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Plasmid Addiction Genes of Bacteriophage P1: *doc*, which Causes Cell Death on Curing of Prophage, and *phd*, which Prevents Host Death when Prophage is Retained

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P1 lysogens of *Escherichia coli* carry the prophage as a stable low copy number plasmid. The frequency with which viable cells cured of prophage are produced is about 10^{-5} per cell per generation. Here we show that a significant part of this remarkable stability can be attributed to a plasmid-encoded mechanism that causes death of cells that have lost P1. In other words, the lysogenic cells appear to be addicted to the presence of the prophage. The plasmid withdrawal response depends on a gene named *doc* (*d*eath *o*n *c*uring), encoding a 126 amino acid protein. Expression of *doc* is not SOS-inducing and killing by Doc is *recA*-independent. In cells that retain P1 the killing is prevented by the product of a gene named *phd* (*p*revent *h*ost *d*eath), encoding a 73 amino acid protein. The genes *phd* and *doc* have been cloned and expressed from a 0·7 kb segment of P1 DNA. The two genes constitute an operon and the synthesis of Doc appears to be translationally coupled to that of Phd. Homologs of the P1 addiction genes are found elsewhere, but *phd* and *doc* are unrelated to previously described genes of other plasmids that also cause an apparent increase in plasmid stability by post-segregational killing.

Keywords: phage P1; post-segregational killing; plasmid stabilization

1. Introduction

Bacteriophage P1 lysogenizes *Escherichia coli* as a low copy number plasmid that is stably maintained by a stringently controlled replicon and an active partition mechanism (reviewed by Yarmolinsky & Sternberg, 1988). Like several other low copy number plasmids, P1 appears to be more stable than can be accounted for by replication control and active partition. The additional stability of some of these plasmids has been attributed to one or more plasmid-encoded mechanisms that selectively kill plasmid-free segregants or their progeny. Means of inducing post-segregational killing are found in F (Ogura & Hiraga, 1983; Bex *et al.*, 1983; Karoui *et al.*, 1983; Brandenburger *et al.*, 1984; Miki *et al.*, 1984*a*,*b*; Jaffé *et al.*, 1985; Golub & Panzer, 1988; Loh *et al.*, 1988), R1 (Bravo *et al.*, 1987; Gerdes *et al.*, 1986, 1990*b*; Thisted & Gerdes, 1992), R100 (Tsuchimoto *et al.*, 1988), R483 and R16 (Gerdes *et al.*, 1990*a*,*b*) and presumably other plasmids that have relevant homologous regions (Golub & Low, 1986; Gerdes *et al.*, 1990*a*).

Two kinds of modules have been described that could account for the post-segregational killing of cured host cells. One is exemplified by the *sok*, *mok*, *hok* genes of R1, the other by the *ccdA*, *ccdB* genes of F. In each case a potential lethal agent is restrained by a relatively unstable gene product that prevents synthesis of the poison or blocks its action. Plasmid loss is followed by decay of the antidote, leaving behind the lethal function to induce cell death. The host killer protein, Hok, and its relatives are membrane proteins that kill by inducing various cellular changes, including membrane leakiness (Gerdes *et al.*, 1986; Poulsen *et al.*, 1992). Synthesis of Hok can be prevented by the

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antisense Sok RNA. This unstable RNA accelerates the turnover of the otherwise stable Hok messenger RNA (Gerdes *et al.*, 1992) and indirectly blocks its translation by blocking translation of the upstream *mok* gene, to which *hok* is translationally coupled (Thisted & Gerdes, 1992). The control of cell death protein, CcdB, kills by poisoning DNA gyrase, leading to double strand breaks in the bacterial chromosome (Bernard & Couturier, 1992). Mutations in DNA gyrase that confer resistance to CcdB have been isolated and described (Bernard & Couturier, 1992; Maki *et al.*, 1992). The DNA scission activity of CcdB appears to be responsible for the capacity of UV-damaged F to elicit an SOS response following introduction into (and subsequent loss from) an unirradiated F^- recipient (Borek, 1958; Rosner *et al.*, 1968; Bailone *et al.*, 1984; Mori *et al.*, 1984). The unstable antidote CcdA can form a complex with CcdB (Tam & Kline, 1989), hypothesized to prevent the lethal interaction between the killer and its target (Maki *et al.*, 1992).

In this study we provide evidence that the P1 prophage harbors a pair of genes that contribute to plasmid stabilization by induction of a lethal response to plasmid loss. The genes that confer *d*eath *o*n *c*uring or *p*revent *h*ost *d*eath when the prophage is retained were named *doc* and *phd*, respectively. In addition, we introduce the idea that all plasmids with the ability to induce post-segregational killing can be thought of as addicting agents†. Comparative studies that we report here indicate that *phd* and *doc* have no obvious homologies in nucleotide or codon sequence with previously described addiction genes, nor does *doc* expression elicit withdrawal symptoms that resemble those produced by Hok or CcdB. Despite these differences we note striking organizational and functional parallels among the known addiction systems.

2. Materials and Methods

(a) *Strains and standard microbiological methods*

Bacterial strains, phages and plasmids used are listed in Table 1. *E. coli* was cultured in Luria broth (Miller, 1972). Ampicillin was used at 100 μg/ml, spectinomycin at 20 μg/ml, kanamycin at 25 μg/ml, and chloramphenicol at 25 μg/ml except that for P1Cm selection the concentration was at 12·5 μg/ml. Transformations, phage crosses, and *in vitro* packaging of DNA were performed by standard procedures (Silhavy *et al.*, 1984). A library of P1 *Sau*3A1 fragments (in pools of average sizes 3 to 5 kb and 5 to 10 kb) cloned into the unique *Bam*HI site within the *int* gene of a λ vector with the immunity of 21 (D69), was a generous gift from Nat Sternberg (O'Regan *et al.*, 1987). Phages with inserts within *int* are unable to excise a cryptic λ prophage located at a secondary attachment site within *galT*. This characteristic was used to identify such phages by a method known as the red plaque test (Enquist & Weisberg, 1976). Lysogens of *int* phages were prepared by supplying the Int protein from a heteroimmune helper, G275. Assays for β-galactosidase were as described by Miller (1972).

(b) *Plasmid nomenclature*

For simplicity we refer to the commonly used plasmid vectors as follows: pB, pBR322; pU, pUC19; pG, pGB2ts. Numbers following these letters refer to the P1 DNA inserts listed in Table 1. Antibiotic resistance determinants are referred to by the 2-letter code: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp/Sm, spectinomycin and streptomycin; Tc, tetracycline.

(c) *Curing of λ (D69) carrying P1 DNA sequences*

Recombination-deficient, non-suppressing strains N205/pACYC184-c1$^+$ (or /P7) lysogenic for various D69-P1-hybrid prophages were concentrated from a log phase culture (grown in TB, 10 mM $MgSO_4$, 0·2% (w/v) maltose), resuspended in 10 mM $mgSO_4$ and infected (or not) with the heteroimmune *int*$^+$ phage G275 at a multiplicity of 3 to 4. The infected cells were diluted in TB and allowed to segregate cured cells for 4 h at 37 °C. Culture samples were spread on LB agar plates previously sparsely seeded with λ*c*I857 R5(amber), that had been P1-modified by growth on BR4 (to avoid restriction by P7) and the plates incubated at 42 °C. Separate plates, to which no challenging phage had been added, received more dilute samples of the culture. Lysogens grow up to make necrotic microcolonies whereas nonlysogens grow into healthy colonies that are essentially unaffected by the occasional infection that occurs during colony development. The percent of cells that were cured was calculated from the ratio of healthy, opaque colonies surviving the λ*c*I857 R5(amber) challenge to colonies recovered on the plates without phage. The method is based on that of Takiff *et al.* (1989).

(d) *Cloning procedures*

Commercially prepared enzymes were used as specified by the suppliers. Plasmid DNA was prepared and analyzed by gel electrophoresis using standard methods (Sambrook *et al.*, 1989). The phosphorylated *Nco*I linker d(pCCCATGGG) from New England BioLabs was used to alter an *Eco*RV site (in pG3) and a *Dra*III site (in pB7) that had been treated with T4 DNA polymerase for 3′ overhang removal (Sambrook *et al.*, 1989).

(e) *Constructions of deletions and insertions*

To map *phd* and *doc*, DNA segments of the plasmids pB7 and pG3 were deleted using exonuclease III (exoIII) and mung bean nuclease (Stratagene Co.). To locate the 3′ end of *phd*, DNA of pB7 was cleaved with *Hin*dIII and *Aat*II (creating exoIII-sensitive and exoIII-resistant protruding ends, respectively). Following partial exonucleolytic digestion and mung bean nuclease treatment, protruding ends were trimmed with T4 DNA polymerase and self-ligation yielded pB8 and pB9. To locate the 5′ end of *phd*, deletions were then generated from pB8

† Since synthesis of the toxic proteins Doc or Hok requires prior synthesis of their relatively unstable antagonists, they may be said to be induced by them. Induction of a stable toxin by a readily eliminated antidote has been suggested as a possible basis for drug addiction (L. Szilard, private communication in 1958; Shuster, 1961). Whether or not morphine addiction can be explained in this way, the model does seem appropriate as an explanation for plasmid addiction.

