Curing of F-like plasmid TP181 by plumbagin is due to interference with both replication and maintenance functions

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F-like plasmid TP181 is particularly susceptible to curing by the naphthoquinone derivative plumbagin, which may attack DNA gyrase. TP181 should provide particular insight into the basis of plasmid elimination, which has application in a number of contexts. Curing was found to be optimal at pH 7.2. An *EcoRI* fragment containing the RF1A replicon of TP181 was joined to a Km^R determinant (giving miniTP181). MiniTP181 had the same increased susceptibility to curing by plumbagin when compared to miniF as TP181 had relative to F. Plumbagin interfered with replication of miniTP181, depressing its copy number and increasing the rate of segregation. Plumbagin also blocked the lethal effect of the *ccd* locus after rifampicin treatment, which mimics production of plasmid-free segregants, so that more of these plasmid-free cells would survive. Restriction mapping and DNA sequence analysis indicated that the *ccd* locus of TP181 is almost identical to that of F but that TP181 lacks the *repC* gene present in F which is needed to activate replication from *oriV*. Thus the sensitivity of TP181 may be due to its dependence on both a replicon which is hypersensitive to perturbation of supercoiling by plumbagin and a host-killing system which is blocked by plumbagin.

Keywords: plasmid curing, plumbagin, rifampicin, *ccd* locus

INTRODUCTION

Bacterial plasmids are extrachromosomal DNA elements which are normally inherited stably as the bacterial host grows and divides. Many plasmids are self-transmissible and if they integrate into the chromosome they can also promote conjugative transfer of chromosomal genes. Many phenotypic traits are encoded by plasmid-borne genes. These include properties such as resistance to antibiotics and heavy metals (Stanisich, 1988). Resistance to antibiotics is once again a major clinical problem and strategies are needed to help combat the spread of antibiotic resistance which is mediated by plasmids.

One strategy to minimize plasmid transmission of antibiotic resistance is to eliminate the plasmids. This process is known as 'curing' and many compounds have been shown to be capable of causing this effect (Caro *et al.*, 1984; Trevors, 1986). Some of these compounds may

cause curing by a rather nonspecific process – they damage and stress the cells so that aberrant cells emerge. Other agents seem to act much more selectively. Acridine orange is an example of this sort. It causes elimination of the sex factor F at concentrations where it has little effect on the bacterial host (Hohn & Korn, 1969). Mutations in the *copB* locus gave resistance to this curing effect and it was suggested that this region might contain the target for acridine orange (Wechsler & Kline, 1980). However, this resistance was probably not very specific and could have arisen simply from the elevated copy number of the mutants, which would decrease the segregation rate even if replication inhibition were observed. Resistance was also found to arise in some temperature-stable revertants of *repts* mutants, suggesting that acridine orange sensitivity is a property of the basic replicon (Lane, 1981). The molecular basis for the sensitivity has not been further defined.

However, it is remarkable that any single agent could cause efficient plasmid loss since detailed analysis of F and other plasmids has revealed multiple plasmid strategies for survival. F and F-like plasmids appear to have two or

The GenBank accession number for the sequence of the TP181 *ccd* region is U51588.

three replicons (Bergquist *et al.*, 1986), which could thwart attempts to block plasmid replication by interfering with just one of these. In addition, F has an efficient partitioning system (Austin & Wierzbicki, 1983; Ogura & Hiraga, 1983a; Mori *et al.*, 1986) as well as systems for killing plasmid-free segregants (Ogura & Hiraga, 1983b; Gerdes *et al.*, 1990). It is therefore worth trying to determine how specific curing agents work and if indeed they can cause curing by attacking just one target.

