INFLUENCE OF BACTERIOPHAGE LAMBDA GENE *P* EXPRESSION ON HOST *ESCHERICHIA COLI* CELLS

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By

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ABSTRACT

Bacterial viruses have been an important tool for molecular biological discoveries. Bacteriophage λ is a bacterial virus that has been intriguing researchers for over five decades. But still, we are yet to answer many questions about bacteriophage λ. λ genes, *O* and *P* play an important role in λ replication initiation. λP outcompetes host DnaC and recruits and directs DnaB helicase to the λ origin of replication, *oriλ*.

My study shows that λP expression is lethal to the host cell. The P-survivor cells were examined and were found to have chromosomal mutations. This led to the possibility that λP protein may be involved in elevating the level of random mutations of the host chromosome. These studies explore this possibility. The influence of P expression on the appearance of chromosomal mutations was assessed by using rifampicin resistance, and auxotrophy as chromosomal targets. Cellular expression of P from a cryptic λ prophage or from a ColE1-type plasmid was employed in this study.

Insertional inactivation of *P* by recombineering knocked out P-lethality and the potential mutator phenotype among the 42°C survivors. The apparent mutator phenotype was also lost in 42°C survivors upon induction of a cryptic prophage with wild-type *O* and an insertion in *P*. When wild-type gene *P* on a ColE1 plasmid was replaced by a deleted *P* or a mutated *P* (P_{π}) gene, the rate of rifampicin resistance among the 37°C cfu was significantly lowered. These observations suggest that there might be a relation between P expression and the appearance of mutations among the P-survivors. My data also support and extend laboratory findings that two missense mutations in host *dnaB* knock out P-lethality and suppress the observed potential mutator phenotype.

ColE1 plasmid loss occurred among the P-survivor cells when P was expressed from a ColE1 plasmid in a wild-type cell. My data suggested that the plasmid-less or cured rifampicin resistant mutants that arose upon thermal induction of P and the 594 rifampicin resistant mutants that arose in the absence of P expression were resistant to P-toxicity. However, my data showed that a 9 base pair deletion in the *rpoB* gene (mutation in *rpoB* confers rifampicin resistance

phenotype to the cells) that affected 4 amino acids was sensitive to P-toxicity which proved that the deletion itself did not make the cells resistant to P-toxicity.

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DEDICATION

I am dedicating this thesis to my parents, Mr. Amitava Banerjee (father) and Mrs. Rina Banerjee (mother). They have sacrificed their whole life for me and today, I am a proud son. I thank them for everything they have done for me.

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LIST OF ABBREVIATIONS AND TERMS

AA	amino acid
Amp	ampicillin
Amp^R	ampicillin-resistant
aroE	*E. coli* gene encoding the enzyme, shikimate dehydrogenase
bio	biotin
bp	base pair
CA	casamino acids
ColE1	Colicinogenic plasmid E1
cos	cohesive ends at either end of the λ vegetative genome; 12 bp ssDNA ends are important in genome circularization
cI	λ gene encoding the λ CI repressor required for maintaining lysogeny
cII	gene for the λ critical lysogenic regulator CII; activates transcription from p_E, p_I and p_{antiQ}
cIII	λ gene encoding CIII participating in establishment of lysogeny
cfu	colony forming units
cro	gene for the λ Cro repressor that binds O_R and O_L to inhibit p_{RM} transcription; transiently induces transcription from p_L and p_R
dnaA	gene for the *E. coli* initiator protein; transcriptional activator

dnaB	*E. coli* gene encoding DnaB helicase required for λ and *E. coli* replication
dnaC	gene for the *E. coli* initiator protein; DnaB loading protein
dnaG	*E. coli* gene for primase; lays down RNA primers which are extended by DNA polymerase III
dnaJ	*E. coli* gene for the DnaJ heat shock protein, chaperone; refolds misfolded proteins; dissociates protein aggregates; removes P from the λ preprimosomal complex
dnaK	*E. coli* gene for the DnaK heat shock protein, chaperone; refolds misfolded proteins; dissociates protein aggregates; removes P from the λ preprimosomal complex
DNA polymerase III	*E. coli* replicative polymerase for synthesizing DNA; the holoenzyme is a complex heteromultimer containing many subunits
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
groP	alleles of *dnaB* that are functional for *E. coli* replication at all temperatures, but are non-functional for λ replication at 42°C; can be suppressed by π mutations in gene *P*
grpD55	An allelic mutation of host *dnaB* gene: 2 missense mutations
grpE	*E. coli* gene for the heat shock protein GrpE; chaperone, refolds

	misfolded proteins; dissociates protein aggregates; removes P from the λ preprimosomal complex
HflB	cellular protease encoded by *E. coli* gene *hflB*; degrades CII
his	histidine
HK022	bacteriophage HK022
HK97	bacteriophage HK97
ilr mutants	Initiation of lambda replication defective mutants of strain Y836 that are defective for λ replication initiation genes *O* or *P* or their gene expression, or have a mutation within *oriλ*
imm	the immunity region; in λ composed of genes *cI, rexA and rexB* and the two operator sites, O_L and O_R
int	gene for the λ Int protein; integrates λ DNA into *E. coli* chromosome via site-specific recombination
IS2	insertion sequence 2
Iteron	four repeated sequence of 19 base pairs each in the origin region of the lambda genome
Kan	kanamycin
Kan^R	kanamycin-resistant
Kb	kilo base pair
Lac^+	*E. coli* cells that can use lactose as energy source
LamB	receptor for phage λ

LB	Luria broth
LDIA	Low Dose Inoculum Assay
LP-7	bacteriophage LP-7
Mal$^-$	*E. coli* fermentation-defective mutants that cannot use maltose as energy source
MIC	minimum inhibitory concentration
MM	minimal medium
N	gene for the λ early antiterminator, N; binds to *nutR* and *nutL* sites on λ mRNA, required for bypass of t_{L1}, t_{R1} and t_{R2} transcriptional terminators
O	λ gene encoding protein O required for replication initiation
O_L	leftward operator sequence – contains binding sites for Cro and CI and overlaps p_L
O_R	rightward operator sequence – contains binding sites for Cro and CI and overlaps p_R
ori	origin of replication
oriC	origin of replication for *E. coli*
oriλ	origin of replication for λ
O-some	the first nucleoprotein complex formed at *oriλ*, composed of O dimers bound to the four iterons of *oriλ*
P	λ gene encoding protein P required for replication initiation

P1	bacteriophage P1
P1 ban	bacteriophage P1 initiator protein Ban
P22	bacteriophage P22
p_{antiQ}	λ promoter for antisense Q RNA production, regulated by CII
p_I	λ promoter for Int production, regulated by CII
p_L	λ promoter under CI regulation for leftward transcription of λ genes *N-cIII-int*
p_R	λ promoter under CI regulation for rightward transcription of λ genes *cro-cII-O-P-Q*
p_{RE}	λ repressor establishment promoter, regulated by CII
PriA	*E. coli* protein initiating the assembly of the primosome which is essential for the replication of phage ΦX174 and ColE1-like plasmid *in vitro*
pfu	plaque forming units
phage	bacteriophage
Q	gene for the λ late antiterminator protein Q; allows transcription to pass through t_R
recA	*E. coli* gene for RecA; essential recombination gene; DNA pairing and strand exchange; RecA binding to ssDNA induces the SOS response
ren	λ gene, function unknown, thought to allow λ to escape Rex

	Exclusion
Replicative Killing (RK)	Cells with a cryptic lambda prophage are killed by the induction of λ replication initiation when shifted from 30°C to 42°C
RK^+ cells	Replicative Killing competent cells
RK^- mutants	Replicative Killing defective cells that plate with equal efficiency at 30°C and 42°C and are defective for λ replication initiation
Rif	rifampicin
Rif^R or rif^R	rifampicin-resistant
rif^S	rifampicin-sensitive
RNA polymerase	required to transcribe mRNA; the holoenzyme is a complex multimer containing many subunits
rpl	Resistant to P-Lethality
rpoB	*E. coli* gene encoding for the β-subunit of RNA polymerase
SSB	Single-stranded DNA binding protein (SSB) encoded by *E. coli ssb* gene
T1	bacteriophage T1
T4	bacteriophage T4
T7	bacteriophage T7
TB	tryptone broth

t_R	rightward termination site
Tet	tetracycline
Tet^R	tetracycline-resistant
tonB	*E. coli* gene encoding an inner membrane protein that extends into the periplasm and is required for the transfer activity of some outer membrane receptors
tRNA	transfer RNA
Ts	temperature sensitive
wt	wild-type
xis	gene for the λ Xis protein; works with Int to exise the prophage from the chromosome
Xyl^-	*E. coli* fermentation-defective mutants that cannot use xylose as energy source
λ	bacteriophage λ
λ*cI*72	λ phage with a mutation in its repressor gene, *cI*
λimm434*P*am3	λ replication defective phage (due to an amber mutation in gene *P*) with an immunity region of the bacteriophage 434
λvir	λ virulent phage; contains a v2 point mutation in O_L and v1v3 point mutations in O_R; operator mutations prevent CI from binding
π	mutations in λ gene *P* enabling λ to grow on GroP *E. coli*

	strains
θ	the theta, circle-to-circle or early mode of λ replication
σ	the sigma, rolling circle or late mode of λ replication
Φ80	bacteriophage Φ80
21	bacteriophage 21
434	bacteriophage 434
82	bacteriophage 82
933W	bacteriophage 933W

1. Introduction

1.1. Summary of Genes and Regulations in Bacteriophage Lambda

Bacteriophage lambda is a bacterial virus which was originally obtained from a clinical isolate of *Escherichia coli*, lysogenic for this phage (Lederberg, 1951). Phage lambda, being a member of the lambda-like (or lambdoid) bacteriophage family, is temperate, *i.e.*, its lifecycle can take either of two developmental pathways, lysis or lysogeny upon infecting a host cell. This is in contrast to lytic phages (eg., T1, T7, T4, etc.) that infect and muliply in the host cells without being able to lysogenize the cell. In the lysogenic mode, λ integrates its double stranded DNA into the host chromosome via site-specific recombination mechanism. λ uses positive control of operons by antitermination (transcription at the p_R promoter past t_{R1} into the λ replication initiation genes *O* and *P* produces O and P proteins); genes that mediate early lytic functions are organized into divergently transcribed operons that are regulated by a single repressor. The repressor is encoded by a gene positioned between the two operons. All lambdoid phage share a common genome arrangement into functional modules that when exchanged within the family, produce fully functional recombinant phage (Brussow and Hendrix, 2002; Hendrix *et al.*, 1999; Wrobel and Wegrzyn, 2002). Other lambdoid phages include Φ80, P22, 434, HK97, 21, 82, 933W, HK022, LP-7, etc. (Campbell, 1971). Lambda has a double stranded DNA (dsDNA) genome of 48,502 base pairs and has an isometric head about 50 nm in diameter and a flexible tail about 150 nm long (Hendrix *et al.*, 1983). The λ tail recognizes the receptor for phage λ, LamB, and the contact between the tail tip and LamB facilitates phage DNA injection into the host. DNA injection is followed by vegetative phage λ growth in the host. Generally lambda is grown on K type hosts of *E. coli*, but other *E. coli* strains can serve as hosts too.

Figure 1. Genetic Map of Bacteriophage Lambda

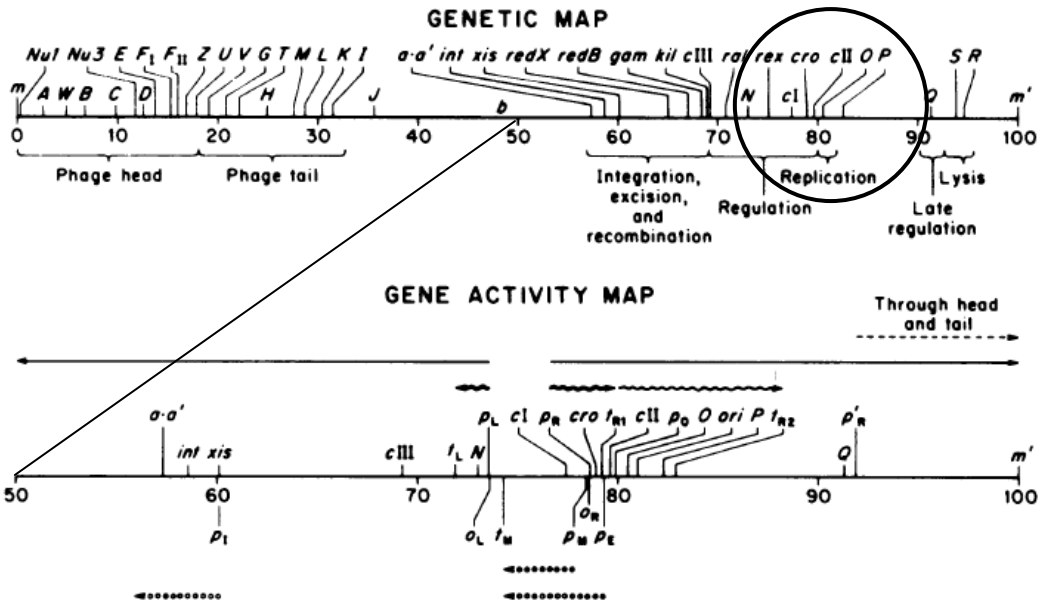


Figure 1. Genetic Map of Bacteriophage Lambda. The upper part of the figure shows groups of genes which carry out vegetative functions like phage head and tail formation; integration, excision and recombination; regulation; replication; late regulation and lysis. The lower part of the figure shows the regulatory sites and their functions. The transcriptional activity is shown by arrows (Echols and Murialdo, 1978).

The genes that I have been studying (*O*, *P* and *cI*) are grouped under regulation and replication in the upper part of Figure 1. In lysogeny, the phage DNA integrates into the host chromosome by site-specific recombination and is passively replicated as a part of the host DNA. λ Int protein aids in the integration of the viral DNA into the host chromosome. The integrated viral DNA is known as a prophage. In lysogeny, the repressor protein, CI (encoded by the gene *cI*) binds to the two operator sites, O_L and O_R. Binding to O_R prevents the expression of

λ replication genes, *O* and *P* from overlapping promoter, p_R. Functional CI is a homodimer composed of two identical polypeptides, each having two domains (Ptashne *et al.*, 1980). Three λ genes play a role in establishing lysogeny, *cI*, *cII* and *cIII*. λ genes *cII* and *cIII* assist gene *cI* in lysogenization. CII protein (97 amino acids long) acts as a transcriptional activator of three promoters, p_E, p_I and p_{antiQ} whose expression helps in the establishment of lysogeny (Kobiler *et al.*, 2002). CIII protein stabilizes the CII protein and in this way, CIII also plays an indirect role in establishing lysogeny.