Table 1
Bacterial strains, phages and plasmids

A. *E. coli K-12 strains*

Strain	Genotype or description	Source, reference
BR4	YMC transduced to carry P1Cm *cry* of Scott (1970) (YMC = *mel*-1 *supE*57 *supF*58)	NIH Collection
BR157	*supD*43 *supD*74(ts) *recA*56 *srl*::Tn*10* *metB*1 *leu*(amber) *trp*(amber) *lacZ*(amber) *galK*(amber) *galE sueC tsx relA*	Yarmolinsky *et al.* (1989)
BR4623	BR157/P1Cm/pACYC184(Km)-c1$^+$	This work
BR4625	BR157/P1Cm r$^-$m$^-$ *repA*103/pACYC184(Km)-c1$^+$	This work
BR5175	SG20250 (λc1*ind sfiA*::*lacZ*)/pG	This work
BR5176	SG20250 (λc1*ind sfiA*::*lacZ*/pG5	This work
DH5	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Bethesda Research Laboratory
DH5α	*supE*44 Δ(*argF-lac*)U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Bethesda Research Laboratory
DJ125	*recA*56 *supE*44 *hsdR thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21	D. K. Chattoraj
MC4100	*araD*139 Δ(*argF-lac*)U169 *rpsL*150 *relA*1 *flbB*5301 *deoC*1 *ptsF*25 *rbsR*	Silhavy *et al.* (1984)
N205	*recA*3 *rpsL*200, *gal*$^+$ revertant of N100 of Gottesman & Yarmolinsky (1968)	NIH Collection
RW842	HfrH *galE* Δ(*pgl attBOB'bio*) *sup*° (λΔ[*int*-G]) ::*galT*	Enquist & Weisberg (1976)
SG20250	*pro*$^+$ transductant of MC4100	S. Gottesman

B. *Bacteriophages*

Phage name	Genotype or description	Source, reference
D69	λ*imm*21 cloning vector	Mizusawa & Ward (1982)
G275	λ*imm*434 Δ(*att*) *int*$^+$ *gal*8 *bio*936 Δ(*galT-bioB*)269	R. W. Weisberg
λc1857(ts) R5(amber)	Thermoinducible λ with suppressible endolysin mutation	NIH collection
λc1*ind sfiA*::*lacZ*	SOS reporter	Huisman & D'Ari (1981) S. Gottesman
λ*imm*21cI h_λ	Immunity 21 selector phage	NIH collection
λ*imm*21cI h_{80}	Immunity 21 selector phage	NIH collection
λ-P1 #76	D69 clone with a P1 DNA insert, mostly fragment *Eco*RI-6	O'Regan *et al.* (1987) N. Sternberg
λ-P1 : *Eco*RI-8	D69 clone with a P1 DNA insert, fragment *Eco*RI-8	N. Sternberg
P1Cm	P1 carrying Tn*9* at IS*1*	Iida & Arber (1980)
P1Cm r$^-$m$^-$ *repA*103	P1 with *repA* mutation suppressible by *supD*	Yarmolinsky *et al.* (1989)
P1Cm c1.100 3.6	Thermoinducible P1 (Rosner, 1972) with a morphogene mutation (Scott, 1968) suppressible by *supD*	This work
P1Cm c1.100 23.115	Thermoinducible P1 (Rosner, 1972) with a morphogene mutation (Walker & Walker, 1983) suppressible by *supD*	This work
P7	Close relative of P1 conferring Ap resistance	Wandersman & Yarmolinsky (1977)

C. *Plasmids previously described*

Plasmid	DNA insert	Vector sequences; cloning site	Drug^r	Source, reference
pACYC184-c1$^+$	*immC*$^+$ Δ*loxP* of P1	pACYC184 (p15A-derivative)	Cm	Yarmolinsky *et al.* (1989)
pACYC184(Km)-c1$^+$	*immC*$^+$ Δ*loxP* of P1	pACYC184 with Km insert in Cm	Km	Yarmolinsky *et al.* (1989)
pB = pBR322	None	ColE1-derivative	Ap, Tc	GenBank no. VB0001
pBR325	None	ColE1-derivative	Ap, Cm, Tc	GenBank no. VB0002
pDRC150	None	P*mac*-31, pU-derivative	Ap	Denise Roberts
pG = pGB2ts	None	pSC101-derivative, ts for replication	Sp/Sm	Clerget (1991); M. Clerget
pU = pUC19	None	ColE1-derivative	Ap	GenBank no. VB0026
pUNG20	14-kb *Hin*dIII fragment of R124	pBR325; *Hin*dIII	Ap, Cm	Firman *et al.* (1985); N. Redaschi
pUNG30	14-kb *Hin*dIII fragment of R124/3	pBR325; *Hin*dIII	Ap, Cm	Firman *et al.* (1985); N. Redaschi

D. *Plasmids constructed in this work, carrying regions of P1* Eco*R1-6 DNA*

Plasmid	DNA insert (see Figs 2 and 3)	Vector sequences; cloning site	Drug^r
pB1	6·8-kb *Eco*RI-6 (entire)	pB; *Eco*RI	Ap, Tc
pB2	3·9-kb *Eco*RI-*Pvu*II	2·29 kb of pB; *Eco*RI-*Pvu*II	Ap
pB3	2·58-kb *Pvu*II-*Eco*RI	2·29 kb of pB; *Eco*RI-*Pvu*II	Ap
pG3	2·58-kb *Pvu*II-*Eco*RI	4·01 kb of pG; *Eco*RI-*Hin*cII	Sp/Sm
pG3E	As pG3, *Nco*I linker at *Eco*RV	4·01 kb of pG; *Eco*RI-*Hin*cII	Sp/Sm
pG3Tn	As pG3, mini-Tn*10* (Km) in *doc*	4·01 kb of pG; *Eco*RI-*Hin*cII	Sp/Sm, Km
pG4	1·03-kb *Hin*cII fragment (ex pB3)	pG; *Hin*cII	Sp/Sm
pB5	1·35-kb *Pvu*II-*Nco*I (blunt)	pB; *Pvu*II	Ap
pG5	1·35-kb *Pvu*II-*Nco*I (blunt)	4·01 kb of pG3; *Eco*RI-*Hin*cII (blunt)	Sp/Sm
pB6	0·91-kb *Pvu*II-*Eco*RV	pB; *Eco*RV	Ap
pB7	0·57-kb *Pvu*II-*Hin*cII	pB; *Eco*RV	Ap
pB7D	As pB7, *Nco*I linker at *Dra*III	pB; *Eco*RV	Ap
pB8 & 9	ExoIII-generated deletions from the *Hin*cII site of pB7	pB; *Eco*RV	Ap

Table 1 *(continued)*

Plasmid	DNA insert (see Figs 2 & 3)	Vector sequences; cloning site	Drug^r
pB10 & 11	ExoIII-generated deletions from the *Pvu*II site in P1 DNA of pB8	pB; *Eco*RV	Ap
pU12	299 bp, coordinates 294 to 592 of Fig. 3, by PCR, with *Eco*RI & *Bam*HI termini	2·66 kb of pU; *Eco*RI-*Bam*HI	Ap
pU13	227 bp, coordinates 366 to 592 of Fig. 3, by PCR with *Bam*HI termini	pU; *Bam*HI	Ap
pU14	674 bp, coordinates 294 to 967 of Fig. 3, by PCR with *Eco*RI & *Bam*HI termini	2·66 kb of pU; *Eco*RI-*Bam*HI	Ap
pG14	674 bp, coordinates 294 to 967 of Fig. 3, by PCR with *Eco*RI & *Bam*HI termini	4·01 kb of pG; *Eco*RI-*Bam*HI	Sp/Sm

after digestion with *Bam*HI and *Sph*I, resulting in pB10 and pB11. An 8 bp *Nco*I linker d(pCCCATGGG) was inserted into the *Dra*III site of pB7, generating pB7D.

To locate *doc*, pG3 DNA was digested with *Eco*RI and the ends so generated were protected from exoIII degradation by filling with deoxy-thioderivatives as recommended by the supplier (Stratagene Co.). The DNA was then digested with *Nco*I (generating an exoIII-sensitive end). Plasmid DNA was recovered after partial exoIII digestion, self-ligated and directly transformed into a host strain (DH5α). Various deletion derivatives were then tested for the presence or absence of Doc activity.

Mutagenesis by insertion of a mini-Tn*10* was as described by Way *et al.* (1984), using λ1105 as a source of a version of the minitransposon that confers kanamycin resistance. The plasmid pG8Tn which is defective in Doc expression was identified as such from among 36 kanamycin-resistant derivatives of pG8.

(f) *DNA sequencing*

The determination of the nucleotide sequence of the P1 DNA fragment encompassing *phd* and *doc* was described earlier (Lehnherr *et al.*, 1991). The nucleotide sequence of newly constructed plasmids and deletion or insertion endpoints were confirmed using standard dideoxy chain termination reactions (Sanger *et al.*, 1977). Double-stranded template DNA was isolated as described by Sambrook *et al.* (1989) and sequenced with Sequenase (*United States Biochemical Corporation*) using standard primers (Bethesda Research Laboratories) or appropriate primers synthesized on a Milligen 8750 oligonucleotide synthesizer. Sequencing reactions at the INRA in Jouy en Josas were performed in a Perkin Elmer apparatus, using the *Taq* DyeDeoxy Terminator Sequencing Kit (Applied Biosystems) and the reaction products were analyzed on an automated DNA sequencer (370A DNA sequencer of Applied Biosystems).

(g) *Protein synthesis* in vitro

To test if *phd* and *doc* encode proteins, the *E. coli in vitro* transcription/translation system of Promega was used. Newly synthesized proteins were radioactively labeled with [^{35}S]methionine and the reaction products were analyzed on an SDS/20% (w/v) polyacrylamide gel (Laemmli, 1970). The gel was fixed in 10% acetic acid, 5% glycerol for 2 h, transferred to a Whatman filter paper, dried at 80°C under vacuum and exposed to a Kodak XAR2 X-ray film for 72 h.

