. If this principle is general and such balanced pairs of interactions are widespread, then one should anticipate the existence of a process that limits the damage caused by translocation, and in the desensitizing effect of TnA we may have found a manifestation of the process. But how widespread the phenomenon is and the extent to which different replicons may interact remain to be established.

The fact that the desensitization is *cis* active is only to be expected since a *trans* action would effectively block translocation. But a *cis* effect does raise problems as to the mechanism. It is often assumed that *trans* effects denote diffusible products, whereas *cis* effects are more readily explained by proposing a site of action of a diffusible product. In the case of the desensitization produced by TnA, one cannot exclude the possibility of a *cis*-active protein. However, for this to be possible, one would have to invoke an enzyme specified by TnA which remained in contact with the replicon of which TnA was a part.

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