Ultraviolet irradiation inactivates the repressor CI, thus, allowing the lytic cycle to predominate. UV induces RecA which causes cleavage of the repressor and allows the phage to switch from lysogeny to lysis. This is known as induction. For my experiments, I have used a repressor with a temperature sensitive mutation, *cI*[Ts]857 in order to be able to induce a prophage by increasing the temperature above 38°C. Upon induction of the prophage, CI is inactivated and λ replication initiation genes, *O* and *P* are de-repressed. λ gene product P "hijacks" the host replication machinery and initiates λ DNA replication. λ *cro* gene encodes Cro protein, which binds to the same operator sites as the repressor. Cro is another phage regulatory protein which is required for the lytic cycle to occur. Lambda has two genes, *N* and *Q* which are positive regulators as they promote the transcription of other λ genes (Campbell, 1971). The N protein promotes the maximum transcription of *O* and *P*. The *Q* gene product increases transcription of the head, tail and lysis genes. Through the action of *int*, *xis* genes, the phage DNA excises from the host chromosome and is then packaged into the head followed by the formation of intact phage particles. Phage burst is achieved by lysing the host cell – an act that kills the cell immediately.

1.1.1. Regulation of Lytic vs. Lysogenic cycle

Phage λ can replicate by either of the two developmental pathways, lysis or lysogeny. Upon injecting its DNA (in double stranded linear form) into the host cell, the DNA circularizes through pairing and ligation of the complementary 12 bp *cos* sequences at either end of the genome (Hershey *et al.*, 1963). Expression of a single protein, CII dictates whether the phage

DNA will undergo lytic cycle or lysogenize, i.e., integrate into the host cell chromosome and passively replicate with the host DNA. CII is known as the 'critical lysogenic regulator' as this protein plays a vital role in establishing lysogeny (Oppenheim *et al.*, 2005). If CII is active, the phage DNA lysogenizes; otherwise, it goes into the lytic cycle. The activity of CII protein is governed by environmental factors. Bacterial protease, HflB destroys CII and this acts as a trigger for the phage DNA to undergo the lytic pathway of phage development and multiplication. Lambda CIII protein protects CII from being degraded. If CII is not degraded, it binds to the promoter p_{RE} and activates the production of CI repressor which then binds to the operator sites, thus, establishing lysogeny.

Both λ CI and Cro proteins bind to the same operator sites, O_L and O_R. Operator occupancy governs the transcription from promoters, p_L and p_R (Ptashne *et al.*, 1980). RecA-mediated cleavage of the CI repressor protein makes the operator sites vacant, thus allowing Cro to bind to the operator sites, thus, preventing further lysogenization. Transcription from p_L and p_R drives the lytic cycle. Int and Xis proteins are transcribed from promoter, p_L and aid in the excision of the phage DNA from the host chromosome. Following excision, almost all of the phage genes are expressed and help in the circularizing of the phage DNA, and packaging it into the phage heads. Intact phage particles are produced and the progeny bursts out of the cell. If CI outcompetes Cro in binding to the O_L and O_R operator sites, transcription from p_L and p_R is blocked, thus, repressing phage lytic growth and maintaining or promoting lysogeny (Ptashne *et al.*, 1980).

1.1.2. Brief Overview of λ Replication

Bi-directional replication of λ DNA involves the complexing of several phage and host proteins at the origin of λ replication site, *oriλ* which consists of four 19 bp iteron sequences followed by an AT-rich sequence. Transcription at the p_R promoter is extended past t_{R1} by the action of the phage encoded antitermination protein N, into the λ replication initiation genes *O* and *P* which produces O and P proteins, respectively, which are essential for the λ replication initiation step.

To initiate replication, first, λ replication initiator protein, O binds to the *oriλ* site forming an O-some (Dodson *et al.*, 1989; Alfano and McMacken, 1989). This structure bends the DNA around itself. Another λ replication initiation protein, P outcompetes host DnaC for binding to host DnaB helicase and this hijacking of the host replication machinery is a vital step in the phage lytic cycle. The P-DnaB complex binds to the O-some, i.e., the *oriλ* site bound by O, forming *oriλ*-O-P-DnaB complex known as the preprimosomal complex (Dodson *et al.*, 1989). The preprimosomal complex is formed by the interaction of lambda O and P proteins. Because P inhibits DnaB helicase activity (Biswas and Biswas, 1987), λP protein is removed from the complex to allow DnaB helicase to unwind the double stranded phage DNA. Activities of host chaperone proteins, DnaJ, DnaK and GrpE are required for the removal of P from the preprimosomal complex to allow DnaB helicase to function. After P removal, DnaB helicase is able to interact with DnaG primase and then the PolIII holoenzyme can start DNA synthesis (Zylicz *et al.*, 1989).

λ replication is bidirectional and forms a θ structure. This is called the θ mode of lambda replication. After several rounds of bidirectional replication, a switch to unidirectional or σ mode of replication occurs, which produces linear concatemeric DNA which upon excision between the *cos* sites, gives rise to the linear λ DNA which is then, packaged into progeny phage proheads.

1.1.3. λ Replication Licensing Factor and its Role in λ Replication Initiation

Stillman coined the term replication licensing factor (Stillman, 1994). For replication of *E. coli*, DnaC protein acts as a replication licensing factor as it brings DnaB helicase to the origin of *E. coli* replication, *oriC*. Without DnaC mediated DnaB targeting to *oriC*, bacterial replication cannot take place. Similarly, for phage λ, the replication initiation protein P plays the role of the replication licensing factor as it outcompetes DnaC for binding to the host DnaB helicase and brings it to *oriλ*, thus, driving λ replication. P also needs to be removed from the site for DnaB helicase activity to occur.

The λ replication initiation protein P is known to interact with lambda O protein (Tomizawa, 1971) which binds to *oriλ* sites (Tsurimoto and Matsubara, 1981); host proteins DnaB (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977; Klein *et al.*, 1980) required as a helicase for chromosomal replication; heat shock proteins DnaJ (Yochem *et al.*, 1978), DnaK (Yochem *et al.*, 1978), GrpE (Zylicz *et al.*, 1987); DnaA (Wegrzyn *et al.*, 1996; Datta *et al.*, 2005b; Datta *et al.*, 2005a), required for *E. coli* replication (acting like λO protein to bind at *E. coli oriC*); and possibly with RNA polymerase (McKinney and Wechsler, 1983).

1.1.4. *E. coli* DnaB helicase

The hexameric DnaB protein is the *E. coli* primary replicative helicase (Lebowitz and McMacken, 1986). DnaB is composed of 470 amino acids and has a molecular weight of 52,265 daltons (Nakayama *et al.*, 1984a). The N-terminal domain of DnaB is involved in protein-protein interactions (Nakayama *et al.*, 1984b; Lu *et al.*, 1996). DnaB interacts with many proteins; such as with DnaA (Sutton *et al.*, 1998), DnaC (Wickner and Hurwitz, 1975), DnaG (Lu *et al.*, 1996), SSB (Biswas *et al.*, 2002), the τ subunit of DNA polymerase III (Gao and McHenry, 2001), λP (Klein *et al.*, 1980), P1 ban (Sclafani and Wechsler, 1981) and *E. coli* RNA polymerase (McKinney and Wechsler, 1983). DnaB forms a complex with DnaC ($DnaB_6$-$DnaC_6$). In the complex between DnaB and DnaC, DnaC acts to target DnaB to the bacterial origin of replication, *oriC*. Because DnaB and DnaC are produced at similar levels, the majority of DnaB in a cell is bound to DnaC (Biswas and Biswas, 1987). DnaB is capable of unwinding extensive stretches of double stranded (ds) DNA (Lebowitz and McMacken, 1986). By using the energy obtained from ATP hydrolysis, DnaB helicase promotes the propagation of replication forks (Lebowitz and McMacken, 1986). McMacken *et al.* reported that DnaB acts as a "mobile promoter" signal for DnaG protein (primase) to produce many RNA primers (McMacken *et al.*, 1977). Others (Kaplan and O'Donnell, 2002; Bujalowski, 2003) showed that DnaB can promote branch migration of a synthetic Holliday junction, suggesting that DnaB may be directly involved in DNA recombination *in vivo*.

1.1.5. Host-Phage Interactions: P-DnaB Genetics and Biochemistry

E. coli replication licensing factor, DnaC binds to DnaB helicase in a 1:1 molar ratio in the presence of ATP or dATP (Kobori and Kornberg, 1982a; Kobori and Kornberg, 1982b; Kobori and Kornberg, 1982c). 20 hexamers of DnaB are present in the cell and are known to form a complex with DnaC (Biswas and Biswas, 1987). λ replication initiation protein, P outcompetes host DnaC for binding to DnaB and directs it to *oriλ* (Konieczny and Marszalek, 1995). This step is crucial for λ replication initiation. Mallory *et al.* showed that when a preformed DnaC-DnaB complex was incubated with λP protein, P-DnaB complex was formed and DnaC was found as a free protein (Mallory *et al.*, 1990). This observation suggested that P was able to dissociate the DnaC-DnaB complex and instead, P was able to form a complex with DnaB.

Two to six monomers of λP bind to each hexamer of host DnaB protein (Mallory *et al.*, 1990; Osipiuk *et al.*, 1993; Learn *et al.*, 1997). DnaB does not unwind dsDNA. P allows DnaB to bind to ssDNA. Learn *et al.* showed that λO protein increases the ssDNA binding activity of P (Learn *et al.*, 1997). It is known that λP interacts with λO protein (Tomizawa, 1971). But, in order to interact with or bind to O protein, P should be bound to DnaB (Alfano and McMacken 1989; Dodson *et al.*, 1989). But, earlier studies (Zylicz *et al.*, 1984; Wickner and Zahn, 1986) indicated that O and P are able to interact irrespective of the presence of host DnaB.

1.1.6. P-killing Phenotype

Rao and Rogers showed that cell killing might be caused by P-mediated titration of DnaB or by some unknown gene to the right of *P* (Rao and Rogers, 1978). This was probably the first time that someone demonstrated P-mediated cell killing. But, no further characterization of their finding was carried out. Klinkert and Klein suggested that P blocked the initiation step of *E. coli* DNA synthesis (Klinkert and Klein, 1979). They reported that host cell division was inhibited by P expression. But, they suggested that P expression did not kill the cells, but slowed down DNA synthesis. Klinkert and Klein reported that due to sequestering of DnaB by P, host cell DNA

synthesis was inhibited (Klinkert and Klein, 1979). In 1982, Tsurimoto *et al.* suggested that loss of cell viability and inhibition of DNA synthesis was due to overexpression of λ *P* gene (Tsurimoto *et al.*, 1982), but no mechanisms for Klinkert and Klein and Tsurimoto *et al.*'s observations were provided. Maiti *et al.* also reported P-mediated host cell killing and showed that P-lethality was not due to the sequestration of DnaB by P (Maiti *et al.*, 1991b). Maiti *et al.* isolated rpl (Resistant to P-Lethality) mutants (Maiti *et al.*, 1991a) which were later found to have mutations in host *dnaA* gene (Datta *et al.*, 2005a). These results suggested that mutations in host gene *dnaA* suppress P-lethality. Our laboratory received an rpl strain from Dr. Mandal which was reported to have a DnaA mutation. However, sequence analysis by our laboratory revealed that it did not have a DnaA mutation (Horbay, 2005). So, Horbay, 2005 was not able to confirm or deny whether mutations in *dnaA* would suppress P-interference, as she named it. Horbay found that P-lethality was reversible in cells exposed to P for up to five hours, so used the term 'P-interference' instead of 'P-lethality/killing' (Horbay, 2005).

1.1.7. History of grpD55 mutations in host *dnaB* gene and π mutations in λ *P* gene

GrpD55 mutations are two missense mutations in host *dnaB* gene and are as follows:

a) Nucleotide position: 4,263,102; Mutation: G to A;
   Amino acid change: V256I
b) Nucleotide position: 4,263,612; Mutation: G to A;
   Amino acid change: E426K
   (Horbay, 2005)

E. coli can use DnaBgrpD55 form of the protein for its replication. But, wild-type phage λ is not able to grow on a grpD55 cell.

Saito and Uchida showed that grpD55 mutations co-transduced with *aroE* at 71.5 minutes and they also suggested *grpD* as a new locus which was required for λ replication initiation (Saito and Uchida, 1977). Later, it was found that the grpD55 mapping was done incorrectly.

Bull and Hayes showed that the grpD55 allele co-transduced with *malF*3089::Tn*10* at 91.5 minutes (Bull and Hayes, 1996). Also, a DnaB-expressing plasmid was able to complement for the grpD55 mutations. This confirmed that grpD55 was an allele of *dnaB* (Bull and Hayes, 1996).

Several host *E. coli* mutants do not allow the growth of wild-type λ phage (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977; Georgopoulos, 1977; Sunshine *et al.*, 1977). The host mutations have been mapped in three important genes required for bacterial DNA replication, *dnaB*, *dnaJ* and *dnaK* (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1978). This phenotype has been referred to as groP$^-$ phenotype. Specific mutations in gene *P* allows λ phage growth on the groP$^-$ mutants. These compensatory mutations in gene *P* have been referred to as π mutations (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977; Georgopoulos, 1977; Sunshine *et al.*, 1977). groPA$^-$ and groPB$^-$ are two classes of groP$^-$ mutants in the *dnaB* gene (Georgopoulos and Herskowitz, 1971). π mutants isolated on groPA$^-$ strains are called πA mutants and π mutants isolated on groPB$^-$ strains are called πB mutants. πB mutants grow both on groPA$^-$ and groPB$^-$ strains. But, πA mutants can only grow on groPA$^-$ strains. Reiser *et al.* reported that πB mutation is an unique kind of mutation mapping close to other πA mutants (Reiser *et al.*, 1983). It is possible to estimate the number of πA mutation sites. The sites are scattered asymmetrically within *P*. The N-terminal of P protein lacks π mutations as it is thought to play a role in interacting with the O protein. π mutations lie on the C-terminal portion of the P protein (Reiser *et al.*, 1983). A group of 4-5 mutants have been shown to be clustered around positions 392-413 while others are scattered towards the part of the *P* gene that corresponds to the C-terminus of the protein (Reiser *et al.*, 1983).

1.2. Role of Host DnaB Protein in ColE1 Plasmid Replication

The colicinogenic plasmid E1 (ColE1) was isolated from *E. coli* cells as a supercoiled DNA molecule with a molecular weight of 4.2×10^6 and as a supercoiled DNA-protein relaxation complex (Clewell and Helinski, 1969). Host DnaB helicase is known to promote the advancement of the replication fork *in vivo* (Lebowitz and McMacken, 1986; McMacken *et al.*, 1977). But, in case of ColE1 replication fork progression, DnaB is unable to dissociate RNA-

DNA hybrids which results in replication fork pausing at silent ColE1 origins (Santamaria *et al.*, 1998). In ColE1 replication, after the formation of the initial part of the new L-strand, synthesis of the new H-strand begins. H-strand is synthesized discontinously in the 5' to 3' direction. Okazaki fragments of about 1000 bases are formed and are subsequenctly linked together. Synthesis of each DNA fragment is initiated at a short primer of RNA. The *dnaG* gene product (DNA primase) transcribes ColE1 to provide this RNA primer. The *dnaB* gene product acts as a mobile promoter which enable DNA primase to initiate transcription (McMacken *et al.*, 1977). DNA polymerase III holoenzyme extends the primers.