3. Results

(a) *Effects of P1 plasmid loss*

In order to assess the effects of P1 plasmid loss on the bacterial host, we first sought a means by which to arrest plasmid replication specifically. Complete arrest of plasmid replication was found to require blockage of both the replicon normally identified with the plasmid state and the lytic (viral) replicon. Conditional blockage of the plasmid replicon was accomplished by use of an amber mutation in the P1 plasmid initiator gene, *repA*, and a thermosensitive suppressor in the bacterial host. The host cells used for this study were supplied with a supplementary source of immunity protein from a cloned P1 *c1* gene (pACYC184(Km)-*c1*$^+$) to assure constant and complete repression of the lytic replicon (Yarmolinsky *et al.*, 1989). In the construct BR4625, which carries both P1Cm *repA*103(amber) and pACYC184(Km)-*c1*$^+$, a shift to the non-permissive temperature (43°C) stopped replication of the P1 prophage. Figure 1a shows that stopping replication of the P1 plasmid also halts promptly the generation of viable progeny bacteria. The control experiment of Figure 1b shows that the temperature shift caused only a transient delay in the replication of isogenic bacteria in which the P1 *repA* gene was wild-type. These results imply that P1 is an addictive plasmid.

Whether or not P1 plasmid replication was permitted, there was a considerable increase in bacterial mass at 43°C as monitored by measurements of absorbance. In Figure 1a, at the temperature non-permissive for P1 replication, the absorbance eventually leveled off at about 70-fold its initial value. In contrast, the number of colony forming units remained constant or showed a modest decrease. Throughout the six hour incubation period essentially all (95%) of these viable bacteria retained P1, as determined by scoring chloramphenicol resistance. We conclude that the arrest of plasmid replication efficiently arrests the generation of viable progeny bacteria, although an increase in bacterial mass still continues for several hours.

Most of the observed mass increase that follows loss of P1 from BR4625 can be accounted for by cell divisions that produce inviable progeny. In this

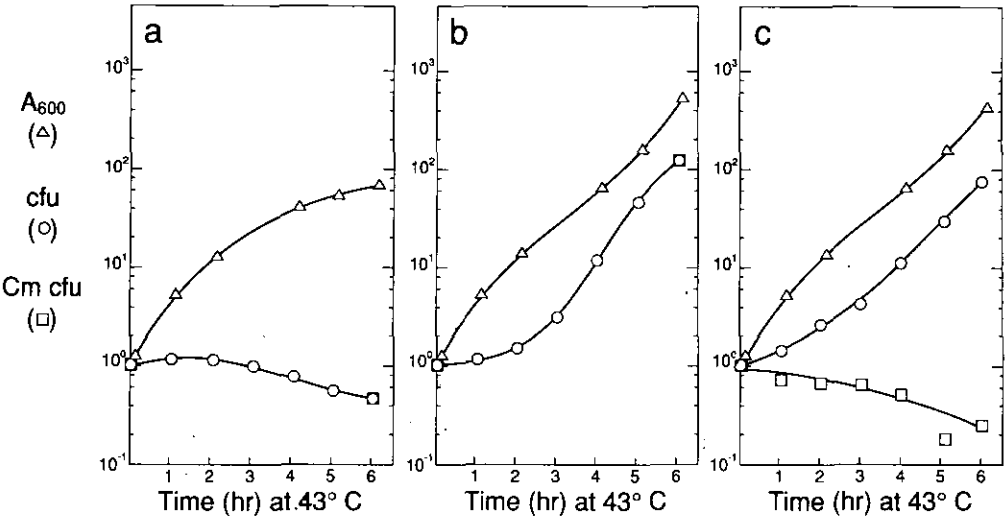

Figure 1. Lethality of P1 loss and its suppression by λ-P1 #76. Cultures of the following bacteria were grown into logarithmic phase at 30 °C in LB supplemented with kanamycin: a, tester strain BR4625 (in which P1Cm *repA*103 (amber) is thermosensitive) and which has been lysogenized with the λ vector D69; b, the *repA*$^+$ control strain, and c, the tester strain lysogenic for λ-P1 #76. Generation times were about 60 min. At time 0 dilutions were made into medium at 43 °C for subsequent measurements of absorbance at 600 nm and of colony forming units (c.f.u.). The cultures sampled for absorbance readings were successively diluted so as to maintain $A_{600} < 0{\cdot}25$ at the time of each sampling. More dilute cultures, initially at approx. 10^3 c.f.u./ml, were sampled for plating on LB agar with either kanamycin (c.f.u.) or chloramphenicol (Cm c.f.u.) and colonies were counted after incubation overnight at 30 °C. P1 lysogens (Cm c.f.u.) where indicated, were scored following transfer of 100 to 200 colonies with toothpicks from LB agar with kanamycin to LB agar with chloramphenicol. Both the absorbance and the colony forming units have been normalized to the $t=0$ point.

strain a fraction of the cells underwent extensive filamentation at 43 °C, with the DNA distributed throughout the filaments (visualized by fluorescence microscopy after staining with 4′,6-diamidino--2-phenyl indole). As noted in a subsequent section, cell filamentation is an exceptional rather than a characteristic symptom of P1 plasmid loss.

(b) *Isolation of a P1 DNA segment that permits bacteria to survive the loss of P1*

The severe response to P1 withdrawal that we observed was presumably due to the loss of one or more P1 genes. We sought to isolate the relevant P1 DNA on the basis of its ability to permit the survival and outgrowth of segregants cured of P1.

Cultures of the *supD*ts *recA* strain BR4625, used above, were lysogenized in four different lots with members of a library of P1 fragments cloned in a λ vector (O'Regan *et al.*, 1987) and the cultures were grown for six hours at 43 °C. It can be seen from Table 2 that during non-selective growth the four lysogenized cultures produced, in varying yield, appreciably greater numbers of bacteria cured of P1 than the tester strain (BR4625) by itself. We estimate that about 5% of cells in the four cultures

Table 2

Selection of λ-P1: phd$^+$ *from a λ-P1 library in* supD*ts strain BR4625*

repA allele of P1Cm	λ-P1 hybrids as prophages	Increment in c.f.u. in 6 h at 43 °C	P1Cm retention (%) Initial	Final
+	—	1300	100	100
103(amber)	—	2	100	52
103(amber)	lot A	89	98	<1
103(amber)	lot B	87	100	2
103(amber)	lot C	58	100	<1
103(amber)	lot D	8	94	4

Strains BR4623 (row 1) and BR4625 (row 2) serve as controls. Cultures of BR4625 (row 3 to 6) were lysogenized in 4 lots and treated as follows: the bacteria were coinfected at 32 °C with members of the λ-P1 library (described in Materials and Methods) and with the heteroimmune helper G275 to permit integration. Lysogens at a sufficient density to grow confluently were selected on plates spread with a 1 : 1 mixture of h_λ and h_{80} *imm*21cI phages. Separate lots of harvested cell paste were diluted to $A_{600}=0{\cdot}001$ in 25 ml LB with kanamycin and grown with aeration at 43 °C, a temperature nonpermissive for replication of P1Cm r$^-$m$^-$ *repA*103 in the *supD*ts host. Samples of the cultures before and after this incubation period were plated on LB agar with kanamycin and scored for colony forming units (c.f.u.) after overnight incubation at 30 °C. Colonies retaining P1Cm were scored by transfer with toothpicks of about 100 colonies from each sample to LB agar with chloramphenicol.

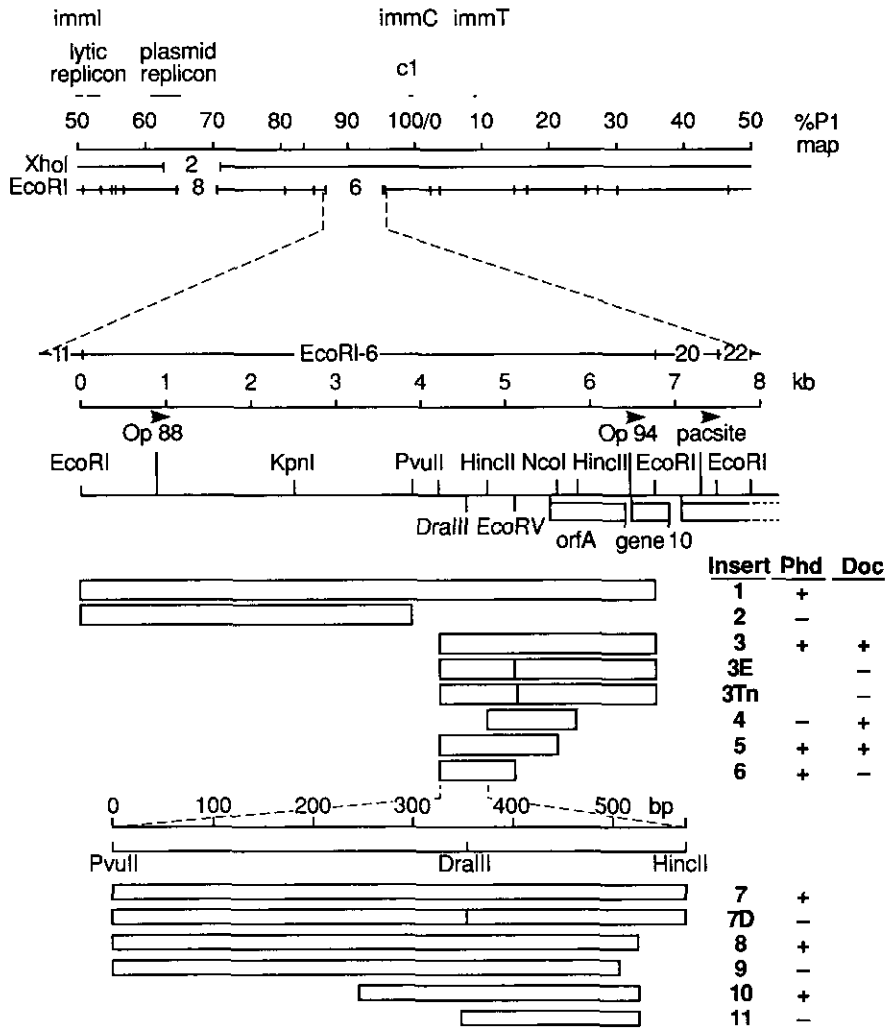

Figure 2. Physical mapping of the determinants of Phd and Doc activities. Successively expanded portions of the P1 physical map (Yarmolinsky & Łobocka, 1993) are displayed with certain landmark genetic features. P1 DNA inserts arbitrarily numbered 1 to 11 are shown in the bottom half of the Figure. D and E indicate an altered *Dra*III or *Eco*RV site, respectively; Tn indicates the insertion of a mini Tn*10* (see Materials and Methods for details). P1 DNA inserts 1 to 3 and 5 to 11 were cloned into pBR322 (for tests of Phd activity) and the products are designated pB plasmids (see Table 1D). P1 DNA inserts 3 to 6 were inserted into the multiple cloning site of pGB2ts (for tests of Doc activity) and the products are designated pG plasmids. The conclusion that pG4 exhibits Doc activity but not Phd activity was deduced from the observation that in order to obtain transformants with this plasmid the presence of a source of Phd (pB6) in the recipient appeared necessary. Other pB clones tested for Phd activity were scored positive if in their presence the loss of P1 from BR4625 or of pG3 or pG5 from DH5 failed to kill bacterial segments. A positive score in the Doc column for inserts 3 and 5 signifies that the generation of viable bacterial segregants cured of pG3 and pG5 was severely hindered by the presence of the P1 DNA carried by these plasmids. Each test was performed at least twice. No entry signifies that none of the above tests were performed.