In this paper we report studies on the compound plumbagin (5 hydroxy-2-methyl-1,4-naphthoquinone), which was observed to eliminate the F-like plasmid TP181 with high frequency (Lakshmi *et al.*, 1986; Bharathi & Polasa, 1991). We show that plumbagin appears to interfere with at least two stability mechanisms.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* K12 strains C600 (*thr-1 leu-6 thi-1 lacY1 supE44 ton-21 galK*), MVIT (C600E. coli C strain C2110 (*polA1 his rha*) were used (all strains were from Professor D. R. Helinski, Department of Biology, UC San Diego, CA, USA). The plasmids used or constructed during the study are given in Table 1. Media used were: L-broth/agar (Kahn *et al.*, 1979), nutrient broth (Difco), Antibiotic Assay Medium no. 3 (Difco), M9 and M9 + Casamino acids (CAA) (Kahn *et al.*, 1979). For selection of antibiotic resistance, media were supplemented with sodium benzylpenicillin (100 µg ml⁻¹ in liquid media and 300 µg ml⁻¹ in agar plates), kanamycin sulphate (50 µg ml⁻¹) tetracycline hydrochloride (20 µg ml⁻¹), streptomycin sulphate (50 µg ml⁻¹).

Isolation, analysis and manipulation of plasmid DNA. Plasmid DNA was isolated by a modification of the alkaline SDS method of Birnboim & Doly (1979). Large-scale plasmid purification was carried out by means of alkaline SDS followed by CsCl/ethidium bromide density-gradient centrifugation. Plasmid DNA was digested with restriction enzymes under conditions recommended by the suppliers and run on 0.8 or 1% (w/v) agarose gel. Bacterial transformations were carried out using the method of Meyer *et al.* (1977). DNA sequencing was

carried out by AltaBioscience using the Dye-terminator method in conjunction with an ABI 373 automated DNA sequencer. Sequences were aligned and analysed using programs of the Wisconsin package (Devereux *et al.*, 1984).

Curing experiments with subinhibitory concentrations of plumbagin. These were carried out as described previously (Lakshmi *et al.*, 1986, 1989). Bacteria from an overnight culture grown under selective conditions were diluted 10³-fold into 1 ml nonselective medium with the appropriate concentration of curing agent. After incubation at 37 °C for 18 h the cultures had again reached saturation and were diluted and plated on L-agar plates. Three hundred colonies were then tested for sensitivity to antibiotic by replica-plating to estimate the proportion of plasmid loss.

Incorporation of [³H]thymidine. Strain MVIT carrying the plasmid was grown overnight in M9 + CAA in presence of selective antibiotic. The culture was diluted and allowed to grow until the OD₆₀₀ reached approximately 0.5 in the same medium. [³H]Thymidine was added to a final concentration of 10 µCi (370 kBq) ml⁻¹ and the culture grown for a further 2 h. Plumbagin at 25 µg ml⁻¹ and 12.5 µg ml⁻¹ was added to the treated samples along with [³H]thymidine and parallel controls with no added plumbagin were maintained. After 2 h, plasmid DNA was extracted, digested with restriction enzymes and run on 0.8% agarose gel to compare the plasmid yield. The incorporation of [³H]thymidine was measured by liquid scintillation counting by cutting the gel slice with plasmid DNA bands, dissolving in water and adding water-miscible scintillant. The corresponding [³H]thymidine incorporation into total bacteria DNA was estimated by precipitating the total DNA from 1 ml culture in 1 ml 10% (w/v) trichloroacetic acid, filtering through a glass-fibre filter (Millipore), washing with ethanol, drying and measuring by liquid scintillation counting as above.