1.3. Escaping Cell Death following λ Cryptic Prophage Induction

A cryptic prophage is a trapped phage DNA which has been deleted for essential λ genes. In my work, I used a strain called Y836 which has a cryptic λ prophage with a temperature sensitive (*cI*[Ts]857) repressor which has been deleted from *int-kil* and rightward from *ren* into *E. coli* (Δ431 deletion) (Hayes, 1991) and this deletion does not allow the prophage to be excised from the host chromosome (as it is deleted for *int* and *xis* genes) and form intact phage particles upon prophage induction. Upon induction of the cryptic prophage at 42°C, λ CI repressor is inactivated which leads to the de-repression of the λ replication initiation genes, *O* and *P*. P outcompetes host DnaC for binding to host DnaB and targets it to *oriλ*. Replication initiation arises from the *oriλ* site in the trapped cryptic prophage, but the prophage is not able to excise itself from the host. This causes replicative stress in the cells, and massive cell killing. This is referred to as Replicative Killing and the phenotype is called RK phenotype. The cells harbouring the trapped cryptic prophage are designated as Replicative Killing Competent (RK^+) cells. Approximately, 1 out of 10^6 cells survive this replicative stress due to host or phage mutations and are therefore known as Replicative killing defective (RK^-) cells (Hayes and Hayes, 1986). Hayes *et al.* isolated two RK^- mutants (denoted as 145d and 101b) which had acquired 13 and 14 point mutations within λ genes *O* and *P* which allowed cell survival and colony formation at 42°C (Hayes *et al.*, 1998). [For details about Replicative Killing and Y836 strain construction, refer to Chu, 2005 and Hayes and Hayes, 1986].

1.4. Auxotrophy and Rifampicin Resistance

Auxotrophs are mutants that are defective in a gene required to synthesize a particular nutrient. Therefore, they are unable to grow in a medium not supplemented with that nutrient. Nutrient gene products or enzymes are parts of important biosynthetic pathways required for cell growth. Defects in any essential nutrient gene block the synthesis of the nutrient and hence, the nutrient has to be supplied in the medium for the auxtrophic cell to grow. For example, a *his*$^-$ cell requires a medium supplemented with histidine to grow. For mutagenesis studies, we have used auxotrophy to screen for a wide variety of mutations.

To screen a more narrow mutation target, we screened for rifampicin resistance. Rifampicin resistance phenoptype is conferred by mutations in *rpoB* gene (4029 bp) which codes for the 1342-amino acid β-subunit of DNA-dependent RNA polymerase (Zillig *et al.*, 1970). DNA-dependent RNA polymerase is a complex multisubunit enzyme that exists in two forms. RNA polymerase core enzyme has a molecular weight of 400 kDa and consists of 5 subunits, including an α dimer (α_2), β subunit, β' subunit and ω subunit. These subunits are converted to a holoenzyme following the binding of one σ subunit. RNA polymerase holoenzyme carries out initiation of transcription at defined promoter sites and core RNA polymerase carries out elongation and termination (Burgess and Travers, 1970; Burgess *et al.*, 1969). Most of the catalytic functions of RNA polymerase is carried out by the β subunit which is encoded by the *rpoB* gene (Jin and Gross, 1989). Kollenda *et al.* obtained rifampicin resistant mutations that were located in 28 and 37 minutes of the *E. coli* chromosome standard map, some distance from the *rpoB* gene at 89.5 minutes (Kollenda *et al.*, 1986). This is probably the only paper that reported rifampicin resistance mutations not linked to the *rpoB* gene, but was never reproduced.

Rifampicin antibiotic has an inhibitory effect on RNA polymerase which leads to inhibition of transcription. Hartmann *et al.* (Hartmann *et al.*, 1967) first showed the inhibitory effect of rifampicin on RNA polymerase from *E. coli*. Later, Johnston and McClure (Johnston and McClure, 1976) showed that rifampicin is specifically involved in the abortive initiation of RNA synthesis by inhibiting the formation of the dinucleotide pppApU and the formation of the second phosphodiester bond. McClure and Cech (McClure and Cech, 1978) showed that the binding of rifampicin to RNA polymerase sterically blocks the translocation of pppApU, thus inhibiting further elongation of the RNA chain. It has been postulated that rifampicin competes

directly or allosterically with the RNA product binding site on RNA polymerase and that the overall inhibition of RNA synthesis by rifampicin is caused by a destabilizing effect on the binding of the intermediate oligonucleotides to the active enzyme-DNA complex (Kessler *et al.*, 1982; Schulz and Zillig, 1981; Wehrli, 1983).

1.5. Luria-Delbrück Fluctuation Test

The Luria-Delbrück Fluctuation Test tested two hypotheses for how mutants arise in bacterial cultures: the random-mutation hypothesis and the direct-change hypothesis. The random-mutation hypothesis predicts that the mutants appeared prior to the addition of the selective agent while the direct-change hypothesis predicts that the mutants appear only in response to the selective agent. Mutants that arose early in a generation yielded greater numbers of mutants in the progeny population. Luria and Delbrück used *E. coli* as the bacterium and bacteriophage T1 as the selective agent. Phage T1 kills wild-type *E. coli*, but mutation in *tonB* (a gene for an inner membrane protein) can make the cells resistant to phage T1 killing. If the cells are spread on an agar plate with the phage only, the resistant bacteria will form colonies. All other cells will be killed. Luria and Delbrück performed two experiments. In their first experiment, they started one culture of bacteria. After incubating the culture, they took out small aliquots and plated them with and without phage T1. This was done to measure the number of resistant mutants and also the total number of cells plated. The fraction of the resistant mutants was calculated.

In their second experiment, they started several small cultures. After incubation, they counted the number of resistant mutants and the total number of cells in each culture. It was seen that in their first experiment, the number of resistant mutants in each aliquot was almost the same. While in their second experiment, a large variation in the number of resistant cells was found. Some had no resistant cells and some had many. Luria and Delbrück referred to the cultures with a large number of resistant cells as ‘jackpot’ cultures. What they said was that the resistant mutants in a ‘jackpot’ culture arose in an early generation. These results proved the random-mutation hypothesis. While the direct-change hypothesis predicts that the results of two

experiments should be the same and no ‘jackpot’ cultures should appear in the second experiment (Luria and Delbrück, 1943; Snyder and Champness, 2003).

1.6. Introductory Hypothesis for the Current Study

Based on the work by Chu (Chu, 2005), it was hypothesized that the overexpression of P can elevate the frequency of cellular chromosomal mutations over a spontaneous level seen in the absence of P expression. To assess the spontaneous and induced mutation frequencies, I used broad (essential nutrient genes) and narrow (*rpoB* gene) chromosomal targets for my study.

2. Materials, Methods and Reagents

2.1. Bacterial and Bacteriophage strains, and Plasmids

2.1.1. *E. coli* Strains

Strain	Relevant Genotype	Source / Reference	Hayes Laboratory Strain #
594	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 IN(*rrnD-rrnE*)1		B10
W3350	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1		B12
594*dnaB*grpD55	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *dnaB*grpD55*malF*3089::Tn*10* Tet^R	Bull and Hayes, 1996	nB295
594*rpoB*$^-$(D10) or Tsc325D10	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *rpoB*Δ1604-1612: CAGGCCGTC: Pro-Gly-Gly-Leu535-538Pro rif^R (moved *rpoB* deletion from sc325D10 into 594)	This work	B323
594sc125A2	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *rpoB*1535: C to A:	This work	B322-594sc125A2

	Ser512Thr rif^{R} (selected from 594 at 25°C)		
594sc325E	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB*1592: C to T: Ser531Phe rif^{R} (selected from 594 at 25°C)	This work	B322-594sc325E
594sc137A2	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB*1586: G to A: Arg529His rif^{R} (selected from 594 at 37°C)	This work	B322-594sc137A2
594sc337D	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB*1714: A to T: Ile572Phe rif^{R} (selected from 594 at 37°C)	This work	B322-594sc337D
Y836	SA500(λ*bio*275*cI*[Ts]857 Δ431) *his*$^{-}$	Hayes and Hayes, 1986	NY1049
Y836*dnaB*grpD55	SA500(λ*bio*275*cI*[Ts]857 Δ431) *his*$^{-}$ *dnaB*grpD55*malF*3089::Tn*10* Tet^{R}	K. Asai (Hayes lab); Hayes *et al.*, 2005	NY1130
594::(*cIII-ren*)$^{\lambda}$	Tn*10* [zbh29 at 17 min] *bio*$^{+}$ transductant: assumed 594 *bio275* (λ*cIII-cI*[Ts]857-*ren*) Δ431 = **	A. Chu (Hayes lab)	NY1057

Y836 *his*$^{+}$	SA500(λ*bio*275 *cI*[Ts]857 Δ431) *his*$^{+}$	Chu, 2005	NY1046
Y836 *P*::*Kan* (Bib11t)	SA500(λ*bio*275 *cI*[Ts]857 Δ431 *O*$^{+}$*P*::*Kan*) *his*$^{-}$ Kan^{R}	S. Hayes	NY1153
Y836 ilr 534c (RK^{-} mutant)	SA500 (λ*bio*275 *cI*[Ts]857 Δ431 *O*$^{+}$*P*$^{+}$) *his*$^{-}$	Hayes and Hayes, 1986	Y870-534c
Y836 ilr 208b (RK^{-} mutant)	SA500 (λ*bio*275 *cI*[Ts]857 Δ431 *O*$^{-}$*P*$^{+}$) *his*$^{-}$	Hayes and Hayes, 1986	MY843-208b
Y836 ilr 223a (RK^{-} mutant)	SA500 (λ*bio*275 *cI*[Ts]857 Δ431 *O*$^{-}$*P*$^{+}$) *his*$^{-}$	Hayes and Hayes, 1986	MY843-223a
Y836 ilr 566a (RK^{-} mutant)	SA500 (λ*bio*275 *cI*[Ts]857 Δ431 *O*$^{+}$*P*::IS2) *his*$^{-}$	Hayes and Hayes, 1986	Y870-566a
594[pSIM6]	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 Amp^{R} *cI*[Ts]857 (contains λ Red genes)	This work and Thomason *et al.*, 2007	P514
594[pcIpR-P-timm]	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R}	C. Hayes (Hayes lab)	P466
594*dnaB*grpD55[pcIpR-P-timm]	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *dnaB*grpD55*malF*3089::Tn*10* *cI*[Ts]857 *P*$^{+}$ Amp^{R}	K. Marciniuk (Hayes lab)	P479
594[pcIpR-ΔP-timm]	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{-}$ Amp^{R}	K. Marciniuk (Hayes lab)	P515

594*dnaB*grpD55[pcIpR-ΔP-timm]	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *dnaB*grpD55*malF*3089::Tn*10* *cI*[Ts]857 *P*$^-$ Amp^R	K. Marciniuk (Hayes lab)	P516
594[pcIpR-P_π-timm]	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 π mutation in *P* Amp^R	K. Marciniuk (Hayes lab)	P505
594*dnaB*grpD55[pcIpR-P_π-timm]	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *dnaB*grpD55*malF*3089::Tn*10* *cI*[Ts]857 π mutation in *P* Amp^R	K. Marciniuk (Hayes lab)	P506
sc325A4	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1585: C to T: Arg529Cys rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325A4
sc325A6	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1595: C to A: Ala532Glu rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325A6
sc325A7	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1527: C to A: Ser509Arg rif^R (selected from 594[pcIpR-P-	This work	P511-sc325A7

	timm] at 25°C)		
sc325A9	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*1687: A to C: Thr563Pro rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325A9
sc325A10	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*1592: C to T: Ser531Phe rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325A10
sc325B1	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*Δ1605-1613: AGGCGGTCT: Pro-Gly-Gly-Leu535-538Pro rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325B1
sc325B2	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB* rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325B2
sc325B3	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB* rif^{R}	This work	P511-sc325B3

	(selected from 594[pcIpR-P-timm] at 25°C)		
sc325B6	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB* rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325B6
sc325B10	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1600: G to T: Gly534Cys rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325B10
sc325C4	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1687: A to C: Thr563Pro rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325C4
sc325C5	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1691: C to T: Pro564Leu rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325C5
sc325C6	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R	This work	P511-sc325C6

	*rpoB*1592: C to T: Ser531Phe rif^R (selected from 594[pcIpR-P-timm] at 25°C)		
sc325C10	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1586: G to A: Arg529His rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325C10
sc325D1	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1574: C to G: Thr525Arg rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325D1
sc325D2	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1576: C to G: His526Asp rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325D2
sc325D4	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 Amp^R *rpoB* rif^R (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325D4

sc325D5	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*1527: C to A: Ser509Arg rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325D5
sc325D6	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*1601: G to T: Gly534Val rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325D6
sc325D8	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*1691: C to T: Pro564Leu rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325D8
sc325D9	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*1565: C to T: Ser522Phe rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)	This work	P511-sc325D9
sc325D10	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*Δ1604-1612:	This work	P511-sc325D10

	CAGGCCGTC: Pro-Gly-Gly-Leu535-538Pro rif^{R} (selected from 594[pcIpR-P-timm] at 25°C)		
sc337A10	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB*1547: A to G: Asp516Gly rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337A10
sc337B1	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB* rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337B1
sc337C1	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB* rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337C1
sc337C6	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB* rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337C6
sc337B8	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB* rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337B8

sc337C4	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB* rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337C4
sc337C5	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB*1691: C to T: Pro564Leu rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337C5
sc337C7	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^{+}$ Amp^{R} *rpoB*1601: G to A: Gly534Asp rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337C7
sc337D1	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB*1714: A to C: Ile572Leu rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337D1
sc337D2	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22 *rpoB* rif^{R} (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337D2
sc337D3	*F*$^{-}$ *lac*$^{-}$3350 *galK*2 *galT*22	This work	P511-

	rpoB rif^R (selected from 594[pcIpR-P-timm] at 37°C)		sc337D3
sc337D6	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *rpoB*1609: G to T: Gly537Val rif^R (selected from 594[pcIpR-P-timm] at 37°C)	This work	P511-sc337D6
594sc125A2 [pcIpR-P-timm]	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1535: C to A: Ser512Thr rif^R	This work	P512-594sc125A2
594sc325E [pcIpR-P-timm]	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1592: C to T: Ser531Phe rif^R	This work	P512-594sc325E
594sc137A2 [pcIpR-P-timm]	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1586: G to A: Arg529His rif^R	This work	P512-594sc137A2
594sc337D [pcIpR-P-timm]	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R *rpoB*1714: A to T: Ile572Phe rif^R	This work	P512-594sc337D
594*rpoB*$^-$(D10) [pcIpR-P-timm] or	*F*$^-$ *lac*$^-$3350 *galK*2 *galT*22 *cI*[Ts]857 *P*$^+$ Amp^R	This work	P530

Tsc325D10 [pcIpR-P-timm]	*rpoB*Δ1604-1612: CAGGCCGTC: Pro-Gly-Gly-Leu535-538Pro rif^{R}		

2.1.2. Bacteriophage Strains

Bacteriophage	Relevant Genotype	Lysate Collection #	Source
λ papa	Wt	1001	Hayes, 1979
λ*cI*72	*cI*$^{-}$	951	Hayes, 1979
λvir	λ*v2v1v3*	260	Hayes, 1979
λ*imm*434*P*am3	*imm*434 *cI*$^{-}$ *P*am3	664	Hayes *et al.*, 1998

2.1.3. Plasmids

pcI-pR-P-timm (constructed by C. Hayes) is a P-expression plasmid (ColE1-type). The promoter, *pR* drives P expression. The repressor protein, CI represses P expression at lower temperatures. pSIM6 plasmid contains λ Red recombination genes under λ *cI*[Ts]857 repressor control (Datta *et al.*, 2006).