gave rise to viable cured progeny, since the average increase in viable cells was about 5% of that observed with the *repA*$^+$ version of P1Cm in the control strain BR4623.

The λ-P1 hybrids present in individual chloramphenicol-sensitive colonies obtained in this experiment were excised and packaged by heteroimmune superinfection. The hybrids were identified as carrying an insert within the *λint* gene by the red plaque test (see Materials and Methods). The tester strain (BR4625) was lysogenized with these phages in a second round of infection. In each case the selected λ-P1 hybrid conferred on BR4625 the capacity to segregate, with increased efficiency, viable bacteria cured of P1. To some extent this segregation can be seen to have occurred even prior to release from selection pressure in lots A and D, a result which suggests the degree to which P1 instability is normally masked. We refer to the selected hybrids that permit this curing as λ-P1 : *phd*$^+$ because of their capacity to prevent host death.

In order to determine which regions of the P1 genome might be represented in the λ-P1 : *phd*$^+$ phages, their DNA was ^{32}P-labeled by nick translation and hybridization was carried out according to Southern (as described by Sambrook *et al.*, 1989), with fragments of P1 DNA separately restricted by

*Eco*RI and *Xho*I (see Fig. 2). At least part of the 7 kb *Eco*RI-6 fragment of P1 was found to be present in each of the λ-P1 : *phd*$^+$ phages tested (data not shown). The phages differed from one another in that some exhibited additional hybridization with flanking *Eco*RI fragments 11 or 20 and 22. None hybridized with the smaller of the two *Xho*I fragments (see Fig. 2).

The later availability of an ordered, overlapping library of P1 DNA cloned in a λ vector permitted us to verify that a clone carrying *Eco*RI-6 and very little additional DNA (λ-P1#76 of O'Regan *et al.*, 1987) allows bacteria that become cured of P1 to survive the loss. Tester strain BR4625 lysogenized with λ-P1#76 is seen in Figure 1c to grow about as well at 43°C as BR4623, the *repA*$^+$ version of BR4625 (Fig. 1b), despite the absence of P1Cm *repA*103(amber) replication. These data suggest that the *phd* function is encoded within *Eco*RI-6, a segment of the P1 DNA that is separated from the basic plasmid replicon by more than 20 kb (see Fig. 2).

(c) *Reassessment of the frequency of P1 loss*

Measurements of the frequency of P1 loss established a curing rate of about 2×10^{-5} per cell per generation in a *rec*$^+$ *E. coli* strain (Rosner, 1972). Subsequent measurements with *rec*$^+$ and *rec* hosts have yielded comparable values (Austin *et al.*, 1981). The evidence presented above that the majority of cured cells lose viability suggests that the frequency of P1 loss has been underestimated.

To obtain a measure of the curing rate unbiased by death of cured cells we have taken advantage of the observation that the viability loss associated with curing *E. coli* of P1 is prevented by a chromosomally integrated λ-P1#76, serving as a stable source of the *phd* product. As in previous studies (Rosner, 1972), cured cells were selected as survivors of thermal induction of prophage P1Cm *c*1.100 at 42°C and their sensitivity to chloramphenicol (to which P1Cm *c*1.100 confers resistance) was confirmed by replica-plating. The P1Cm *c*1.100 carried either of two suppressor-sensitive mutations in phage morphogenes. As these phages were incapable of plating on the non-suppressing host used in the experiment, reinfection was prevented.

An approximately sevenfold increase in the measured loss frequency of P1 as a consequence of preventing the death of cured segregants with λ-P1#76 is reported in Table 3. We conclude that the addiction genes of P1 confer on it an apparent stability that significantly supplements the stability provided by the fully functional primary replication and partition mechanisms of the plasmid.

(d) *Linkage of* phd *to gene(s) responsible for death on curing*

A close linkage between *phd* and the gene(s) responsible for the cell death attendant upon loss of P1 was demonstrated by showing that bacteria lysogenic for λ-P1 hybrids that carry *phd* were refractory to a procedure for curing the lysogens. The hybrid prophages were excised from the host chromosome by heteroimmune superinfection with an *int*$^+\Delta att\lambda$ phage. Cells from which the repressed prophage (and superinfecting phage) had been lost were allowed to accumulate. Colonies derived from cured survivors of superinfection were recognized by their failure to complement for growth a λ with a defect in the endolysin gene R (see Materials and Methods). A relative paucity of viable segregants cured of the λ-P1 prophages was taken as evidence for the expression from them of *doc*, a death on curing function.

Three λ-P1 hybrid prophages were tested: a λ-P1 hybrid that had been selected for its expression of *phd*$^+$, the hybrid λ-P1#76, which we had shown to be *phd*$^+$, and a λ carrying the *Eco*RI-8 fragment of P1 as an insert. *Eco*RI-8 was tested because it encompasses the P1 origin of plasmid replication and a close linkage of addiction genes to the origin of plasmid replication has been found in both F (Ogura & Hiraga, 1983) and R1 (Bravo *et al.*, 1987). The λ vector, D69, served as a control. Bacteria carrying either of the *phd*$^+$ λ-P1 hybrids were cured by heteroimmune superinfection with $<1\%$ the efficiency of bacteria carrying the λ vector alone or $<2\%$ the efficiency of bacteria carrying λ-P1 : *Eco*RI-8. Additional experiments suggest that the close relative of P1, P7, when present as prophage, can allow cells cured of λ-P1#76 to survive. We interpret this result to mean that P7 possesses a Phd function that can substitute for the Phd function of P1, although P7 is not entirely homologous to P1 in the region corresponding to *Eco*RI-6 (Meyer *et al.*, 1986). We conclude that P1,

Table 3

Effect of chromosomally integrated λ-P1 : phd$^+$ *on the frequency of segregation of viable cells cured of P1*

Unselected plasmid prophage	Curing rate per cell per generation ($\times 10^5$) No integrated prophage	Integrated λ-P1#76
P1Cm *c*1.100 3.6	3 (2–8)	17 (12–21)
P1Cm *c*1.100 23.115	2 (2–5)	17 (16–20)

Derivatives of recA3 strain N205 were grown at 30°C on LB agar with chloramphenicol to select for retention of the indicated P1 prophage. For each determination cell suspensions were made of 5 young colonies. These were streaked onto LB agar plates at 30°C and grown for about 21 generations (24 to 27 h). An entire colony from each streak was resuspended in TMG buffer (Silhavy *et al.*, 1984) and appropriate dilutions were plated on pre-warmed agar at 30°C and 42°C. Colony formers were counted. Curing rates shown are the median of 5 determinations of the fraction of bacteria surviving thermal induction divided by the number of generations of non-selective growth prior to the final platings. The range of values obtained for P1 in N205 (column 1) showed no overlap with those tested in N205 (λ-P1#76) (column 2). At 30°C, the temperature at which the segregation of cured cells was allowed to proceed, colony size appeared unaffected by the presence of the plasmid prophages, singly or together with λ-P1#76, although the latter markedly reduced colony size at 42°C.

and presumably P7, encode *phd* and *doc* genes closely linked to each other, but not to the plasmid origin of replication. This conclusion is confirmed by more direct mapping experiments described below.

(e) *Localization of* phd

Our effort to localize the genes *phd* and *doc* was facilitated by the availability of the DNA sequence of part of *Eco*RI-6 (GenBank Accession No. M95666) and a restriction site map of the remainder (Citron *et al.*, 1989; Lehnherr *et al.*, 1991). The sequencing had been performed in the course of studies of the activation of late gene transcription by the product of gene *10*, which is encoded largely within *Eco*RI-6. These studies demonstrated a toxicity of both the C1-controlled gene *10* product and the N-terminal portion of it that is encoded within *Eco*RI-6 (Lehnherr *et al.*, 1991, 1992). For this reason we used a recipient expressing C1 repressor, from a pACTY184-*c*1$^+$ plasmid, in our cloning of the *Eco*RI-6 fragment into pBR322.

Introduction of the pBR322-*Eco*RI-6 construct, pB1 (for plasmid nomenclature see Materials and Methods and Table 1), into tester strain BR4625 permitted viable segregants cured of P1 to be generated at a temperature that is non-permissive for the maintenance of the thermosensitive P1 prophage. Under identical conditions a control strain BR4625 that had been transformed with pBR322 became *non-viable following the loss of P1. The results* obtained with the *Eco*RI-6 clone are similar to those obtained with the slightly larger P1 segment that is present in λ-P1#76 (see Fig. 1) and confirm our surmise that Phd activity can be expressed from *Eco*RI-6.