Rifampicin-induced killing of bacteria with plasmids. The cultures with plasmids were grown overnight at 37 °C in selective conditions. The cells were diluted 1:100 into non-selective medium, grown for 2 h, and then rifampicin was added at 200 µg ml⁻¹. Sampling for viable count was done at 10–15 min intervals after diluting in saline solution. The effect of plumbagin on rifampicin-induced killing was determined by incorporating a subinhibitory concentration of plumbagin along with

Table 1. Plasmids used or constructed in the study

Plasmid	Replicon	Size (kb)	Selectable markers*	Reference/source
TP181	RepFIA	98.2	Ap, Cm, Km, Sm, Su, Tc	Willshaw <i>et al.</i> (1978)
pML31	RepFIA	16	Km	Lovett & Helinsky (1976)
pBR322	pMB1	4.63	Ap, Tc	Bolivar <i>et al.</i> (1977)
pDF11	ColE1/RepFIA	22	Km	Figurski <i>et al.</i> (1978)
pOU82	IncFII	12	Pn	Gerdes <i>et al.</i> (1985)
pRK2501	IncP	11.0	Km, Tc	Kahn <i>et al.</i> (1979)
pLAK54	pMB1/RepFIA	14.6	Ap, Tc	This study
pLAK77	pMB1/RepFIB	11.6	Ap, Tc	This study
pLAK100	pMB1	11.6	Ap, Km, Tc	This study
pLAK105	RepFIA	16.5	Km	This study
pLAK107	RepFIB	14.5	Km	This study

* Antibiotic resistance markers: Ap, ampicillin/penicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; Pn, penicillin resistance; Sm, streptomycin resistance; Su, sulphonamide resistance; Tc, tetracycline resistance.

rifampicin in the sample. Controls with only rifampicin and plumbagin respectively were included simultaneously to estimate the effect of plumbagin alone on viability.

RESULTS

Construction of miniTP181 plasmids

In order to identify the basis of curing by plumbagin we wished to determine whether the enhanced susceptibility to curing observed for TP181 was also associated with mini derivatives containing the primary replicon and stability functions. Therefore DNA of the 98.2 kb plasmid was digested with *EcoRI* and the fragments were separated by electrophoresis on a 0.8% agarose gel. Previous studies had shown that miniF replicons hybridize with *EcoRI* fragments of 9 kb and 6.5 kb from TP181 (Bergquist *et al.*, 1982, 1986), which we thus presumed to be equivalents of the f5 and f7 fragments of F plasmids carrying the primary and secondary replicons (RF1A and RF1B). The fragments were purified and cloned separately into the *EcoRI* site of pBR322. Transformation into *E. coli* strain C2110 (*polA*) allowed the selection of derivatives which had acquired the DNA PolI-independent TP181 replicons. pLAK54 and pLAK77 were identified as containing the 9.5 kb and 6.5 kb fragments of TP181 respectively with the two replicons. The identity of both fragments as corresponding to the expected replicon was confirmed by

DNA sequencing from the ends of the fragments using primers which hybridized to the flanking vector sequences (see below).

MiniTP181 retains the increased sensitivity to plumbagin

To obtain selectable miniTP181 derivatives that could be studied in a *PolA*⁺ host we joined the relevant replicon fragments to an *EcoRI* fragment (from pDF11) containing a Km^R determinant. pLAK105 and pLAK107 contained the primary (RF1A) and secondary (RF1B) replicons respectively (Bergquist *et al.*, 1986). These miniTP181 derivatives were tested for their sensitivity to curing with plumbagin and the activity was compared with miniF plasmid pML31 in a range of media. The results (Table 2) showed that pLAK107 with the RF1B replicon was very unstable even in the absence of plumbagin, as expected from a study of the equivalent replicon of F (Lane & Gardner, 1979). pLAK105 with the RF1A replicon was stable in the absence of plumbagin. Addition of sub-inhibitory concentrations of plumbagin increased the rate of loss of pLAK107 slightly but had a dramatic effect on pLAK105, indicating that this miniTP181 derivative retains the sensitivity to curing by plumbagin. The curing was highest in Antibiotic Assay Medium no. 3 followed by nutrient broth and M9+CAA, which all showed similar levels of curing that were significantly better than those in L-broth and M9 medium. The primary miniTP181 replicon in pLAK105 was consistently and significantly more sensitive to curing with plumbagin than pML31. This was consistent with the earlier observation that TP181 was more sensitive to curing than the F plasmid (Lakshmi *et al.*, 1986). It suggested that study of pLAK105 may provide an explanation for the particular sensitivity of TP181 to plumbagin.