2.2. Reagents, Media and Growth Conditions

2.2.1. Solid Support Media

a) TBA: Tryptone agar plates include 11 grams of Bacto agar, 10 grams of Bacto tryptone and 5 grams of NaCl per liter (Hayes *et al.*, 2005).
b) LBA: Luria broth plates include 11 grams of Bacto agar, 10 grams of Bacto tryptone, 5 grams of Bacto yeast extract and 5 grams of NaCl per liter.
c) MM: Minimal media plates include 11 grams of Bacto agar, 100 ml of 10X M9 salts [a], 16 ml of 25% glucose, 0.1 ml of 1M $CaCl_2$, 1 ml of 1M $MgSO_4.7H_2O$ and 0.2 ml of 0.3% ferric citrate per liter (Arber *et al*., 1983).
d) MM+his: Minimal media plates supplemented with histidine include 11 grams of Bacto agar, 100 ml of 10X M9 salts [a], 16 ml of 25% glucose, 0.1 ml of 1M $CaCl_2$, 1 ml of 1M $MgSO_4.7H_2O$, 0.2 ml of 0.3% ferric citrate per liter and histidine at a final concentration of 0.1 mg/ml.
e) MM+his+bio: Minimal media plates supplemented with histidine include 11 grams of Bacto agar, 100 ml of 10X M9 salts [a], 16 ml of 25% glucose, 0.1 ml of 1M $CaCl_2$, 1 ml of 1M $MgSO_4.7H_2O$, 0.2 ml of 0.3% ferric citrate per liter, histidine at a final concentration of 0.1 mg/ml and biotin at a final concentration of 1 μg/ml.
f) MM+CA: Minimal media plates supplemented with casamino acid [not vitamin free] include 11 grams of Bacto agar, 100 ml of 10X M9 salts [a], 16 ml of 25% glucose, 0.1 ml of 1M $CaCl_2$, 1 ml of 1M $MgSO_4.7H_2O$, 0.2 ml of 0.3% ferric citrate, 0.01 M Tris per liter and 0.3% casamino acid (1% casamino acids have also been used - mentioned with the data table).
g) TB+Amp: Tryptone broth plates supplemented with ampicillin include 11 grams of Bacto agar, 10 grams of Bacto tryptone, 5 grams of NaCl per liter and ampicillin at a final concentration of 50μg/ml.
h) TB+Rif: Tryptone broth plates supplemented with rifampicin include 11 grams of Bacto agar, 10 grams of Bacto tryptone, 5 grams of NaCl per liter and rifampicin at a final concentration of 100μg/ml.

i) TB+Kan: Tryptone broth plates supplemented with kanamycin include 11 grams of Bacto agar, 10 grams of Bacto tryptone, 5 grams of NaCl per liter and kanamycin at a final concentration of 50μg/ml.

j) TB+Tet: Tryptone broth plates supplemented with tetracycline include 11 grams of Bacto agar, 10 grams of Bacto tryptone, 5 grams of NaCl per liter and tetracycline at a final concentration of 15μg/ml.

k) TA: Molten TB top agar includes 10 grams of Bacto tryptone, 6.5 grams of Bacto agar and 5 grams of NaCl per liter.

[a] 10X M9 salts (Arber *et al.*, 1983)

70 grams Na_2HPO_4

30 grams KH_2PO_4

5 grams NaCl

10 grams NH_4Cl

1 liter deionized water

Note: Deionized water was used in the plates.

2.2.2. Liquid Growth Media

a) TB: Tryptone broth includes 10 grams of Bacto tryptone and 5 grams of NaCl per liter. Deionized water is used. The broth is stirred until the ingredients dissolve, poured into bottles, and autoclaved for 30-45 minutes (Hayes *et al.*, 2005).

b) LB: Luria broth includes 10 grams of Bacto tryptone, 5 grams of Bacto yeast extract and 5 grams of NaCl per liter. Deionized water is used. The broth is stirred until the ingredients dissolve, poured into bottles, and autoclaved for 30-45 minutes.

c) SOB media (2% w/v Bacto tryptone, 0.5% Yeast extract and 10 mM NaCl)+ Mg (in 200 ml SOB, add 2 ml 1M $MgCl_2.6H_2O$ and 2 ml 1M $MgSO_4.7H_2O$) was used for subculturing for making competent cells for electroporation.

d) SOC media (in 20 ml SOB media + Mg, add 144 µl of 25% glucose – final concentration of glucose is 1.8µg/ml) was added to the electroporated/pulsed cell-DNA mixture.

The liquid cultures were incubated in a shaking water bath adjusted to the desired temperature. The plates were incubated inverted in a closed temperature controlled air incubator.

2.2.3. Antibiotics

a) A stock of 50 mg/ml ampicillin is used. 50 mg of ampicillin sodium salt was dissolved in 1 ml of sterile water and was mixed well until ampicillin was dissolved. The stock solution was filter sterilized and was stored in -20°C. 1 ml of 50 mg/ml ampicillin was added to 1 liter of media to get a final concentration of 50µg/ml (Miller, 1992).
b) A 50 mg/ml stock of rifampicin is used. 100 mg of rifampicin was dissolved in 2ml of methanol. Approximately, 5 drops of 10N NaOH was added to facilitate the antibiotic to dissolve. The stock solution was filter sterilized and the container (eppendorf tube) was wrapped with a foil as rifampicin is light-sensitive. 2 ml of 50 mg/ml rifampicin was added to 1 liter of media to get a final concentration of 100µg/ml. Freshly made rifampicin stock was added to the media and also freshly made media plates supplemented with rifampicin were used for the experiments (Miller, 1992).
c) A stock of 50 mg/ml kanamycin is used. 100 mg of kanamycin was dissolved in 2 ml of sterile water and was mixed well until kanamycin was dissolved. The stock solution was filter sterilized and stored in -20°C. 1 ml of 50 mg/ml kanamycin was added to 1 liter of media for final concentration of 50µg/ml (Miller, 1992).
d) A stock of 15 mg/ml tetracycline is used. 15 mg of tetracycline is dissolved in 0.5 ml of concentrated ethanol and 0.5 ml of sterile water. The container (eppendorf tube) is wrapped with a foil. 1 ml of 15 mg/ml tetracycline was added to 1 liter of media for final concentration of 15µg/ml (Miller, 1992).

2.2.4. Buffers

a) Φ80 buffer (0.01 M Tris pH 7.6 and 0.1 M NaCl) was utilized for cell culture and phage dilutions (Hayes *et al.*, 2005).
b) TE buffer (0.01 M Tris-HCl pH 7.6-8 and 0.01 M Na_2EDTA) was used for storage of DNA.
c) TE* buffer (0.01 M Tris-HCl pH 7.6-8 and 0.001 M Na_2EDTA) was used for manipulation of DNA (Hayes *et al.*, 2005).
d) TBE buffer (1 M Tris pH 8, 1 M Boric acid and 0.02 M Na_2EDTA) was used to make agarose gels and as running buffer during electrophoresis.

2.3. Methods and Protocols in Molecular and Microbial Genetics

2.3.1. Plasmid DNA Isolation

Plasmid DNA was isolated using EZ-10 Spin Column Plasmid DNA Kit (BioBasic) and QIAprep Spin Miniprep Kit (50) – Cat. No.: 27104 (Qiagen), as per included instructions.

2.3.2. DNA Agarose Gel Electrophoresis

0.8% agarose gels were made using TBE buffer and agarose (purchased from Sigma). Gels were run at approximately 90 V for up to 1.5 hours. DNA band sizes were estimated using a 1 Kb DNA ladder purchased from Invitrogen. The 1 Kb DNA ladder produces DNA bands at 10 Kb, 8 Kb, 6 Kb, 5 Kb, 4 Kb, 3 Kb, 2 Kb, 1.5 Kb, 1 Kb and 0.5 Kb. The ladder contains equimolar mixtures of each fragment, and was utilized to estimate the mass of unknown DNA samples by comparing unknown band intensities to those of the known bands.

2.3.3. Preparing Competent Cells and Transformation of *E. coli*

A log phase subculture was pipetted into an eppendorf tube and was centrifuged for 2 minutes at 12 K. The supernatant was discarded and the cell pellet was gently resuspended in one-half of the original volume of 0.01M NaCl. The resuspended cell-NaCl mixture was centrifuged as before. The supernatant was discarded and the pellet was gently resuspended in one-half of the original volume of 0.03M $CaCl_2$. The tube was held on ice for 30 minutes. Centrifuging was done as before. The supernatant was discarded and the cell pellet was gently resuspended in $1/10^{th}$ of the original volume of 0.03M $CaCl_2$. The cells are now competent for transformation and were held on ice (for a very short period). 0.2 ml of the competent cells was mixed with 5 µl of the plasmid DNA. The cell/DNA mixture was held on ice for 60 minutes and then, heat shocked at ~42-43°C for 90 seconds in a heat block. The cell-DNA mixture was chilled on ice for 2 minutes and then, 800 µl of TB was added to the transformation mixture to make the total volume ~1 ml. The solution was incubated for ~1½ hours in a 25°C or 30°C shaking water bath (as per requirement) with very slow and gentle shaking. The transformation mixture was diluted and plated on TB+50µg/ml Amp selection plates. In most cases, the transformation mixture was also plated on rich TB plates for determining the frequency of appearance of Amp^R transformants. Incubation was done at 25°C or 30°C depending on the cell type. Incubation varied from 48-72 hours depending on the growth rate of the transformants.

2.3.4. Restriction Digestion Analysis

All restriction enzymes were purchased from New England Biolabs (NEB) and used as directed.

2.3.5. Strip Streaking of Phage for Single Plaques

0.3 ml of host cells mixed with 3 ml of warm tryptone top agar was plated onto a rich tryptone agar plate. The agar was allowed to solidify for 10-15 minutes. With a pipette, 0.05 ml (or, a lesser volume) of phage lysate was dropped close to the edge of the plate lawn (spot phage lysate labelled in Figure 2). Using autoclaved paper strips, streaking was performed as indicated

in Figure 2 below. A fresh paper strip was used for each strip 1, 2, 3 and 4. The 4th paper strip was used 3 times; 4a, 4b and 4c as shown in Figure 2 below. Only the edge of the spot phage lysate or end of previous strip, for 1, 2 and 3 was touched. Strip 4 was run across the stripped area of 3 as illustrated in the figure below. This method is a fast way of obtaining single plaques if a phage count (pfu/ml) is not needed.

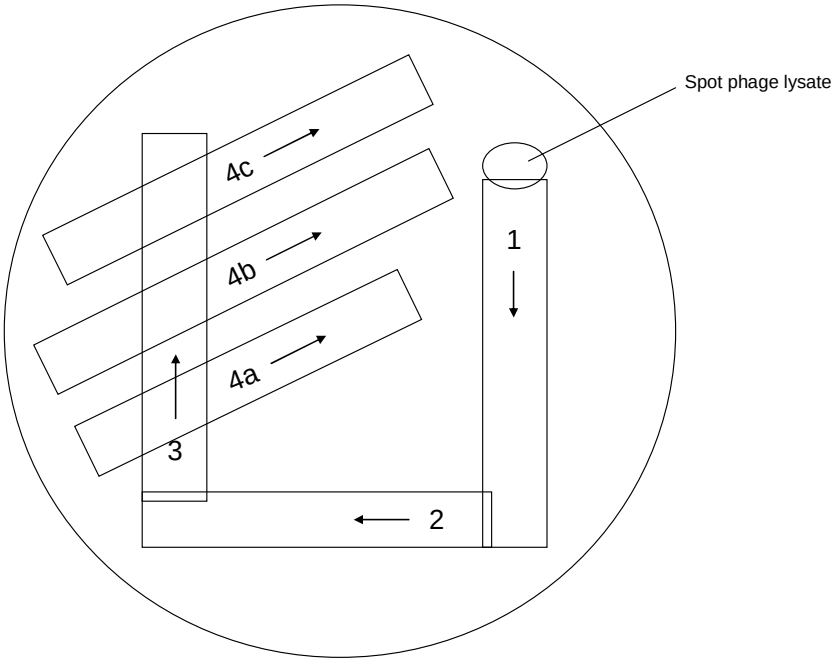


Figure 2. Strip Streaking of Phage for Single Plaques. The small ellipse in the figure represents the spot phage lysate and the rectangles represent the path used to dilute the spotted phage by the individual paper strips.

2.3.6. Preparation of a Standard Phage Lysate

20 ml LB was pipetted into a 125 ml culture flask. 200 µl of 1M $MgCl_2$, 200 µl of 1M Tris-HCl (pH 7.5) and 200 µl of 1M $CaCl_2$ (each at 0.01M final concentration) were added to LB. One flask was made per lysate. The flask was kept in a 39°C air incubator for pre-warming. An individual (single; i.e., one) plaque was picked using a glass 5" pipette attached to a pipetter aid and was suspended into 0.5 ml of Φ80 buffer in a 1.5 ml eppendorf tube. (The top of the pipetter aid should be raised before coring the isolated plaques.) The solution was pipetted up and down several times to ensure that the plaque was submerged in the buffer. The plaque-buffer was incubated for ~15 minutes in 39°C air incubator. The eppendorf tube was centrifuged at 13K for 2 minutes and then, the top 0.4 to 0.5 ml was carefully decanted. While the plaque-buffer solution is incubating, 0.3 ml overnight cell culture was mixed with 20 µl of 1M $MgCl_2$, 20 µl of 1M $CaCl_2$ and 20 µl of 1M Tris-HCl (pH 7.5) in another 1.5 ml eppendorf tube. The 0.4 to 0.5 ml of phage-plug eluate was added to the above mixture and the resulting solution was gently vortexed and then, the solution was incubated for 15 minutes in a 37°C air incubator. The cell/phage-eluate mixture was added to the 20 ml LB that was pre-warming at 39°C. The flask was placed in a 39°C shaking water bath (speed at 4.5) and was incubated for 3 to 6 hours. The mixture was monitored for cell growth and lysis. The lysate was poured into a 30 ml Corex tube and was spun at 8 to 10K for 10 minutes. The supernatant was decanted into a sterile bottle containing 0.3 ml chloroform (to kill any contaminating cells). The bottle was shaken and was stored in the refrigerator/cold box. The phage was titered to determine the number of pfu/ml.