Further localization of *phd* was achieved by applying the above-mentioned test for Phd activity to fragments of *Eco*RI-6 that were cloned in pBR322 and grown in a *recA* host (DH5α) carrying pACYC184-*c*1$^+$. In this way *phd* was localized to the *Pvu*II-*Eco*RI fragment of pB3 and then to the *Pvu*II-*Nco*I moiety of this fragment (pB5, see Fig. 2). Fragments 3 and 5 were also cloned into the pSC101-based thermosensitive vector, pGB2ts. The resulting constructs (pG3 and pG5, respectively) express Doc in addition to Phd. Following thermally induced loss of the plasmids, the cells cease dividing after about five generations. We have used various bacterial strains harboring either pG3 or pG5 as relatively simple indicators with which to monitor DNA fragments cloned into pBR322 for Phd activity. Using either of these indicators or the original BR4625 tester strain, we found that pB7, which contains a 574 bp *Pvu*II-*Hin*cII fragment, expressed Phd activity. The cloned region in pB7 includes all but the last two codons of a 73-codon orf.† This orf is preceded by a potential ribosome binding site and a promoter sequence (denoted Pr92, based on its location in the P1 map; Yarmolinsky & Lobocka, 1993) and is followed by a tandem pair of stop codons (Fig. 3). The homology score of Pr92 relative to an optimal *E. coli* consensus promoter was calculated using the computer program TargSearch (Mulligan *et al.*, 1984). The obtained score of 52·1% lies between the values for *lac*P1 of *E. coli* (49·7%) and P*bla* of pBR322 (52·7%) and suggests a promoter of moderate strength.

Polymerase chain reactions were used to amplify specific DNA fragments in order to test directly the function of the 73-codon orf and Pr92. The plasmid pU12, containing a 299 bp P1 fragment extending from positions 294 to 592 (see Fig. 3), including the promoter and the 73-codon orf, expressed Phd activity in *trans*. A second plasmid pU13, containing just the promoterless 73-codon orf (positions 366 to 592) showed no activity. When the latter fragment was cloned under the control of a hybrid maltose-lactose promoter, *mac*-31(Vidal-Ingigliardi & Raibaud, 1985) in pDRC150, Phd activity was regained. The P1 DNA of pB7 was subjected to limited exonucleolytic digestion as described in Materials and Methods. The resulting deletion end-points (shown in Figs 2 and 3) were identified by sequencing (pB8 through pB11). The potential promoter sequence, Pr92 is deleted in pB11, a plasmid that lacks Phd activity, whereas both the promoter and the activity are retained in pB10. The predicted ribosome binding site sequence *seems also to be important for Phd expression, since* the tandem insertion of two 8 bp *Nco*I linkers d(pCCCATGGG) at the cut and trimmed *Dra*III site in the region of the ribosome binding site renders pB7D defective in Phd activity. These results allow us to conclude that the 73-codon orf encodes Phd and is expressed from Pr92.

The plasmid pB9 fails to express Phd activity because of the replacement of the C-terminal 24 codons of the 73-codon orf by 17 codons of the pBR322 vector (Fig. 3). The retention of the activity in pB8, in which the C-terminal 19 codons of the 73-codon orf are replaced by 77 codons of pBR322 (originally from Tn*3*), indicates that the activity does not reside in this C-terminal region of Phd.

(f) *Localization of* doc

As already noted, *the 1·35 kb DNA fragment 5* (Fig. 2) is a segment of *Eco*RI-6 from which both *phd* and *doc* can be expressed. Within fragment 5 two similarly oriented orfs downstream of the 73-codon *phd* gene suggest themselves as possible candidates for *doc*, on the assumption that *doc* encodes a protein. The middle, 126-codon orf can be interrupted conveniently at a unique *Eco*RV site. Plasmid pG3E was generated by insertion of an 8 bp *Nco*I linker d(pCCCATGGG) at this site in pG3, and plasmid pG6 by deletion of P1 DNA distal to the *Eco*RV site. Neither pG3E nor pG6 express Doc activity, as no post-segregational lethality was observed at 42°C, a temperature non-permissive for

† Abbreviation used: orf, open reading frame.

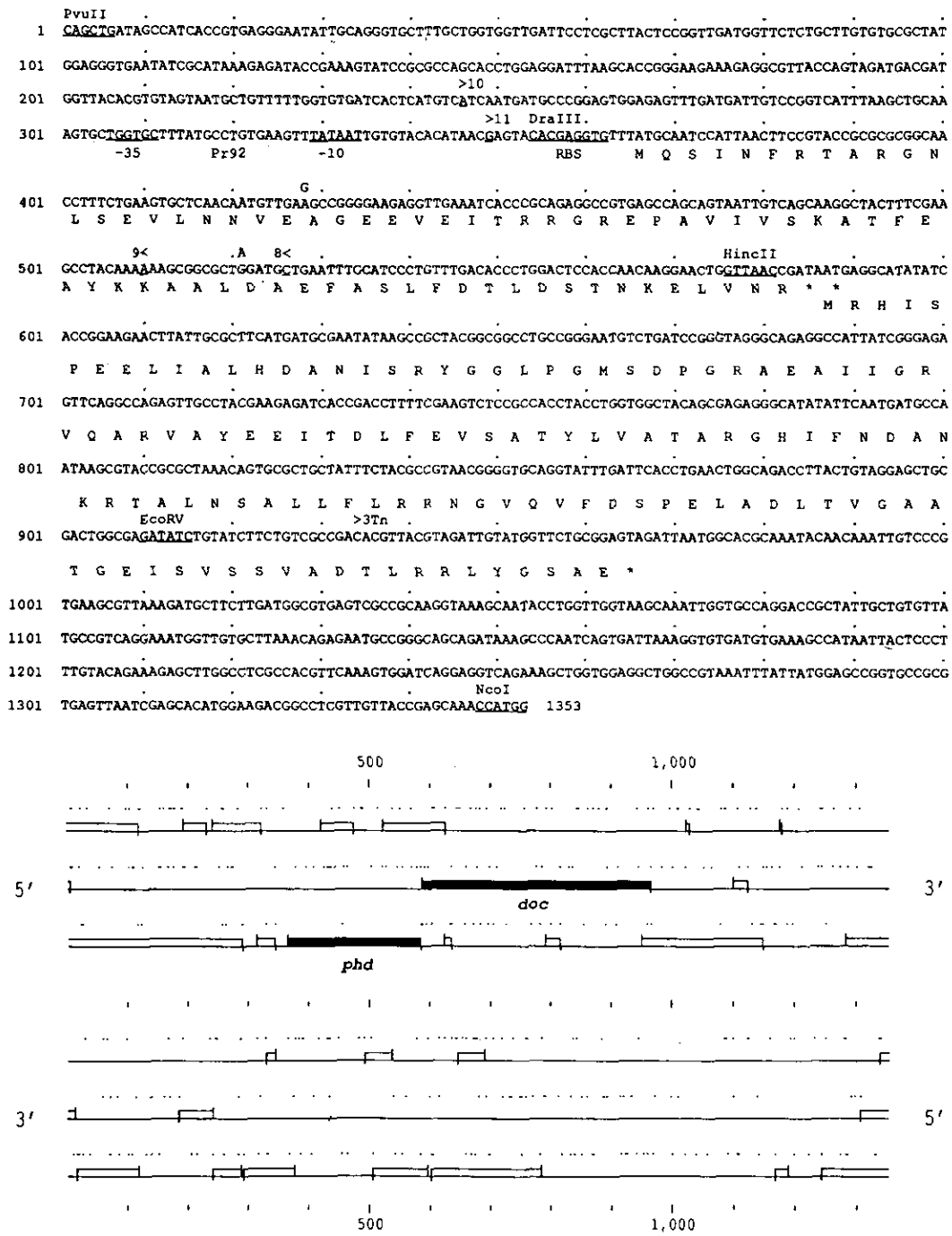

Figure 3. Nucleotide sequence in the region of the P1 addiction genes. Top panel: the sequence shown is excerpted from the GenBank entry M95666 and renumbered so that the first nucleotide of the *Pvu*II restriction site corresponds to nucleotide number 1 and the last nucleotide corresponds to the end of the *Nco*I site. The −10 and −35 regions of the putative addiction promoter, Pr92, are underlined. The set of nucleotides corresponding to a ribosome binding site is marked RBS. Amino acid sequences of predicted polypeptides of *phd* and *doc* are shown below the sequences using the conventional 1-letter code. Restriction sites referred to in the text are indicated by underlining. The 2 bases of R124/3 which are non-identical to those of P1 in the region between coordinate 297 and the *Hinc*II site at the end of *phd* are written above the corresponding bases of the P1 DNA sequence. Note that neither alters the deduced amino acid sequence. Bottom panel: the orfs initiated from ATG codons are displayed in all 6 reading frames. Those to which we assign the names *phd* and *doc* are filled in. Dots denote the positions of codons that are designated as rarely used in *E. coli* by the program Ecohigh.Cod with a threshold of 0·00 (Devereux *et al.*, 1984).

the maintenance of pG plasmids. The fact that we could maintain pG3 in the absence of a source of C1 protein suggests that pG3 failed to express toxic amounts of truncated gene *10* protein. In the experiments involving carriers of pG3 or its derivatives, the bacteria were grown without a source of C1 protein. Insertion of a mini-Tn*10* transposon in pG3, followed by screening for loss of Doc activity,

led to the isolation of pG3Tn. The transposon was found to have inserted 21 bp to the right of the *Eco*RV cleavage site, still within the 126-codon orf (Figs 2 and 3). To test if the 66-codon orf downstream of the 126-codon orf is also a part of the addiction cassette, the plasmid pG14 was constructed (Table 1). This plasmid carries a 674 bp fragment of P1 DNA, encompassing the putative promoter sequence, the 73-codon *phd* gene and the 126-codon orf, but lacks all but the first five codons of the 66-codon orf. In an assay for Doc activity pG14 scores positive, indicating that the 66-codon orf is dispensable. These results show that the 126-codon orf is *doc*.