Table 2. Curing by plumbagin of miniTP181 and miniF plasmids in different media

Medium	Plasmid	Plasmid loss (%)*		
		[Plumbagin] ($\mu\text{g ml}^{-1}$):		
		0	12.5	25
Antibiotic assay medium no. 3	pLAK105	0	34	47
	pLAK107	24	36	42
	pML31	0	10	25
L-broth	pLAK105	0	13	24
	pLAK107	27	29	38
	pML31	0	5	14
Nutrient broth	pLAK105	0	29	44
	pLAK107	23	22	39
	pML31	0	8	19
M9 medium	pLAK105	0	13	26
	pLAK107	26	30	39
	pML31	1	5	11
M9 + CAA	pLAK105	0	26	45
	pLAK107	26	36	46
	pML31	0	9	20

* Plasmid loss is estimated as total loss during a 10^5 increase (approximately 17 generations). Overnight cultures were diluted 10^5 and allowed to grow back to saturation. The experiment was repeated twice and the figures presented are the mean of these results. The relative levels of stability in different media were reproducible.

Effect of pH of the medium on curing

The influence of pH of the medium on curing against pLAK105 and pLAK107 was compared in Antibiotic Assay Medium in the range from pH 6.8 to 8.2. There was very little curing activity at pH 8.0 and above. The curing frequency increased with decrease in pH up to pH 7.0, the optimum being pH 7.2, where a maximum of 58% curing over approximately 15 generations was observed with a plumbagin concentration of $25 \mu\text{g ml}^{-1}$.

Plumbagin differentially blocks [³H]thymidine incorporation into CCC plasmid DNA

To study the effect of plumbagin on replication of plasmid TP181, pLAK105 and pLAK107 were transformed into thymine auxotrophic strain MVIT and the amount of [³H]thymidine incorporation into covalently closed circular (CCC) plasmid DNA and total DNA was compared in the presence and absence of plumbagin. Plasmids pBR322, pOU82 and pRK250, which are not cured or are cured with very low frequency by plumbagin, were also included as controls. The results (Table 3) showed a

Table 3. Effect of plumbagin treatment on incorporation of [³H]thymidine into CCC plasmid and total DNA

Plasmid	[Plumbagin] ($\mu\text{g ml}^{-1}$)	Incorporation of [³ H]thymidine [c.p.m. (ml culture) ⁻¹]		Proportion of [³ H]thymidine in CCC DNA
		CCC DNA	Chromosomal DNA	
pLAK105	0	15×10^3	12×10^5	0.012
	12.5	6.9×10^3	15×10^5	0.005
	25.0	2.9×10^3	5.5×10^5	0.006
pLAK107	0	33×10^3	5.5×10^5	0.061
	12.5	30×10^3	5.5×10^5	0.054
	25.0	15×10^3	2.7×10^5	0.054
pRK2501	0	11×10^3	7.1×10^5	0.015
	12.5	11×10^3	6.5×10^5	0.017
	25.0	9.1×10^3	5.5×10^5	0.016
pBR322	0	59×10^3	7.4×10^5	0.078
	12.5	46×10^3	4.9×10^5	0.095
	25.0	39×10^3	4.0×10^5	0.098
pOU82	0	11×10^3	8.4×10^5	0.013
	12.5	15×10^3	5.2×10^5	0.029
	25.0	10×10^3	4.9×10^5	0.021