2.3.7. Streaking of Bacterial Cells for Single Colonies

Method 1: A loopful of bacterial cells was streaked on top of an agar plate (concentrated cells labelled in Figure 3). After sterilizing the loop, vertical streaks (patch 2 in Figure 3) were made across the concentrated cells as shown in the figure below. The loop was sterilized again. Then, a horizontal streak 3 was made across patch 2. After sterilizing the loop again, a vertical streak 4 was made across the horizontal streak 3. The loop was sterilized. Finally, streaks were made across vertical streak 4 (see Figure 3) to obtain isolated single colonies. This technique is a fast way to obtain single colonies if a cell count (cfu/ml) is not needed.

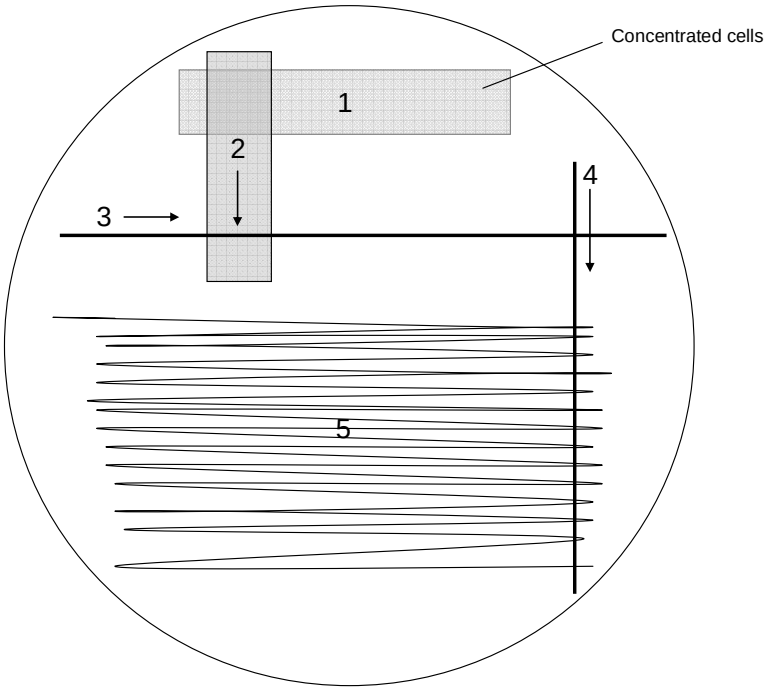


Figure 3. Streaking of Bacterial Cells for Single Colonies (Method 1). Rectangles 1 and 2 represent patch of cells. Lines 3 and 4 represent streaks in the direction of the arrows.

Method 2: A loopful of bacterial cells was streaked on top of an agar plate (as in Method 1). After sterilizing the loop, a downward vertical streak was made across the concentrated cells. The loop was flipped or turned to the other side and multiple streaks were made throughout the plate across the vertical streak to obtain isolated single colonies. This method is quicker than Method 1 as less sterilization steps are needed.

2.3.8. Cross Streaking Immunity Assay

The loop was initially flamed for sterilization. After cooling the loop in agar (6-8 stab times), a loopful of λ*cI*72 phage was applied as a vertical streak about a 1/3rd of the way across the plate. After flaming the loop and cooling it, λvir was applied as a parallel streak 2/3rd of the

way across the plate. The lysate solutions were allowed to dry on the agar surface by opening the lids slightly. After loop sterilization, a single colony of a cell was touched and was streaked (about 1 inch) across the dry streak of λ*c1*72. The steps were repeated for the dry streak of λvir. The plates were incubated at 30°C for 16 hours (or, overnight). For each single colony assayed, the above steps were repeated (Figure 3). After the incubation period, it was noted whether the streaked cells had been lysed by the phages.

Also, using a sterile loop, a single colony of the cell was touched and then the cell was streaked on two fresh TB plates (labeled 30°C and 42°C). These plates were then incubated at their labeled temperatures for 16 hrs (or, overnight). The growth of the cells at 30°C and 42°C (lysogens will be induced at 42°C and thus, no growth will be observed) were recorded.

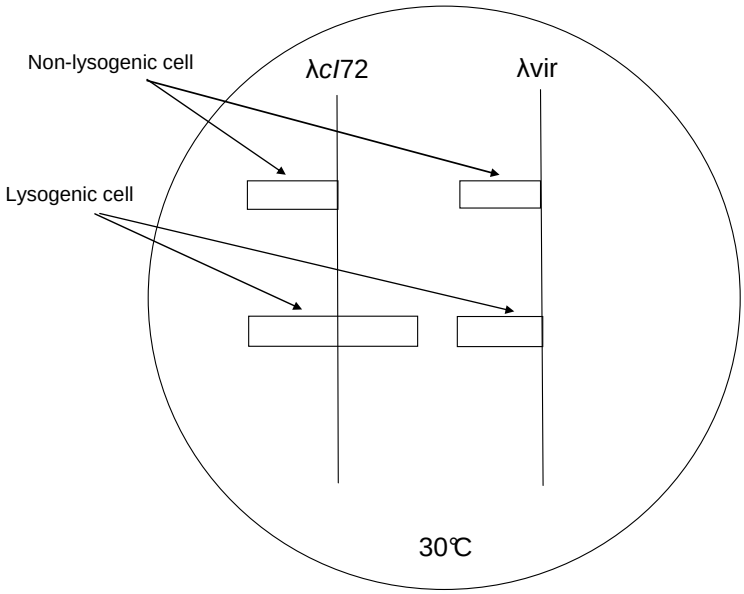


Figure 4. Cross Streaking Immunity Assay. The vertical lines represent streaks of λ*cI*72 and λvir phages. Phage λ*cI*72 lyses non-lysogenic cells. Lysogenic cells are immune to λ*cI*72. Both lysogens and non-lysogens are lysed by phage λvir.

2.3.9. Stab Assay for Auxotrophy and Plasmid Retention

Colonies arising on rich TB plates at 30°C were picked with sterile toothpicks and were stabbed onto minimal media plates. The stabbed minimal media plates were incubated at 30°C to check whether any spontaneous auxotrophs arose within the starting assay cells. Similarly, the colonies arising on rich TB plates at 42°C were picked with sterile toothpicks and stabbed onto two minimal media plates. One plate was incubated at 30°C and another at 42°C (to determine whether the mutations are temperature sensitive). In both the above cases, stabbing was also done on rich TB plates to ensure transfer of cells. The incubation time is 48 hours. Potential auxotrophs were picked from the TB control plate and streaked onto a minimal media plate for auxotrophy confirmation. Streaking was also done on a minimal media plate supplemented with biotin to find out if the mutants were biotin auxotrophs.

Note: Histidine was added to minimal media plates for Y836 *his*$^-$ and Y836 *his*$^-$ derived cells.

Colonies that arose on rich TB plates at 25°C, 30°C, or 37°C were stabbed onto TB+50μg/ml Amp plates using a sterile toothpick to test ampicillin resistance, i.e., evidence cells carry a plasmid. In the above case, stabbing was also done on rich TB plates to ensure transfer of cells. The plates were incubated at 25°C for 48-72 hours.

2.3.10. Replicative Killing Assay

RK^+ cells were tested for their ability to plate at equal efficiency on rich TB agar, minimal media agar supplemented with histidine (MM+his), and minimal media plates supplemented with casamino acid (MM+CA) at 30°C. At this temperature, the prophage genes remain repressed and therefore, there is no replicative killing of the cells. A late log phase or almost stationary phase culture was centrifuged in a Corex centrifuge tube at 5K for 5 minutes. The supernatant was discarded. The pellet was resuspended in 10 ml of Φ80 buffer and was re-centrifuged at 5K for 5 minutes. The rich media supernatant was discarded. The pellet was resuspended in 2 ml of Φ80 buffer (10^0 culture dilution). Then, the culture was diluted and plated on rich TB, minimal media plates supplemented with histidine and minimal media plates

supplemented with casamino acid at 10^{-7} and 10^{-8} plating dilutions. The plates were incubated at 30°C for 48 hrs. The titers obtained are used as a control for the next assay to find the extent of cell killing at 42°C.

For this assay, the same procedure (mentioned above) was followed using the overnight culture and plating was done at 10^{-1}, 10^{-2} and 10^{-3} plating dilutions. The plates were prewarmed for 2 hours and incubated at 42°C for 48 hours. The RK^- frequency and the extent of cell killing was found using the cell titer at 42°C and the control cell titer at 30°C.

2.3.11. Re-repression Assay

594[pcIpR-P-timm] cells were grown in TB containing ampicillin at a final concentration of 50μg/ml at 25°C for ~40-44 hours (to stationary phase). 2 ml of the cells was centrifuged at 6K for 6 minutes in a 15 ml Corex tube. The supernatant was discarded and the cell pellet was resuspended in 2 ml of Φ80 buffer. The diluted cells were plated at 25°C, 30°C and 37°C on TB plates. Incubation periods were ~48 hours for 30°C and 37°C plates, and ~72 hours for 25°C plates. There were 3 other sets of plates on which diluted cells were plated which were initially incubated at 37°C for 1 hour, 2 hours and 6 hours and then, were shifted to 30°C. Before shifting the plates to 30°C, the plates were cooled on ice. All the plates that were incubated at 37°C were prewarmed for ~3½ hours. The total incubation period for the plates that were incubated at 37°C for 1, 2 and 6 hours and then, were shifted to 30°C was ~48 hours.

The colonies that arose on rich TB plates at 25°C, 30°C, 37°C and 37°C plates shifted to 30°C were picked with sterile toothpicks and were stabbed onto 2 plates, namely, TB+Amp and TB plates. Then, these stabbed plates were incubated at 25°C for ~48 hours to test for plasmid retention.

2.3.12. Low Dose Inoculum Assay (LDIA)

A culture from a single colony of 594[pcIpR-P-timm] cells was grown (in TB supplemented with ampicillin at a final concentration of 50μg/ml) at 25°C in a shaking water bath. 0.5 ml of the stationary phase culture of 594[pcIpR-P-timm] cells was diluted to 10^{-7} culture dilution in Φ80 buffer. 0.1 ml aliquots from the 10^{-7} culture dilution (i.e., 10^{-8} plating dilution) were added to each of the 40 tubes containing 1 ml of TB. The diluted cells added to the tubes were also plated on TB plates which were incubated at 30°C or 25°C to find out the tentative number of cells added to each tube. All 40 tubes were allowed to shake at 25°C for 48 hours at a high speed until there was visible cell growth. All 40 tubes had growth to stationary phase. The tubes were vortexed well and 0.1 ml of the cells (10^{-1} plating dilution) from each tube was plated on two TB+100μg/ml Rif plates. One plate was incubated at 25°C and the other at 37°C. Cells from a few tubes were titered on TB plates at 25°C and 37°C and their average titer was used to determine the frequency of rifampicin resistance at 25°C and 37°C for all cultures. The 37°C plates were prewarmed for 2 hours. The plates at 37°C were incubated for 48 hours and the ones at 25°C were incubated for 72 hours.

For 594 cells, 0.5 ml of a stationary phase overnight culture was diluted to 10^{-7} culture dilution in Φ80 buffer. 0.1 ml aliquots from the 10^{-7} culture dilution (i.e., 10^{-8} plating dilution) were added to each of the 4 tubes containing 1 ml of TB. The diluted cells added to the tubes were also plated on two TB plates to find out the tentative number of cells added to each tube. One of the spread plates was incubated at 25°C and the other was incubated at 30°C for 48 hours. All 4 tubes were allowed to shake at 30°C for 48 hours until there was visible cell growth. All 4 tubes had growth to stationary phase. After vortexing each culture tube, 0.1 ml of the cells (10^{-1} plating dilution) from each of the 4 cultures was plated on two TB+100μg/ml Rif plates. One plate was incubated at 25°C and the other at 37°C. Two tubes having cell growth were titered on TB plates to determine the frequency of rifampicin resistance at 25°C and 37°C. The 37°C plates were prewarmed for 2 hours. All the plates were incubated for 48 hours.

2.3.13. Isolation of Independent Rifampicin Resistant Mutants

One rif^R mutant (that arose from a rif^S cell, 594[pcIpR-P-timm]) from each culture tube of Low Dose Inoculum Assay was streaked or stabbed on fresh TB+100μg/ml Rif plates at 25°C for confirmation of rifampicin resistance. Incubation period was 48-72 hours depending on cell growth. Some cells took more time to grow on fresh TB+Rif plates than others.

For 594 rif^R mutants, one rif^R mutant (that arose from wild-type 594 cells) from each culture tube was streaked for single colonies on a TB+100μg/ml Rif plate which was then, incubated at 25°C for 48 hours.

2.3.14. Phage Plating Technique

0.1 ml of the phage lysate was diluted in Φ80 buffer and was plated on a rich TB plate with 0.3 ml of the host cells and 3 ml of warm tryptone top agar. The plates were incubated overnight at 30°C or 37°C.

2.3.15. Cell Spreading Technique

A volume of the overnight culture was centrifuged at 6K for 6 minutes and the supernatant was discarded. The cell pellet was resuspended in the same volume of Φ80 buffer (10^0 culture dilution). The cells were then diluted and plated on media plates as per requirement.

2.3.16. Preparation of Competent Cells and Electroporation of DNA into *E. coli*

A single colony of 594[pSIM6] was picked and inoculated in 10 ml TB containing ampicillin at a final concentration of 50μg/ml. The culture was shaken at 30°C overnight. A small volume of the overnight culture of 594[pSIM6] cells was subcultured in a volume of SOB media + Mg and was shaken at ~30°C for ~4 hours to mid-log phase. An aliquot of the subculture was centrifuged at 6K for 6 minutes (1X wash) in a Corex tube. The supernatant was discarded and the cell pellet was resuspended in the same volume of Φ80 buffer. 0.1 ml of the

resuspended cells was plated on TB+Rif plates (10^{-1} plating dilution). The cells were also diluted and plated on TB and TB+Amp plates (10^{-7} plating dilution). This was done to determine the initial number of Amp^R 594[pSIM6] cells present in the subculture. The rest of the 594[pSIM6] subculture was transferred to a 42°C shaking water bath and was incubated for 15 minutes with slow shaking. The flask containing cells was then swirled in an ice bath and was allowed to sit in the ice bath for 5 minutes. 10 ml aliquot of the cells was taken into a cold 30 ml Corex tube. The cells were centrifuged at 7K for 7 minutes. The supernatant was discarded. 1 ml of ice cold 10% glycerol was added/tube to the cell pellet (obtained by centrifuging 10 ml cells). The mouths of the tubes were sealed with parafilm and the pellet was resuspended by rolling the tube gently. When the pellet was resuspended, an additional 10 ml of ice cold 10% glycerol was added/tube and the cells were mixed gently. The cells were centrifuged again at 7K for 7 minutes. The supernatant was discarded. 1 ml ice cold 10% glycerol was added/tube to the cell pellet. The mouths of the tubes were sealed with parafilm and the pellet was resuspended by rolling the tube gently. The samples were then pipetted into cold 1.5 ml eppendorf tubes (cooled to -20°C) and were centrifuged at 12K for 1 minute. The supernatant was discarded and the pellet was resuspended in 100µl final volume of ice cold 10% glycerol. The cells were mixed by finger tapping. The cells are then competent for electroporation. 50µl of the competent cells was mixed with 2.5µl of the *rpoB* DNA containing the mutation (actual *rpoB* PCR product sequenced was used). The cell-DNA mixture was pipetted into a cold electroporation cuvette (cooled to -20°C before use) and the cuvette was put into an electroporator and the mixture was pulsed. (Electroporator was warmed up for a while before use.) A blank was also pulsed which only had cells and no DNA (control). After pulsing, the TC and kV values were recorded for each electroporation. Immediately after pulsing, 1 ml of SOC media was added to the mixture in the cuvette and the whole mixture+SOC was immediately pipetted out into a 2 ml eppendorf tube (cooled to -20°C before use). The tube was immediately incubated in a 30°C shaking water bath and shaken gently at a very low speed for ~3 hours. Then, a small volume of electroporated mixture was pipetted into an eppendorf tube and centrifuged at 12K for 2 minutes. The supernatant was discarded and the cell pellet was resuspended in the same volume of Φ80 buffer (10^0 culture dilution). This step was done to remove the rich SOC media. Plating was done on TB+100µg/ml Rif selection plates (blank was also plated). TB plating for blank and at least one strain were done as controls. Incubation of all plates was done at 30°C for ~48-62 hours.