How is the *doc* message translated? The absence of a recognizable ribosome binding site associated with *doc* and the overlap between the first stop codon following *phd* and the ATG start codon of *doc* implies that the translation of the *doc* message is coupled to that of its preceding partner.

(g) *Genes* phd *and* doc *encode proteins*

The plasmids pU12 and pU14 contain *phd* and *phd/doc*, respectively, under the control of the indigenous promoter Pr92 (see Table 1). To address the question of whether *phd* and *doc* encode proteins, these two plasmids, and pUC19 as a control, were used as templates in transcription/translation reactions *in vitro* (see Materials and Methods). The result of one such experiment is shown in Figure 4. The control plasmid pUC19 served as template for the synthesis of two proteins: pre-β-lactamase and a truncated β-galactosidase from *lacZ'*, which have molecular masses of 31·6 kDa and 12·1 kDa, respectively. The smaller of the two proteins was absent from the pU12 and pU14 lanes presumably due to the presence of a DNA fragment cloned into the polylinker sequence of pUC19, which in most cases disrupts the *lacZ'* gene. A single novel protein is determined by pU12. The position of the protein band on the 20% polyacrylamide gel is in good agreement with the expected molecular mass for Phd of 8·1 kDa. Besides Phd, a second protein is expressed from pU14. This protein most likely corresponds to Doc (expected molecular mass: 13·6 kDa) despite its slightly greater mobility than the 12·1 kDa product of *lacZ'*. The intensities of the bands on the X-ray film were used to quantify the amounts of proteins synthesized. On a molar basis about 40 times more Phd was made than (unprocessed) pre-β-lactamase and about 14 times more Phd than Doc. The dependence of *doc* expression on prior translation of *phd* message and the fact that *doc* contains 16 rare codons, while *phd* contains only one (see Fig. 3, bottom panel), could account for a pronounced bias.

(h) *SOS induction and* doc *expression*

Is *doc* expression SOS-inducing? The experiment shown in Figure 5 was designed to answer this question. Expression of *doc* was initiated in a strain

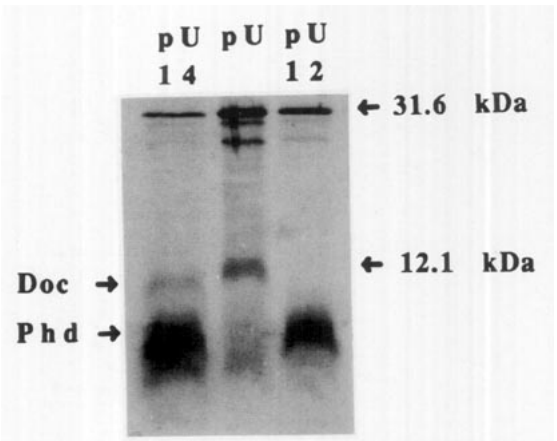

Figure 4. Autoradiograph showing the [^{35}S]methionine radiolabeled products of *in vitro* transcription/translation reactions using as templates the plasmids pU14 and pU12, which encode the P1 functions *phd/doc* and *phd*, respectively, and the vector pU. Arrows to the right indicate the positions of the 31·6 kDa pre-β-lactamase and the 12·1 kDa truncated β-galactosidase specified by the pU vector DNA. Arrows to the left mark the protein bands most likely corresponding to Phd and Doc, specified by the P1 DNA inserts.

(BR5176) that carries the *phd* and *doc* genes on the thermosensitive vector pGB2ts (pG5) by raising the temperature from 30 °C to 41 °C. Surviving bacteria were scored as colony formers on plates incubated at 30 °C. The number of plasmid-carrying cells was seen to increase about tenfold during the first hour of growth at 41 °C, presumably due to dilution of pre-existing plasmid copies. Beyond this point, despite a significant increase in the absorbance of the culture at 600 nm (A_{600}), the generation of any additional colony-forming units (c.f.u.) was prevented. As in experiments shown in Figure 1a, all the colony formers retained pG5. A control culture (BR5175), which initially harbored the pGB2ts vector (pG) without any P1 DNA insert, continued its exponential growth uninterruptedly.

Microscopic examination of the cells five hours after the shift to 41 °C revealed that the increase in absorbance of the BR5176 culture was not the result of filamentation, as might have been expected if *doc* expression had induced an SOS response. Instead the cells appeared to be appreciably shorter than cells of the uninhibited BR5175 control (data not shown). Prior to the temperature shift no differences were discerned between cells from the two cultures.

Both strains used in this experiment carried a *sfiA* :: *lacZ* fusion on a non-inducible λ prophage (Huisman & D'Ari, 1981) which permitted SOS induction to be monitored by peroidic measurements of β-galactosidase. At the times of sampling for these determinations additional samples were taken to medium containing the SOS-inducing, DNA-alkylating agent methanemethyl sulfonate to assess the capacity for SOS induction during the next hour. We see from

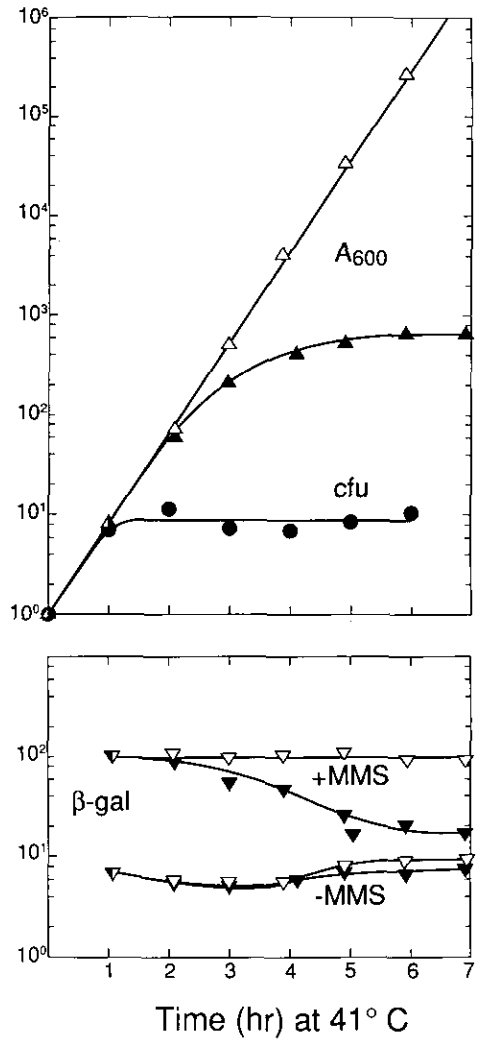

Figure 5. Lack of induction and diminished inducibility of the SOS response following *doc* expression. Overnight cultures of Δ*lac*(λc*Iind*⁻*sfiA* ::*lacZ*) strain BR5175, which carries pGB2ts (pG) and BR5176, which carries the pGB2ts-*phd*⁺*doc*⁺ plasmid pG5, were grown with agitation at 30°C in LB supplemented with spectinomycin to assure plasmid retention. The cultures were diluted into broth without antibiotic, grown into logarithmic phase at 30°C, and at the start of the experiment diluted into prewarmed broth at 41°C. Serial dilution maintained the A_{600} <0·2. Samples were taken for absorbance (A_{600}) measurements and for plating on LB agar at 30°C with and without spectinomycin. Data obtained using BR5175 are represented by open symbols, using BR5176 with filled symbols. The relative numbers of colony forming units (c.f.u.) of BR5176 without spectinomycin are plotted. Both the absorbance and the colony forming units have been normalized to the $t=0$ point. Very similar results to these were obtained with both strains when spectinomycin was present in the agar (data not shown). In the case of BR5175, the c.f.u. without spectinomycin (not shown) and A_{600} increased exponentially in parallel. Assays of β-galactosidase (β-gal) were performed on samples of the cultures both directly and 1 h after incubation at 41°C with methanemethyl sulfonate (MMS, final concentration 0·025%) and the results are expressed in Miller units (Miller, 1972).

Figure 5 that the loss of pG itself (from BR5175) neither induces an SOS response nor affects SOS-inducibility. A loss (from BR5176) of the addicting plasmid pG5 also appears not to induce an SOS response; on the contrary, the capacity for such an induction is gradually weakened as the cells lose their viability.

(i) *A homologous* phd *gene in the IncFIV plasmids R124 and R124/3*

A search of the GenBank/EMBL data base for DNA sequences homologous to at least part of the region of *Eco*RI-6 that includes *phd* and *doc* revealed the presence of such a region in the IncFIV plasmid R124/3. The homology extends from coordinate 297 of Figure 3 (upstream of Pr92) to at least coordinate 581 (corresponding to the limit of the R124/3 sequence published by Price *et al.* (1989)) and to the penultimate codon of *phd*, with only two mismatches in this interval. These two mismatches occur within *phd*, but neither alters the amino acid composition of the gene product. The homology between P1 and R124/3 presumably extends downstream of *phd* as judged by the strong signal obtained when a portion of R124/3 was hybridized under stringent conditions with a P1 probe encompassing *doc* (data not shown). These results prompted us to test clones of the P1-homologous region of R124/3 (and its relative R124) for the presence of a Phd-sensitive addiction response.

To test for *doc* activity, a 3·4 kb fragment of pUNG20, containing the putative addiction cassette of R124, was cloned into pG. The resulting plasmid did not elicit a lethal response upon plasmid loss, indicating that either no *doc* homolog or only a mutated version of the gene was present. On the other hand, a bacterial strain harboring the plasmids pUNG20 or pUNG30 and pG5 survived the heat-induced loss of pG5, indicating that the former two plasmids contain a functional *phd* gene.