significant decrease in the plasmid yield in plumbagin-treated samples for pLAK105. When [³H]thymidine incorporation into CCC plasmid DNA was expressed as a percentage of total incorporation it could be seen that at both concentrations of plumbagin there was a significant differential effect on pLAK105, a two- to threefold decrease being observed. Given that the copy number of IncFI replicons is very low (approximately one per chromosome) a decrease of this magnitude could result in there not being enough plasmid copies for efficient transmission to daughter cells. When the effect on plasmids pBR322, pOU82 and pRK2501 was examined in the same way it was clear that they were not differentially affected – incorporation into plasmid DNA remained at about the same percentage of total incorporation at both plumbagin concentrations. The fact that concentrations of plumbagin which do not inhibit bacterial growth have a significant effect on incorporation of [³H]thymidine into CCC DNA suggests that a primary target for this antimicrobial agent could be DNA replication, consistent with DNA gyrase being its target. An alternative explanation would be that plumbagin is causing DNA gyrase to convert a large proportion of the CCC DNA to open circular form and that we are simply not recovering it by the plasmid isolation method employed here. DNA topology can affect many processes, including replication initiation, transcription and formation of complexes such as those likely to form at the centromere-like sequence required for partitioning. At present our data do not distinguish between these possibilities. Whatever the explanation, these results identify an aspect of fundamental properties where TP181 clearly is much more sensitive to plumbagin than either the bacterial chromosome or other plasmids.

Plumbagin blocks host cell killing induced by rifampicin

As plumbagin was observed to eliminate TP181 and also interfere with DNA replication or topological form, it was of interest to investigate its influence on the other plasmid maintenance systems since F-like plasmids have several loci which may minimize loss even if replication efficiency drops. These functions include active partition (*sopABC*) (Austin & Wierzbicki, 1983) and site-specific resolution (*resD*) (Lane *et al.*, 1986) as well as systems which couple replication and cell proliferation (*ccd*) (Ogura & Hiraga, 1983a). The *ccd* locus, located at coordinates 42.84–43.6 of miniF, includes two genes, *ccdB* and *ccdA*, which code for a poison and antidote playing an important role in plasmid maintenance by killing plasmid-less segregants (Miki *et al.*, 1984). CcdB protein causes cell death and induction of the SOS response when its action is not prevented by CcdA (Jaffe *et al.*, 1985). CcdB acts by attacking DNA gyrase, which may also be the target for plumbagin (Bernard & Couturier, 1992; Bernard *et al.*, 1993).

The lethal effect of genes which have their effect when the plasmid has been lost is generally inducible by rifampicin, which mimics the loss of plasmid (Gerdes *et al.*, 1990). We expected the same to be true for the effect of the *ccd* locus. Preliminary tests with the standard C600 strain used in these experiments showed that the viable count did not decrease during incubation with rifampicin ($200 \mu\text{g ml}^{-1}$) if the bacteria were subsequently diluted and plated on medium without rifampicin. However, when F, TP181, miniF or miniTP181 were present we observed a marked host lethality was induced although the effect observed was least with miniTP181 (Fig. 1). Since *ccd* is the only

Fig. 1. Effect of plumbagin on the rifampicin-inducible killing phenotype of (a) TP181, (b) pLAK105 (miniTP181), (c) pML31 (miniF) and (d) control plasmid pLAK107 (TP181 RepFIB fragment lacking known killer system) in *E. coli* C600. The data points are the means of duplicates within one experiment. The differences were reproducible between experiments, which were repeated twice. ○, Plus rifampicin; □, plus plumbagin; △, plus rifampicin and plumbagin.

miniF locus known to be induced by loss of plasmid it seems very likely that this was causing the lethal effect and would be the basis for the effect with miniTP181 as well. pLAK107 showed no rifampicin-induced effect, as expected since the *F* *Eco*RI fragment 7 with RepFIB replicon is not associated with such a function. The response of C600(pLAK107) to rifampicin also serves as a control to demonstrate that the rifampicin-induced effect is dependent on the presence of specific plasmids. The difference in the magnitude of the lethal effect with pML31 and pLAK105 suggests the possibility that the *ccd* genes of TP181 are not as potent as those of *F*. The difference between the lethal effect of TP181 and miniTP181 (pLAK105) suggests that other loci in addition to *ccd*, for example *bok/sok* homologues, are contributing a large part of the TP181 rifampicin-induced lethal effect. The addition of plumbagin at subinhibitory concentration (25 µg ml⁻¹) along with rifampicin blocked the rifampicin-induced killing function of TP181, pML31 and miniTP181 plasmid pLAK105 (Fig. 1a, b, c). Plumbagin had no influence on the response of C600(pLAK107) since it showed no rifampicin-induced lethality in the first place (Fig. 1d). Thus plumbagin may have some rather general effects on rifampicin-induced effects since it blocks most of the lethality exerted by TP181 but it also seems to have specific interactions with *ccd*-like systems. This may potentiate its curing activity by interfering with the ability to kill plasmidless segregant clones.