2.3.17. P Complementation Assay

The phage λimm434*P*am3 has an amber mutation in *P* and therefore, it only grows on host cells which can complement for P or on a nonsense suppressor strain [a] like TC600. 594 has been used as a negative control for this assay while suppressor strain, TC600 has been used as a positive control.

0.1 ml of the λimm434*P*am3 phage lysate was diluted in Φ80 buffer and was plated on a rich TB plate with 0.3 ml of the host cells and 3 ml of warm tryptone top agar. The plates were incubated overnight at 37°C or 30°C (as per requirement). For determining P expression from plasmid containing cells, the cells were grown in TB containing ampicillin at a final concentration of ~50μg/ml in a ~25°C shaking water bath for ~48 hours.

[a] Nonsense suppressor strain: A bacterial strain / cell which has a mutation within the anticodon of a normal tRNA, enabling the mutated tRNA to recognize and insert an amino acid at a stop codon.

3. Results

3.1. P-killing and the involvement of host DnaB helicase

Viability of *E. coli* cells upon λP expression was studied in this work. The involvement of host DnaB helicase in P-killing was not clear. λP is known to outcompete host DnaC for binding to DnaB helicase and this hijacking of the *E. coli* replication machinery by bacteriophage λ might explain cell killing upon P expression. In order to study cell killing upon P expression, the following P-expression systems were employed:

a) P-expression from a cryptic λ prophage:

Upon shifting cells containing the cryptic λ prophage and expressing wild-type P from 30°C to 42°C, cells were killed at a high frequency (Figure 5 and Supplemental Table 2). Y836, Y836 *his*$^{+}$, 594::(*cIII-ren*)$^{\lambda}$ and Y836 ilr *O*223a*P*$^{+}$ are wild-type P-expressing strains which all exhibited cell killing at 42°C. Insertional inactivation of *P* in Y836 *O*$^{+}$*P*::*Kan* cells and IS2 insertion in *P* in Y836 ilr *O*$^{+}$*P*::IS2 cells suppressed cell killing at 42°C which suggested that some level of P-expression was toxic/lethal to the cells. GrpD55 mutations in DnaB suppressed cell killing in spite of P being expressed from the cryptic λ prophage. This suggested that P-DnaB interaction was necessary for P-killing.

Figure 5. Cell survival at 42°C w.r.t. 30°C

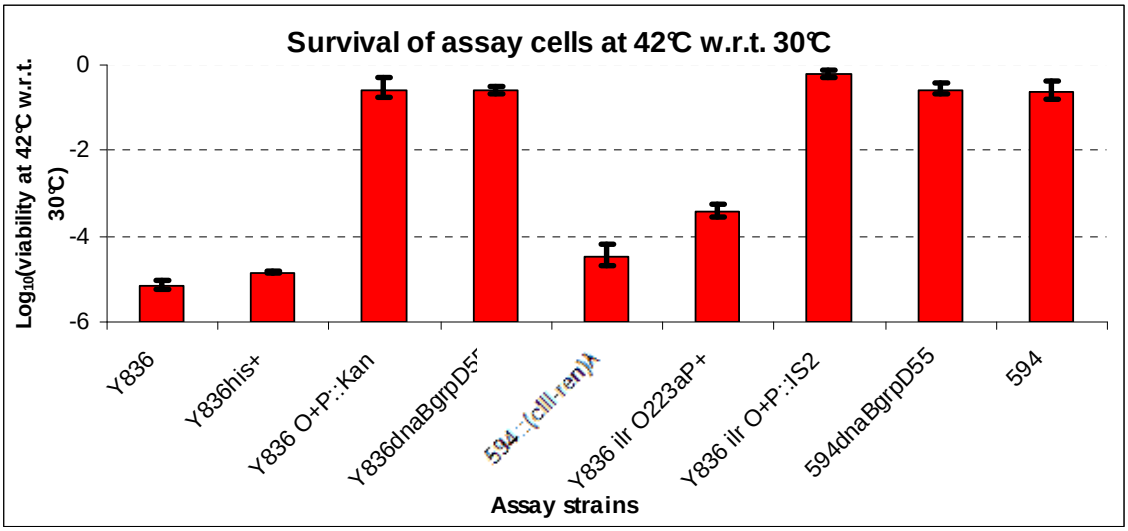


Figure 5. Cell survival at 42°C w.r.t. 30°C. Assay cell cultures were washed and the cell pellets were resuspended in Φ80 buffer. The cells were diluted and plated on rich TB plates in duplicates that were incubated at 30°C and 42°C. Viability at 42°C w.r.t. 30°C = Cell titer on TB at 42°C / Cell titer on TB at 30°C. [See actual data in Supplemental Table 2.]

b) P expression from a plasmid:

594[pcIpR-P-timm] cells were killed by an approximately 200 fold when shifted from 25°C to 37°C (Figure 6 and Supplemental Table 3). At 37°C, only trace amounts of P was expected to be expressed from the plasmid because there was leaky expression of P at 37°C even when the CI repressor was active. This was probably because the plasmid system used had only the O_R operator and therefore, the P-expression system was not tight. The repressor, CI functions tightly when both O_L and O_R operators are present (Ptashne *et al.*, 1980). Also, unpublished data from our laboratory showed that phage λ*cI*72 did not grow on a 594 strain containing a P-expression plasmid under a *cI*[Ts]857 repressor (594[pcIpR-P-timm] cells) at 37°C which suggested that the repressor, CI was still active at 37°C. GrpD55 mutations in DnaB suppressed the cell killing phenotype which suggested that P-DnaB interaction was essential for P-killing.

Figure 6. Cell survival at 37°C and 30°C w.r.t. 25°C

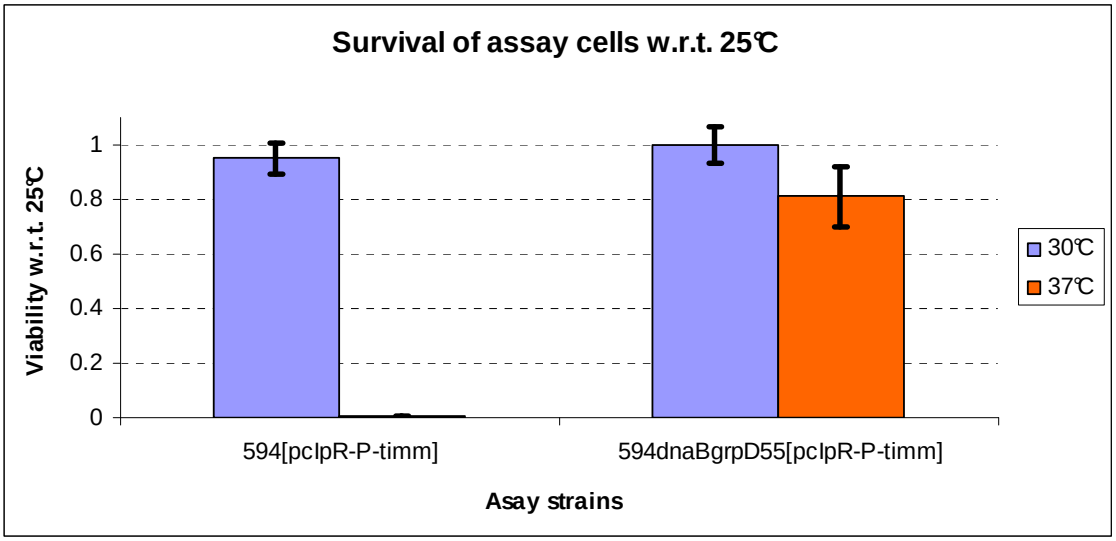


Figure 6. Cell survival at 37°C and 30°C w.r.t. 25°C. Assay cells were washed and the cell pellets were resuspended in Φ80 buffer and were diluted. The diluted cells were plated on rich TB plates that were incubated at 25°C, 30°C and 37°C. Viability at 30°C or 37°C w.r.t. 25°C = Cell titer on TB at 30°C or 37°C / Cell titer on TB at 25°C. [See actual data in Supplemental Table 3.]

3.2. Determining whether the cells can recover, i.e., start growing when P is removed from the cells

Host cells are killed upon P expression (Section 3.1). It was asked whether P inhibits cells from growing or does P kill the cells. 2 ml of 594[pcIpR-P-timm] stationary phase cells were centrifuged at 6K for 6 minutes in a Corex tube and the cell pellet was resuspended in the same volume of Φ80 buffer. The diluted cells were plated at 25°C, 30°C and 37°C on TB plates. Incubation period was ~48 hours for 30°C and 37°C plates and ~72 hours for 25°C plates. There were 3 other sets of plates on which diluted cells were plated which were initially incubated at 37°C for 1 hour, 2 hours and 6 hours and then, were shifted to 30°C. Plasmid retention was determined by stab assay of the colonies arising on rich TB plates at 25°C, 30°C, 37°C and 37°C plates shifted to 30°C. Upon shifting the 594[pcIpR-P-timm] cells from 37°C to 30°C after 1 or 2

hours, the cells were able to recover from the P-stress and start to grow again. 6 hours of cellular exposure to P and then shifting cells back to 30°C decreased viability by 150-fold and plasmid retention by about half (Figures 7 and 8 and Supplemental Table 4). So, it can be inferred that after a certain period of cellular exposure to P, cells are not able to grow even if they are shifted back to a permissive temperature. But, shorter P exposures prevent cell growth, but upon shifting to 30°C, cell starts growing again. Hence, P-lethality can be renamed as P-inhibition (Horbay, 2005).

Figure 7. Re-repression (of P) assay – analysis of viability

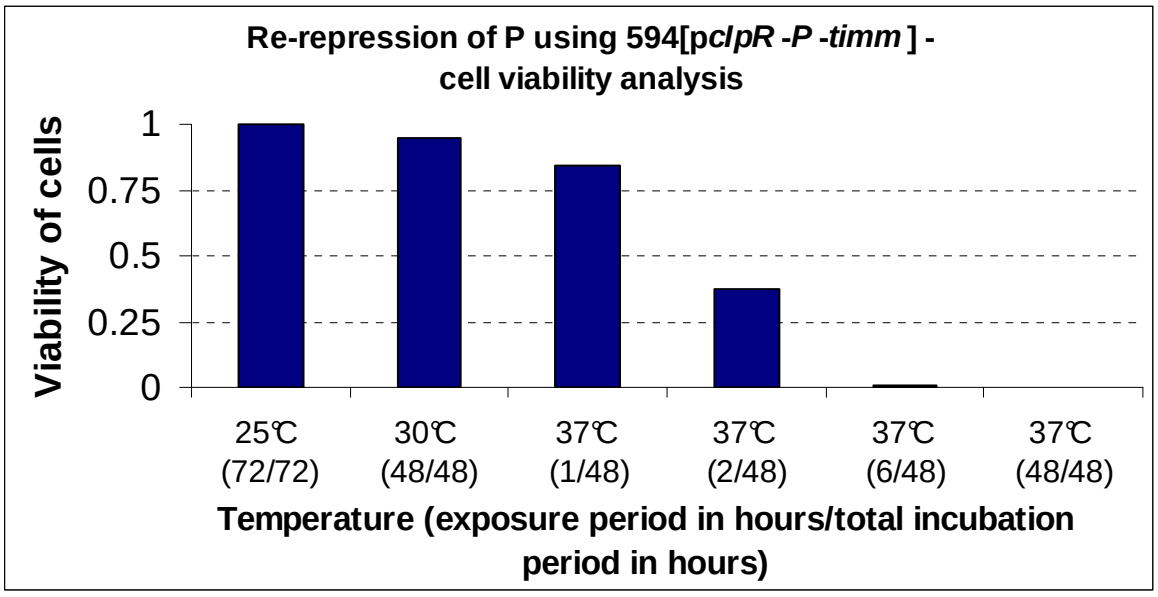


Figure 7. Re-repression (of P) assay – analysis of viability. A volume of stationary phase cells of 594[pcIpR-P-timm] were washed and the cell pellet was resuspended in the same volume of Φ80 buffer. The diluted cells were plated on TB plates that were incubated at 25°C, 30°C and 37°C (controls). 3 sets of plates were also plated with the same cells at 37°C which after 1, 2 and 6 hours, respectively were cooled on ice for a while and were then shifted back to 30°C. Viability on rich TB plates was noted after the incubation period. Viability of cells at 25°C, 30°C or 37°C = Cell titer (cfu/ml) at 25°C, 30°C or 37°C / Cell titer (cfu/ml) at 25°C. Viability of cells after shifting 37°C plates to 30°C after 1 or 2 or 6 hours = Cell titer (cfu/ml) after shifting 37°C plates

to 30°C after 1 or 2 or 6 hours / Cell titer (cfu/ml) at 25°C. [See actual data in Supplemental Table 4.]