4. Discussion

Parasitic relationships are commonly reinforced by addictive mechanisms that place in jeopardy the life of a host that succeeds in ridding itself of the parasite. Lysogenic or colicinogenic bacteria that are cured of a prophage or Col factor become vulnerable to the phage or colicin from which they were formerly protected (Lwoff, 1953; Frédéricq, 1957). Our attention is directed here to a mechanism of addiction involving a more elusive lethal factor, one which acts from within, as do the products of certain selfish, potentially lethal genes in higher organisms (Bull *et al.*, 1992).

Evidence for addiction of a bacterium to a harbored plasmid can be traced to a number of early findings. In a study of the R factor ColV*Btrp*, Koyama & Yura (1975) suggested that acquired interdependency between host and plasmid might be of general occurrence among plasmids that

appear to be stringently maintained. This interpretation has been given to reports of stress responses in cells that have received UV-damaged plasmids of various kinds (Borek & Ryan, 1958; Devoret & George, 1967; Monk, 1969), P1 among them (Rosner *et al.*, 1968; MacQueen & Donachie, 1977). The first of these reports described the indirect induction of prophage in lysogenic bacteria that received by conjugation a UV-damaged F (Borek & Ryan, 1958). Subsequent work showed that the damage sustained by an F that is capable of inducing an SOS response appears sufficient to prevent F maintenance and that, in the case of both F and P1, the SOS induction can be prevented by the presence of a homologous undamaged plasmid (Borek & Ryan, 1958; Rosner *et al.*, 1968). The relevant SOS-inducing function of F was eventually identified as a member of an addiction module that augments the apparent stability of the plasmid. Moreover, the wide distribution of addiction modules among low copy number plasmids came to be recognized.

Our motivation for undertaking this study was partly to determine the extent to which the maintenance functions of P1 resemble or differ from those of other low copy number plasmids and partly to resolve a specific question: Are indirect inductions by P1 and by F symptoms of analogous plasmid distress (Rosner *et al.*, 1968) that can be attributed to the expression of addiction genes, as suggested by Mori *et al.* (1984)?

Our results make it clear that whatever mechanism elicits SOS induction by UV-irradiated P1, it is not the same mechanism as for UV-induced loss of F. Whereas F loss leads to cell filamentation and SOS induction, the symptoms of P1 loss lead to a reduction in cell size and a decreased capacity for SOS induction. The relatively weak indirect induction that UV-damaged P1 mediates is most likely analogous to the SOS induction observed in bacteria infected with a suitable UV-damaged phage (D'Ari & Huisman, 1982) or phasmid (Sommer *et al.*, 1991) that are capable of initiating replication. This conjecture is supported by the observation that indirect induction mediated by P1 depends upon retention of the capacity to initiate DNA replication (Yarmolinsky & Stevens, 1983). A resident P1 prophage can prevent an introduced P1 plasmid from initiating viral or plasmid replication. Damaged DNA can be processed by replication to yield single-stranded DNA, a known inducer of the SOS response (Sassanfar & Roberts, 1990).

The compact P1 addiction module we describe here is composed of an antidote and a killer gene. In this respect it resembles all previously described addiction systems (see Introduction). However, the P1 genes exhibit no obvious nucleotide or codon sequence homology to any of the known systems. The target of the killer protein Doc remains to be identified. There is nothing to suggest that the target of Doc is DNA gyrase, the known target of CcdB (Bernard & Couturier, 1992; Maki *et al.*, 1992), and, given that Doc expression fails to induce an SOS response, it seems clear that neither DNA gyrase nor any other enzyme is converted by Doc into a lethal endonuclease.

The *ccdA ccdB* genes of F are closely linked to the plasmid origin of replication, as are the *pemI pemK* addiction genes of R100 (Tsuchimoto *et al.*, 1988). Not so the *phd doc* genes of P1 (see Fig. 2). On the other hand, all three gene pairs are of similar small size and, not surprisingly, arranged so that the order of transcription is antidote before killer. The diminutive *phd* and *ccdA* genes (73 and 72 codons, respectively) even tolerate loss or replacement of extensive regions without loss of antidote activity. They differ, however, in that the amino acids deleted from the corresponding proteins are C-terminal in Phd and N-terminal in CcdA (see pB8 of Fig. 2; Bernard & Couturier, 1991).

The design of addiction modules offers protection against inadvertent expression of the killer protein in various ways. Translational coupling assures that translation of the Doc messenger RNA is preceded by translation of the antidote to Doc. Further, as the efficiency of the coupling is presumably less than 100%, a bias in favor of antidote synthesis would result. This strategy is apparently not employed by the *ccdA ccdB* gene pair (Bex *et al.*, 1983), but the expression of Hok and its relatives does depend on translational coupling (Gerdes *et al.*, 1992; Thisted & Gerdes, 1992). Furthermore, the transit of ribosomes across the *doc* portion of the *phd* doc transcript must be selectively hindered by the abundance of rare codons in *doc* (Fig. 3). If the results of the *in vitro* transcription/translation experiments (Fig. 4) are any indication of the situation *in vivo*, the concentration of Phd in P1 lysogens is far greater than that of the poison it antagonizes. An excess of antidote over poison might be needed to assume the well-being of carriers of the addicting plasmid.

For the addiction mechanism to perform its function, the accumulated antidote must be subject to turnover. Upon plasmid loss the antidote should be degraded faster than the killer, in order to allow the latter to attack its target. In the case of the analogous proteins PemI of R100 and CcdA of F, this turnover is accomplished by the action of the bacterial protease encoded by *lon* (Tsuchimoto *et al.*, 1992; Couturier, personal communication). In a separate communication from this laboratory evidence will be provided that a protease other than Lon is implicated in the functioning of the P1 addiction mechanism.

Taken together our observations indicate the extent to which the addiction module of P1 differs from those previously described and the extent to which similar organizational principles enter into its design. It has been pointed out that for industrial purposes the expression of a single killer gene is not sufficient to assure the sterility of a culture in which that gene is expressed (Fox, 1989). Nature being no less exigent, it is not surprising that certain plasmids (e.g. F and R1) are endowed with addiction modules of more than one type. The finding that the

P1 addiction gene *phd* is present in the unrelated plasmid R124 (and R124/3), is suggestive evidence for the existence of a traffic in the P1 addiction module among plasmids. The range of bacterial hosts in which this commodity is useful to the plasmids which carry it remains to be seen.

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References

Austin, S., Zeise, M. & Sternberg, N. (1981). A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell*, **25**, 729–736.

Bailone, A., Brandenburger, A., Lévine, A., Pierre, M., Dutreix, M. & Devoret, R. (1984). Indirect SOS induction is promoted by UV-damaged miniF and requires the miniF *lynA* locus. *J. Mol. Biol.* **179**, 367–390.

Bernard, P. & Couturier, M. (1991). The 41 carboxy-terminal residues of the miniF plasmid CcdA protein are sufficient to antagonize the killer activity of the CcdB protein. *Mol. Gen. Genet.* **226**, 297–304.

Bernard, P. & Couturier, M. (1992). Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J. Mol. Biol.* **226**, 735–745.

Bex, F., Karoui, H., Rokeach, L., Drèze, P., Garcia, L. & Couturier, M. (1983). Mini-F encoded proteins: identification of a new 10·5 kilodalton species. *EMBO J.* **2**, 1853–1861.

Borek, E. & Ryan, A. (1958). The transfer of irradiation-elicited induction in a lysogenic organism. *Proc. Nat. Acad. Sci., U.S.A.* **44**, 374–377.

Brandenburger, A., Bailone, A., Lévine, A. & Devoret, R. (1984). Gratuitous induction. *J. Mol. Biol.* **179**, 571–576.

Bravo, A., de Torrontegui, G. & Diaz, R. (1987). Identification of components of a new stability system of plasmid R1, ParD, that is close to the origin of replication of this plasmid. *Mol. Gen. Genet.* **210**, 101–110.

Bull, J. J., Molineux, I. J. & Warren, J. H. (1992). Selfish genes. *Science*, **256**, 65.

Citron, M., Velleman, M. & Schuster, H. (1989). Three additional operators, Op21, Op68, and Op88, of bacteriophage P1. Evidence for control of the P1 *dam* methylase by Op68. *J. Biol. Chem.* **264**, 3611–3617.

Clerget, M. (1991). Site-specific recombination promoted by a short DNA segment of plasmid R1 and by an homologous segment in the terminus region of the *Escherichia coli* chromosome. *New Biol.* **3**, 780–788.

D'Ari, R. & Huisman, O. (1982). DNA replication and indirect induction of the SOS response in *Escherichia coli*. *Biochimie*, **64**, 623–628.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**, 387–395.

Devoret, R. & George, J. (1967). Induction indirecte du prophage λ par le rayonnement ultraviolet. *Mutat. Res.* **4**, 713–725.

Enquist, L. W. & Weisberg, R. A. (1976). The red plaque test: a rapid method for identification of excision defective variants of bacteriophage lambda. *Virology*, **72**, 147–153.

Firman, K., Price, C. & Glover, S. W. (1985). The *Eco*R124 and *Eco*R124/3 restriction and modification systems: cloning the genes. *Plasmid*, **14**, 224–234.

Fox, J. L. (1989). Contemplating suicide genes in the environment. Conditional lethal mutations may minimize risk when engineered microbes are released into the environment. *ASM News*, **55**, 259–261.

Frédéricq, P. (1957). Colicins. *Annu. Rev. Microbiol.* **11**, 7–22.

Gerdes, K., Rasmussen, P. B. & Molin, S. (1986). Unique type of plasmid maintenance function: post-segregational killing of plasmid-free cells. *Proc. Nat. Acad. Sci., U.S.A.* **83**, 3116–3120.

Gerdes, K., Paulsen, L. K., Thisted, T., Nielsen, A. K., Martinussen, J. & Andreasen, P. H. (1990*a*). The *hok* killer gene family in Gram-negative bacteria. *New Biol.* **2**, 946–956.