Conservation and divergence of miniTP181 and miniF

The difference in the sensitivity of miniTP181 and miniF to curing by plumbagin made it interesting to determine the extent of homology/divergence between the two plasmids. A range of restriction sites were mapped in the

Fig. 2. Comparison of the restriction maps of the *Eco*RI fragments with the RF1A replicons from TP181 and *F*. Note that all the sites shown at the left end of *F* are missing in TP181. Below are indicated the locations of protein-coding regions (arrows above and below the line) of miniF as well as *cis*-acting regions (solid blocks on the line). Nomenclature is according to Kline (1985). The mapping of these regions is based on the nucleotide sequences and functional analysis: *pifA*, Rotman *et al.* (1983); *repC/pifC*, Caughey *et al.* (1986), Miller & Malamy (1986), Tanimoto & Iino (1984); *oriV*, Eichenlaub *et al.* (1977), Lane *et al.* (1984); *ccdAB*, Ogura & Hiraga (1983b); *resD*, Lane *et al.* (1986); *oriS*, Tolun & Helinski (1982); *repE*, Murotsu *et al.* (1981), Tolun & Helinski (1982); *copB*, Murotsu *et al.* (1981); *sopABC*, Austin & Wierzbicki (1983), Ogura *et al.* (1983a). Thus the region where miniTP181 does not line up with miniF covers the *oriV* *repC* region.

TP181 segment of pLAK105 and compared with the *F* DNA in pML31. This showed that restriction maps could be aligned easily throughout the *ccd*, *rep* and *sop* regions but that the 2 kb region adjacent to (upstream of) *ccd*, encoding the RepC protein in *F*, is not conserved in TP181 (Fig. 2). This was confirmed by DNA sequencing

Fig. 3. Dot-matrix comparison (using the program COMPARE with a window size of 21 and a stringency of 14) of part of the DNA sequence of miniF and miniTP181 showing conservation in and downstream of the *ccd* locus (coordinates 3000–3600) and divergence in the *oriV/repC* region (coordinates 1400–2900).

from the ends of the miniTP181 *EcoRI* fragment. At the downstream ends the DNA sequence (463 bp) matched that of F with 86% identity, indicating that TP181 and F possess highly related but not identical sequences and that the *EcoRI* site at the end of miniTP181 is in exactly the same place as in miniF. No homology was found for the sequence at the other end of the *EcoRI* fragment, confirming the conclusion from the restriction map comparison.

Since an important locus for TP181 sensitivity to curing by plumbagin may be the *ccd* locus, it was of interest to determine whether it shows obvious differences from the F *ccd* region. Therefore the region was sequenced using synthetic oligonucleotides which were initially designed by consideration of the known F sequences. The results of the sequencing showed that at the DNA level there was approximately 95% sequence identity with F. The promoter, ribosome-binding sites and transcriptional terminator of the *ccd* genes are all highly conserved. At the level of amino acid sequences CcdA and CcdB show 90.4 and 98% sequence identity respectively. This high degree of conservation is consistent with the similar interference of plumbagin with the rifampicin-induced killing associated with the *ccd* loci of TP181 and F.