Figure 8. Re-repression (of P) assay – analysis of plasmid retention

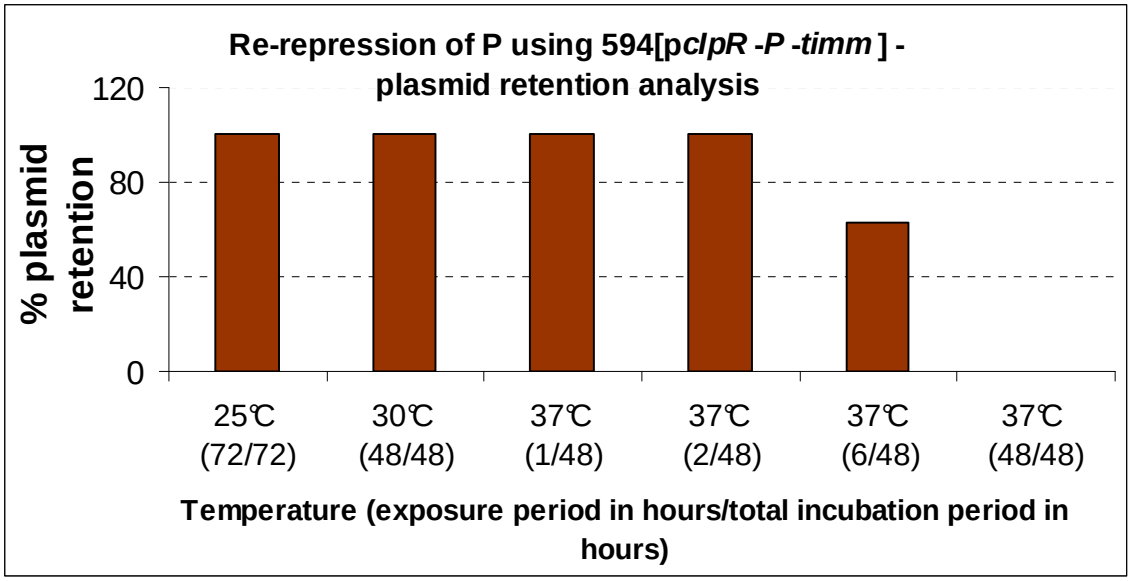


Figure 8. Re-repression (of P) assay – analysis of plasmid retention. A volume of stationary phase cells of 594[pcIpR-P-timm] were washed and the cell pellet was resuspended in the same volume of Φ80 buffer. The diluted cells were plated on TB plates that were incubated at 25°C, 30°C and 37°C (controls). 3 sets of plates were also plated with the same cells at 37°C which after 1, 2 and 6 hours, respectively were cooled on ice for a while and were then shifted to 30°C. Cfu obtained at different temperatures on rich TB plates were picked with a sterile toothpick and stabbed onto a rich TB plate supplemented with ampicillin at a final concentration of 50μg/ml and also on a TB control plate. Incubation of the stabbed plates was done at 25°C. % plasmid retention among cfu that arose at a particular temperature = (Number of cfu that were able to grow on TB+Amp plates / Total number of cfu stabbed) x 100. [See actual data in Supplemental Table 4.]

3.3. Preliminary results of Chu (Chu, 2005) that were reproduced in this work

3.3.1. Efficiencies of plating of starting RK^+ cells at 30°C and rare RK^- mutants at 42°C on rich TB and minimal media (MM) agar plates

Chu showed that auxotrophs arise at a very high frequency among the RK^- mutants and the auxotroph formation is linked to λ gene expression (Chu, 2005). Y836 cells containing the cryptic λ prophage are Replicative Killing competent (RK^+) cells. Y836 *his*$^+$ transductants were constructed by transducing the *his*$^+$ allele into Y836 cells (Chu, 2005). The cryptic λ prophage was transduced into wild-type 594 cells (Chu, 2005) and the resultant λ^+ transductants, i.e., 594::(*cIII-ren*)$^\lambda$ were also RK^+ cells. Upon cryptic λ prophage induction in Y836, Y836 *his*$^+$ and 594::(*cIII-ren*)$^\lambda$, RK^- mutants arose on minimal media (MM) agar plates at a lower frequency than on rich TB plates (Table 2). This was assessed by comparing the cell titers on MM and rich TB plates at 42°C after centrifuging the cultures at 6K for 6 minutes and resuspending the cell pellet in the same volume of Φ80 buffer. The resuspended cells were diluted and plated on rich TB and MM plates at 30°C and 42°C. Table 1 shows control 30°C cell titer data, and the titers of the starting cells were roughly the same on both MM and TB plates at 30°C. This assay is of utmost importance because the current study depends on the validity of the results obtained by Chu (Chu, 2005). Hence, these assays were reproduced and as expected, they yielded the same results (Tables 1 and 2).

Table 1. Efficiency of plating of assay strains on rich (TB) and minimal medium (MM) at 30°C

Strain	Experiment #	Titer on TB at 30°C [a]	Titer on MM at 30°C [a]	Titer on TB / Titer on MM	Mean ± Standard error
Y836 *his*$^-$ (MM+his plates used)	1	2.6×10^8	7.4×10^8	0.35	0.7 ± 0.35 [b]
	2	1.71×10^9	1.64×10^9	1.04	
Y836 *his*$^+$	1	1.5×10^9	1.5×10^9	1	1
594::(*cIII-ren*)$^\lambda$	1	1.6×10^9	1.2×10^9	1.33	1.06 ± 0.101
	2	1.6×10^9	1.5×10^9	1.07	
	3	5.6×10^8	5.7×10^8	0.98	
	4	1.4×10^9	1.64×10^9	0.85	
594	1	1.2×10^9	1.3×10^9	0.92	1.08

[a] Assay cells were plated on rich TB and MM (or, MM+his for Y836 *his*$^-$ cells) agar plates at 30°C and the cell titers on the above plates were determined.

[b] Standard error has been calculated by taking 2 sets of data into consideration.

Table 2. Frequency at which RK$^-$ mutants arose on rich TB and MM agar plates at 42°C

Strain	Experiment #	RK$^-$ frequency at 42°C [a]		Frequency on TB / Frequency on MM	Mean ± Standard error
		TB	MM		
Y836 *his*$^-$ (MM+his plates used)	1	1.69×10^{-5}	8.65×10^{-7}	20	12.7 ± 2.7
	2	1.12×10^{-5}	7.3×10^{-7}	15	
	3	1.35×10^{-5}	8.65×10^{-7}	16	
	4	1.65×10^{-5}	1.05×10^{-6}	16	
	5	8.71×10^{-7}	2.47×10^{-7}	4	
	6	1.27×10^{-6}	2.41×10^{-7}	5	
Y836 *his*$^+$	1	1.27×10^{-5}	1.27×10^{-6}	10	8.5 ± 1.2
	2	1.67×10^{-5}	2.53×10^{-6}	7	
	3	1.47×10^{-5}	2.47×10^{-6}	6	
	4	1.33×10^{-5}	1.2×10^{-6}	11	

[a] Assay cells were plated on rich TB and MM agar plates at 42°C. Survivors at 42°C were designated as RK$^-$ mutants. RK$^-$ frequency on rich TB plates = Titer on TB at 42°C / Titer on TB at 30°C and RK$^-$ frequency on MM plates = Titer on MM at 42°C / Titer on MM at 30°C.

3.3.2. Assessing if the inclusion of Casamino acids (not vitamin free) in MM agar suppresses the drop in RK^- frequency

On addition of Casamino acids (not vitamin free) to minimal medium, the drop in cell titer at 42°C was suppressed which suggested that nutrient gene mutations might be present among the rare RK^- survivors (Tables 3 and 4).

Table 3. Efficiency of plating of RK^+ culture cells on rich TB and MM+CA at 30°C (Control for Table 4)

Strain	Experiment #	Titer on TB at 30°C [a]	Titer on MM+CA at 30°C [a]	Titer on TB / Titer on MM+CA	Mean ± Standard error
Y836 *his*$^-$	1	6×10^8	5.5×10^{8} [b]	1.09	1.02 ± 0.075 [d]
	2	1.5×10^9	1.6×10^{9} [c]	0.94	

[a] Y836*his*$^-$ cells were plated on rich TB and MM+CA agar plates at 30°C and the cell titers on the above plates were determined.

[b] Used 1% final concentration of Casamino acid (not vitamin free).

[c] Used 0.3% final concentration of Casamino acid (not vitamin free).

[d] Standard error has been calculated by taking 2 sets of data into consideration.

The difference between Table 2 and Table 4 is that in Table 2, the RK^- frequency on MM is lower than that on TB plates at 42°C whereas in Table 4, the RK^- frequency on TB is lower than that on MM+CA at 42°C which indicates that MM+CA recovers more RK^- mutants than TB.

Table 4. The recovery of RK$^-$ mutants is enhanced on MM by the addition of CA

Strain	Experiment #	RK$^-$ frequency at 42°C [a]		Frequency on TB / Frequency on MM+CA	Mean ± Standard error
		TB	MM+CA		
Y836 *his*$^-$	1	6.33×10^{-6}	1.24×10^{-5} [b]	0.51	0.59 ± 0.08
	2	4.5×10^{-6}	8.91×10^{-6} [b]	0.51	
	3	3.87×10^{-6}	5.13×10^{-6} [c]	0.75	

[a] Y836 *his*$^-$ cells were plated on rich TB and MM+CA agar plates at 42°C. RK$^-$ frequency on rich TB plates = Titer on TB at 42°C / Titer on TB at 30°C and RK$^-$ frequency on MM+CA plates = Titer on MM+CA at 42°C / Titer on MM+CA at 30°C.

[b] Used 1% final concentration of Casamino acid (not vitamin free).

[c] Used 0.3% final concentration of Casamino acid (not vitamin free).

3.3.3. Important controls established by Chu (Chu, 2005)

Chu found that Y836 did not have intrinsic mutator activity. It was also ruled out that the drop in RK$^-$ frequency on MM relative to rich TB was linked to the *bio* operon (due to deletion in λ fragment). In other words, addition of biotin to minimal medium did not suppress the drop in RK$^-$ frequency. Also, the drop in RK$^-$ frequency and the appearance of auxotrophs among the RK$^-$ mutants was not linked to the SOS gene products (Chu, 2005).

Chu showed that pcI$^+$ plasmid expressing wild-type CI repressor at 30°C and 42°C completely suppressed replicative killing in Y836, Y836 *his*$^+$, 594::(*cIII-ren*)$^\lambda$ and W3101::(*cIII-ren*)$^\lambda$ cells (Chu, 2005). This suggested that the cell killing was linked to λ gene expression. Hayes *et al.* reported that grpD55 mutations (2 missense mutations) in host *dnaB* gene suppressed λ replication initiation from *oriλ* (Hayes *et al.*, 2005). Moreover, grpD55 mutations completely suppressed replicative killing (or, RK phenotype) (Chu, 2005). But, grpD55 mutations did not completely suppress the drop in RK$^-$ frequency on minimal media relative to rich media which suggested that "DNA lesions preventing colony formation at 42°C likely appear within the population of Y836*dnaB*grpD55 cells induced for λ gene expression" (Chu, 2005). In other words, Chu concluded that auxotroph formation was not linked to replicative killing or, auxotroph formation did not require actual replication initiation from *oriλ* (Chu, 2005). λ gene expression triggered auxotroph formation (Chu, 2005). Auxotrophy was a very broad target for mutagenesis. A small target, rifampicin resistance was also briefly studied by Chu and similar links to λ gene expression were found (Chu, 2005). Chu showed that auxotroph formation was linked to λ *P* gene expression from a cryptic λ prophage (Chu, 2005). Since some RK$^-$ strains used by Chu were not sequenced (for λ genes *O* and *P*) then, the assay of the strains had to be repeated and confirmed. Also, further work was necessary to ascertain whether the λ gene product P could influence the apparent mutator phenotype, which we define as ancillary mutations within *E. coli* chromosome that arise during selection at 42°C for RK$^-$ mutants.

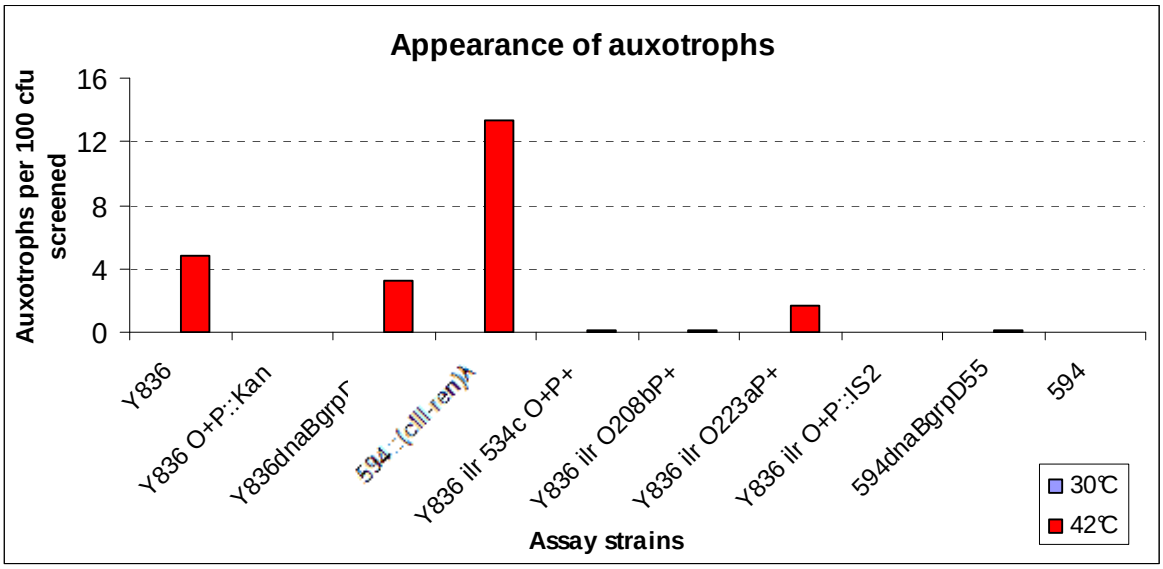
3.4. Determining whether λ gene product(s) is/are responsible for triggering auxotrophic mutations

Chu linked formation of auxotrophs with λ *P* gene expression from a cryptic λ prophage (Chu, 2005). In this study, actual colony forming units (cfu) arising on rich TB plates were picked with sterile toothpicks and stabbed onto minimal media plates for auxotrophy confirmation. This is a better way of looking for auxotrophs as each individual colony can be analysed. Stab assays of Y836, 594::(*cIII-ren*)$^\lambda$ and Y836*dnaB*grpD55 were done by Chu (Chu, 2005) and we repeated these assays for these strains in Figure 9 and Supplemental Table 5 for

confirmation. The results were similar to those of Chu (Chu, 2005). GrpD55 mutations in *dnaB* gene suppressed auxotroph formation to some extent (Figure 9 and Supplemental Table 5).

In this study, four isolated and sequenced Replicative Killing defective (RK^-) mutants, and one Y836 mutant with a kanamycin insertion in *P* (constructed by recombineering by S. Hayes) were employed. The RK^- mutants, Y836 ilr *O*208b P^+ and Y836 ilr *O*223a P^+ have spontaneous mutations in O-origin site that prevent λ replication initiation. Y836 ilr O^+P::IS2 RK^- mutant has a spontaneous IS2 insertion in *P*. Y836 ilr 534c O^+P^+ strain is RK^- but sequenced as O^+P^+, suggesting that the mutation preventing λ replication initiation is in a chromosomal gene, possibly *dnaA* or in another λ gene. Since Y836 ilr 534c O^+P^+ strain complemented for P, i.e., λimm434*P*am3 phage grew on this strain, it was inferred that in Y836 ilr 534c O^+P^+ cells, all host genes required for λ replication were functioning properly. Upon shifting a Y836 strain with a kanamycin insertion in *P* (Y836 O^+P::*Kan*) from 30°C to 42°C, no auxotrophs arose among the 42°C cfu. Moreover, the isolated and sequenced RK^- mutants, Y836 ilr *O*208b P^+, Y836 ilr *O*223a P^+ and Y836 ilr 534c O^+P^+ yielded auxotrophs at 42°C unlike the P^- strain, Y836 ilr O^+P::IS2 (Figure 9 and Supplemental Table 5). These results agreed with Chu's findings (Chu, 2005) that the appearance of auxotrophs was linked to λ *P* gene expression.