Gerdes, K., Thisted, T. & Martinussen, J. (1990*b*). Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1: *sok* antisense RNA regulates formation of a *hok* mRNA species correlated with killing of plasmid-free cells. *Mol. Microbiol.* **4**, 1807–1818.

Gerdes, K., Nielsen, A., Thorsted, P. & Wagner, E. G. M. (1992). Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turnover of the stable Hok, SrnB, and PndA effector messenger RNAs. *J. Mol. Biol.* **226**, 637–649.

Golub, E. I. & Low, K. B. (1986). Unrelated conjugative plasmids have sequences which are homologous to the leading region of the F factor. *J. Bacteriol.* **166**, 670–672.

Golub, E. I. & Panzer, H. A. (1988). The F factor of *Escherichia coli* carries a locus of stable plasmid inheritance *stm*, similar to the *parB* locus of plasmid R1. *Mol. Gen. Genet.* **214**, 353–357.

Gottesman, M. E. & Yarmolinsky, M. B. (1968). Integration-negative mutants of bacteriophage lambda. *J. Mol. Biol.* **31**, 487–505.

Huisman, O. & D'Ari, R. (1981). An inducible DNA replication-cell division mechanism in *E. coli*. *Nature (London)*, **290**, 797–799.

Iida, S. & Arber, W. (1980). On the role of IS*1* in the formation of hybrids between bacteriophage P1 and the R plasmid NR1. *Mol. Gen. Genet.* **177**, 261–270.

Jaffé, A., Ogura, T. & Hiraga, S. (1985). Effects of the *ccd* function of the F plasmid on bacterial growth. *J. Bacteriol.* **163**, 841–849.

Karoui, H., Bex, F., Drèze, P. & Couturier, M. (1983). Ham22, a mini-F mutation which is lethal to host cell and promotes *recA* dependent induction of lambdoid prophage. *EMBO J.* **2**, 1863–1868.

Koyama, A. H. & Yura, T. (1975). Plasmid mutations affecting self-maintenance and host growth in *Escherichia coli*. *J. Bacteriol.* **122**, 80–88.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680–689.

Lehnherr, H., Guidolin, A. & Arber, W. (1991). The bacteriophage P1 gene *10* encodes, a *trans*-activating factor required for late gene expression. *J. Bacteriol.* **173**, 6438–6445.

Lehnherr, H., Velleman, M., Guidolin, A. & Arber, W. (1992). Bacteriophage P1 gene *10* is expressed from a promoter-operator sequence controlled by C1 and Bof proteins. *J. Bacteriol.* **174**, 6138–6144.

Loh, S. M., Cram, D. S. & Skurray, R. A. (1988). Nucleotide sequence and transcriptional analysis of a third function (Flm) involved in F-plasmid maintenance. *Gene*, **66**, 259–268.

Lwoff, A. (1953). Lysogeny. *Bacteriol. Rev.* **17**, 269–337.

MacQueen, H. A. & Donachie, W. D. (1977). Intracellular localization and effects on cell division of a plasmid blocked in deoxyribonucleic acid replication. *J. Bacteriol.* **132**, 392–397.

Maki, S., Takiguchi, S., Miki, T. & Horiuchi, T. (1992). Modulation of DNA supercoiling activity of *Escherichia coli* DNA gyrase by F plasmid proteins. Antagonistic actions of LetA (CcdA) and LetD (CcdB) proteins. *J. Biol. Chem.* **257**, 12244–12251.

Meyer, J., Stålhammar-Carlemalm, M., Streiff, M., Iida, S. & Arber, W. (1986). Sequence relations among the IncY plasmids p15B, P1, and P7 prophages. *Plasmid*, **16**, 81–89.

Miki, T., Chang, Z.-T. & Horiuchi, T. (1984a). Control of cell division by sex factor F in *Escherichia coli* II. Identification of genes for inhibitor protein and trigger protein on the 42.84–43.6 F segment. *J. Mol. Biol.* **174**, 627–646.

Miki, T., Yoshioka, K. & Horiuchi, T. (1984b). Control of cell division by sex factor F in *Escherichia coli*. I. The 42.84–43.6 F fragment couples cell division of the host bacteria with replication of plasmid DNA. *J. Mol. Biol.* **174**, 605–625.

Miller, J. H. (1972). *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mizusawa, S. & Ward, D. F. (1982). A bacteriophage lambda vector for cloning with *Bam*HI and *Sau*3AI. *Gene*, **20**, 317–322.

Monk, M. (1969). Induction of phage λ by transferred irradiated ColI DNA. *Mol. Gen. Genet.* **106**, 14–24.

Mori, H., Ogura, T. & Hiraga, S. (1984). Prophage induction caused by mini-F plasmid genes. *Mol. Gen. Genet.* **196**, 185–193.

Mulligan, M. E., Hawley, D. K., Entriken, R. & McClure, W. R. (1984). *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase activity. *Nucl. Acids Res.* **12**, 789–800.

Ogura, T. & Hiraga, S. (1983). Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc. Nat. Acad. Sci., U.S.A.* **80**, 4784–4788.

O'Regan, G. T., Sternberg, N. L. & Cohen, G. (1987). Construction of an ordered, overlapping, library of bacteriophage P1 DNA in λD69, *Gene*, **60**, 129–135.

Poulsen, L. K., Larsen, N. W., Molin, S. & Andersson, P. (1992). Analysis of an *Escherichia coli* mutant strain resistant to the cell-killing function encoded by the *gef* gene family. *Mol. Microbiol.* **6**, 895–905.

Price, C., Lingner, J., Bickle, T. A., Firman, K. & Glover, S. W. (1989). Basis for changes in DNA recognition by the *Eco*R124 and *Eco*R124/3 type I DNA restriction and modification enzymes. *J. Mol. Biol.* **205**, 115–125.

Rosner, J. L. (1972). Formation, induction, and curing of bacteriophage P1 lysogens. *Virology*, **48**, 679–689. (The P1CM*clr*100 of this article is the P1Cm c1.100 of subsequent publications.)

Rosner, J. L., Kass, L. R. & Yarmolinsky, M. B. (1968). Parallel behavior of F and P1 in causing indirect induction of lysogenic bacteria. *Cold Spring Harbor Symp. Quant. Biol.* **33**, 784–789.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci., U.S.A.* **74**, 5463–5467.

Sassanfar, M. & Roberts, J. W. (1990). Nature of the SOS-inducing signal in *Escherichia coli*. *J. Mol. Biol.* **212**, 79–96.

Scott, J. R. (1968). Genetic studies on bacteriophage P1. *Virology*, **36**, 564–574.

Scott, J. R. (1970). A defective P1 prophage with a chromosomal location. *Virology*, **40**, 144–151.

Shuster, L. (1961). Repression and de-repression of enzyme synthesis as a possible explanation of some aspects of drug action. *Nature (London)*, **189**, 314–315.

Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984). *Experiments in Gene Fusions.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sommer, S., Leitaõ, A., Bernardi, A. & Devoret, R. (1991). Introduction of a UV-damaged replicon into a recipient cell is not a sufficient condition to produce an SOS-inducing signal. *Mutat. Res. DNA Repair*, **254**, 107–117.

Takiff, H. E., Chen, S.-M. & Court, D. L. (1989). Genetic analysis of the *rnc* operon of *Escherichia coli*. *J. Bacteriol.* **171**, 2581–2590.

Tam, J. E. & Kline, B. C. (1989). The F plasmid *ccd* autorepressor is a complex of CcdA and CcdB proteins. *Mol. Gen. Genet.* **219**, 26–32.

Thisted, T. & Gerdes, K. (1992). Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1. Sok antisense RNA regulates *hok* gene expression indirectly through the overlapping *mok* gene. *J. Mol. Biol.* **223**, 41–54.

Tsuchimoto, S., Ohtsubo, H. & Ohtsubo, E. (1988). Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. *J. Bacteriol.* **170**, 1461–1466.

Tsuchimoto, S., Nishimura, Y. & Ohtsubo, E. (1992). The stable maintenance system *pem* of plasmid R100: degradation of PemI protein may allow PemK protein to inhibit cell growth. *J. Bacteriol.* **174**, 4205–4211.

Vidal-Ingigliardi, D. & Raibaud, O. (1985). The *mac* promoters: functional hybrid promoters activated by the *malT* product and repressed by the *lacI* product. *Nucl. Acids Res.* **13**, 1163–1172.

Walker, J. T. & Walker, D. H., Jr (1983). Coliphage P1 morphogenesis. Analysis of mutants by electron microscopy. *J. Virol.* **45**, 1118–1139.

Wandersman, C. & Yarmolinsky, M. B. (1977). Bipartite control of immunity conferred by the related heteroimmune plasmid prophages, P1 and P7 (formerly ϕAmp). *Virology*, **77**, 386–400.

Way, J. C., Davis, M. A., Morisato, D., Roberts, D. E. & Kleckner, N. (1984). New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene*, **32**, 369–379.

Yarmolinsky, M. B. & Lobocka, M. B. (1993). Bacteriophage P1. In *Locus Maps of Complex Genomes* (O'Brien, S. J., ed.), 6th edit. pp. 1.50–1.61. Cold Spring Harbor Laboratory Press, NY.

Yarmolinsky, M. B. & Sternberg, N. (1988). Bacteriophage P1. In *The Bacteriophages* (Calendar, R., ed.), pp. 291–438, Plenum Press, NY.

Yarmolinsky, M. B. & Stevens, E. (1983). Replication-control functions block the induction of an SOS response by damaged P1 bacteriophage. *Mol. Gen. Genet.* **192**, 140–148.

Yarmolinsky, M. B., Hansen, E. B., Jafri, S. & Chattoraj, D. K. (1989). Participation of the lytic replicon in bacteriophage P1 plasmid maintenance. *J. Bacteriol.* **171**, 4785–4791.

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