We extended the sequence upstream and downstream from the *ccd* locus. This showed that downstream the high level of homology continues through into the *resD* locus, thought to encode a resolvase that may be involved in multimer resolution as part of plasmid maintenance (Lane *et al.*, 1986). Dot-matrix comparison (Fig. 3) shows that upstream the homology continues for approximately 500 bp but then disappears at the start of the *repC* gene, which has been shown to be essential for the function of the primary replication origin *oriV* in F (Tanimoto & Iino, 1984). This thus defines the point of divergence between F and TP181 which is evident from the restriction map (Fig. 2). Searching the rest of known F sequence as well as the complete sequence database

available failed to reveal the identity of the extra DNA present at this upstream end of miniTP181.

DISCUSSION

In this paper we have shown that plumbagin blocks replication and interferes with the lethal effect of the *ccd* locus after induction by addition of rifampicin. How can these activities be reconciled with the known effects of plumbagin? Plumbagin is a plant-derived naphthoquinone reported to induce topoisomerase II-mediated DNA cleavage as well as activating superoxide dismutase and acting as a weak intercalating agent (Durga *et al.*, 1992, 1990; Farr *et al.*, 1985; Fuji *et al.*, 1992; Krishnaswamy & Purushothaman, 1980; Liu, 1989). The rifampicin-induced killing of host bacteria by miniF and miniTP181 is most likely to be due to the *ccd* locus since no other loci that are known to be activated by plasmid loss (cessation of plasmid-determined gene expression) are known to be present on miniF. The killing works through the release of the activity of CcdB, which is a poison of DNA topoisomerase II (Bernard & Couturier, 1992). It is possible that the interference with this rifampicin-induced lethality by plumbagin is due to it causing a conformational change in topoisomerase at subinhibitory concentrations so that it is no longer so sensitive to the action of CcdB. The availability of both purified proteins should allow this hypothesis to be tested.

However, since the interference of plumbagin with rifampicin-induced killing is similar for both F and TP181 it seems unlikely that this effect explains the increased sensitivity of TP181 to curing by plumbagin. Our studies on incorporation of [³H]thymidine into CCC DNA suggest that the difference is probably due to the sensitivity of TP181 either to replication inhibition by plumbagin or to conversion to open circular form due to nicking by DNA gyrase inhibited by plumbagin. We favour the idea that replication is affected either directly or indirectly because our physical analysis of TP181 shows that it does not encode the *repC* gene which is present in miniF. When *repC* is intact in miniF, replication is initiated from a region designated *oriI* located at kb coordinate 42.6 (Lichenlaub *et al.*, 1977). Deletion of *repC* apparently activates a silent origin, *oriS*, located at coordinate 44.4 kb which depends on *repE* (Manis & Kline, 1978; Figurski *et al.*, 1978). Thus the mini-replicon of pTP181 may use the *RepE/oriS* replicon rather than the *RepE/RepC/CcdB/oriV* replicon on which F appears to rely. We propose that this *ori2* replicon may be more sensitive to changes in supercoiling brought about by subinhibitory concentrations of plumbagin. Alternatively the intercalating activity of plumbagin may attack a particularly sensitive site which affects *oriS* but not *oriV*. Surveys of the replicons present in F-like plasmids have not used probes which would allow us to check how many lack the *repC-oriV* region and therefore to predict which plasmids should be particularly sensitive to plumbagin.

The dual action of plumbagin against plasmid replication or topological form and lethality to plasmid-free segregants may well explain the curing activity of this

compound. Neither action on its own might be very effective. The rate of loss of plasmids lacking the *ccd* locus should be low (less than 10^{-2} per generation) because there are auxiliary stability functions, including the *sop* active partitioning locus, which reduce the loss rate to well below what would be expected for random partition. Similarly, interference with replication may result in poor segregation but if segregants die then it will just result in slower growth of the population. The fact that both effects can be achieved at concentrations of plumbagin well below those which cause a significant effect on bacterial growth suggests that plumbagin may have subtle molecular effects which could be exploited if the structure of its target binding site(s) were known.

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