Figure 9. Stab assay to look for auxotrophs among the 42°C cfu



Fold cell killing at 42°C	$9x10^5$	153	2668 [a]	10^5	0	0	4762	0	7	6

Figure 9. Stab assay to look for auxotrophs among the 42°C cfu. Cfu that arose on rich tryptone agar plates at 42°C were picked with a sterile toothpick and stabbed to duplicate MM or MM+his plates incubated at 30°C and 42°C and a control rich TB agar plate incubated at 30°C. Colonies that were not able to grow on MM or MM+his agar plate at either 30°C or 42°C were auxotrophs. Chu (Chu, 2005) and this study identified some of the auxotrophs to be Ts conditional auxotrophs. [See actual data in Supplemental Table 5.] When shifted from 30°C to 42°C, Y836 cells underwent $9x10^5$-fold cell killing, Y836 O^+P::*Kan* cells underwent 153-fold cell killing, Y836*dnaB*grpD55 cells underwent 2668-fold cell killing, 594::(*cIII-ren*)$^{\lambda}$ cells underwent 10^5-fold cell killing, Y836 ilr *O*223a P^+ cells underwent 4762-fold cell killing, 594*dnaB*grpD55 cells underwent 7-fold cell killing, 594 cells underwent 6-fold cell killing and no cell killing was observed in Y836 ilr *O*208b P^+, Y836 ilr 534c O^+P^+ and Y836 ilr O^+P::IS2 cells on rich TB plates.

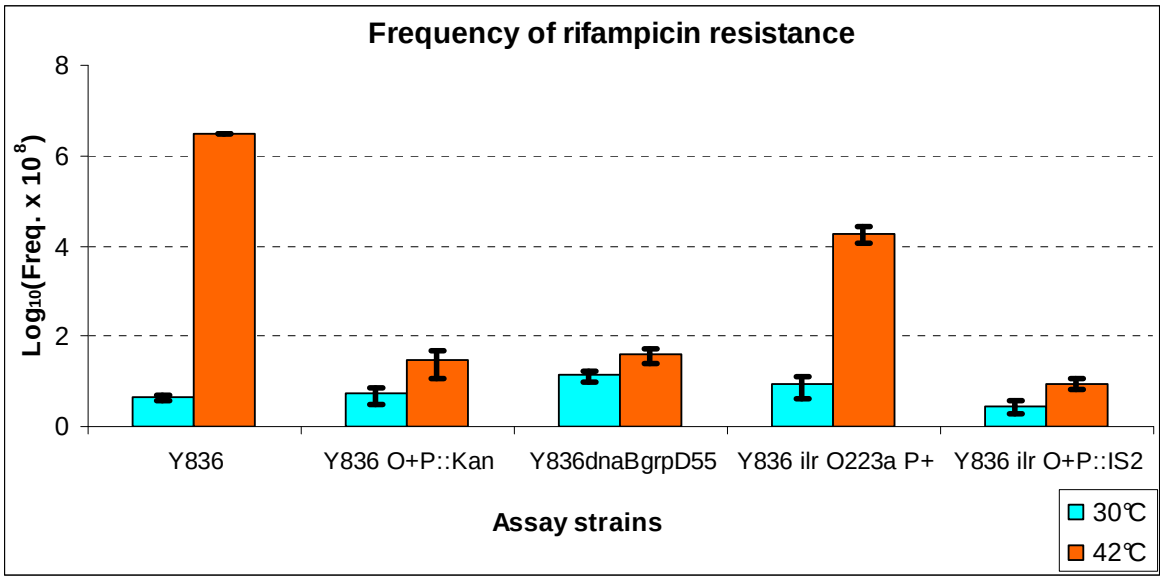
[a] In these assays, Y836*dnaB*grpD55 cells underwent cell killing when shifted from 30°C to 42°C for some unknown reasons. Other assays done with the same strain (data shown in Figures 5 and 10) did not undergo any cell killing when shifted from 30°C to 42°C which agreed with Chu's findings (Chu, 2005). Also, 594*dnaB*grpD55[pcIpR-P-timm] cells did not undergo cell killing when shifted from 25°C to 37°C (data shown in Figures 6 and 11) which proved that grpD55 mutations in DnaB knocked out P-killing.

3.5. Assessing whether the apparent mutator activity can be demonstrated using another target and whether host DnaB is required for the potential mutator activity

To assess whether the apparent mutator activity can be demonstrated by employing an independent selection (other than screening for auxotrophs), rifampicin resistance was employed as another possible target for potential mutator activity. Unlike auxotrophy, rifampicin resistance is a narrow target and is conferred by *rpoB* gene mutations. Chu showed that Y836 (O^+P^+) exhibited a high frequency of rifampicin resistance at 42°C when compared to 30°C (Chu, 2005). Chu also showed that grpD55 mutations in *dnaB* gene suppressed the increase in rifampicin resistance frequency (Chu, 2005). The same results were reproduced in this work for confirmation (Figure 10 and Supplemental Table 6).

As a part of this work, it was found that insertional inactivation of *P* in Y836 O^+P::*Kan* cells suppressed rifampicin resistance frequency by approximately 10^5-folds at 42°C in comparison to Y836 P^+ cells. Sequenced RK^- mutant, Y836 ilr *O*223a P^+ showed an approximately 2000-fold increase in rifampicin resistance frequency from 30°C to 42°C. In a comparison between Y836 ilr *O*223a P^+ and Y836 ilr O^+P::IS2 cells, the inactivation of *P* by an IS2 insertion resulted in an approximately 2000 fold drop in rifampicin resistance frequency at 42°C (Figure 10 and Supplemental Table 6). It was inferred that the increase in the frequency of rifampicin resistance is linked to λP expression.

Figure 10. Induction assay of cells for determining rifampicin resistance frequency at 42°C vs. 30°C



Cell killing at 42°C	+	-	-	+	-

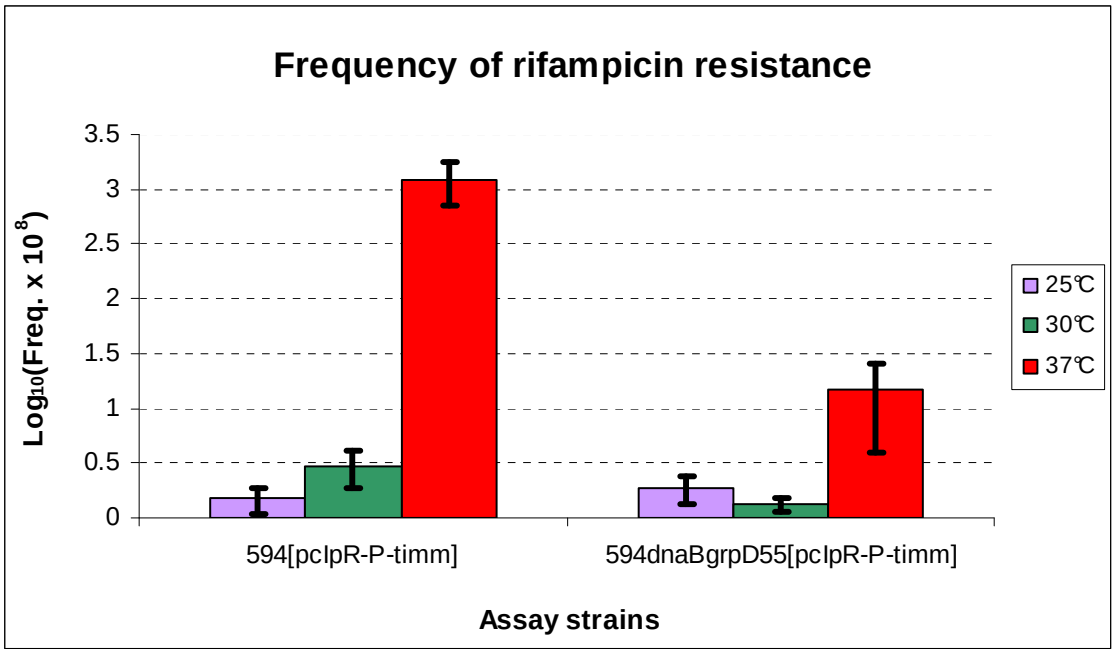
Figure 10. Induction assay of cells for determining rifampicin resistance frequency at 42°C vs. 30°C. A volume of late-log to stationary phase cells were washed once and the cell pellet was resuspended in the same volume of Φ80 buffer. The diluted cells were plated on rich tryptone agar plates and tryptone agar plates supplemented with rifampicin at a final concentration of 100μg/ml at 30°C and 42°C for 48 hours. The 42°C plates were prewarmed for ~2 hours. Frequency of rifampicin resistance at 30°C = Number of rif^R cfu/ml on TB+Rif plates at 30°C / Number of cells (cfu/ml) on TB plates at 30°C. Frequency of rifampicin resistance at 42°C = Number of rif^R cfu/ml on TB+Rif plates at 42°C / Number of cells (cfu/ml) on TB plates at 42°C. [See actual data in Supplemental Table 6.] When shifted from 30°C to 42°C, Y836 cells underwent $4x10^5$-fold cell killing, Y836 *O*$^+$*P*::*Kan* cells underwent 7-fold cell killing, Y836*dnaB*grpD55 cells underwent 5-fold cell killing, Y836 ilr *O*223a *P*$^+$ cells underwent $2x10^4$-fold cell killing and Y836 ilr *O*$^+$*P*::IS2 cells underwent 3-fold cell killing on rich TB plates.

3.6. Determining if P expression from a plasmid exhibits the potential mutator activity

It was asked whether P expression from a plasmid exhibited the potential mutator activity. This was a much more accurate way to pinpoint P and investigate whether P was the cause of the apparent mutator activity as the plasmid only expressed P under a *cI*[Ts] repressor.

A volume of each of the stationary phase cells of 594[pcIpR-P-timm] and 594*dnaB*grpD55[pcIpR-P-timm] were centrifuged and the cell pellets were resuspended in Φ80 buffer. Serial dilutions of the cells were performed and were plated on TB+100μg/ml Rif and control TB plates at 25°C, 30°C and 37°C for 48 hours. It was found that 594[pcIpR-P-timm] showed an approximately 800-fold increase in rifampicin resistance frequency at 37°C (in comparison to 25°C) (Figure 11). This suggested that the increase in rifampicin resistance frequency is linked to λP expression. GrpD55 mutations in host *dnaB* gene suppressed rifampicin resistance frequency at 37°C by approximately 80-fold in comparison to wild-type 594[pcIpR-P-timm] cells (Figure 11 and Supplemental Table 7) which suggested that P-DnaB interaction was required for the potential mutator activity.

Figure 11. Induction assay of pcIpR-P-timm transformants of wild-type 594 and 594*dnaB*grpD55 cells for determining rifampicin resistance frequency at 25°C, 30°C and 37°C



Cell killing at 30°C & 37°C	-	+	-	-

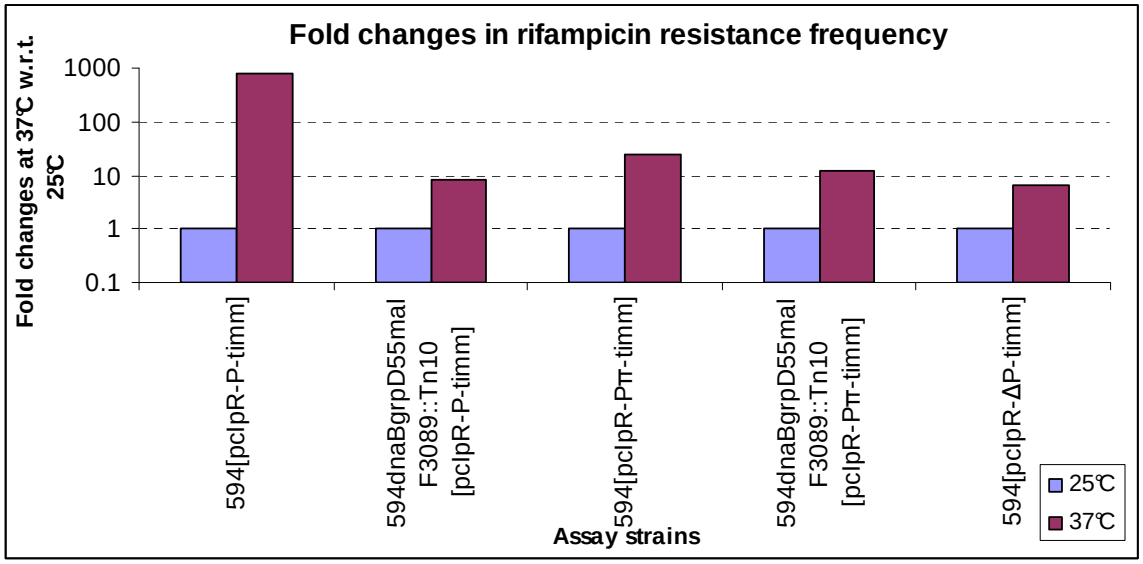
Figure 11. Induction assay of pcIpR-P-timm transformants of wild-type 594 and 594*dnaB*grpD55 cells for determining rifampicin resistance frequency at 25°C, 30°C and 37°C. A volume of stationary phase cells were washed once and the pellet was resuspended in the same volume of Φ80 buffer. (For 594[pcIpR-P-timm] cells, 5X concentrated cells were used and so, 5 ml cells were washed once and the cell pellet was resuspended in 1 ml of Φ80 buffer.) The diluted cells were plated on rich tryptone agar plates and tryptone agar plates supplemented with rifampicin at a final concentration of 100μg/ml at 25°C, 30°C and 37°C for 48 hours. The 37°C plates were prewarmed for ~2 hours. Frequency of rifampicin resistance at 25°C, 30°C or 37°C = Number of rif^R cfu/ml on TB+Rif plates at indicated temperature / Number of cells (cfu/ml) on TB plates at the same indicated temperature, i.e., either 25°C, 30°C or 37°C. [See actual data in Supplemental Table 7.] When shifted from 25°C to 30°C, no cell killing was observed in 594[pcIpR-P-timm] and 594*dnaB*grpD55[pcIpR-P-timm] cells on rich TB plates. When shifted from 25°C to 37°C,

594[pcIpR-P-timm] cells underwent 624-fold cell killing and no cell killing was observed in 594*dnaB*grpD55[pcIpR-P-timm] cells on rich TB plates.

3.7. Is P-DnaB interaction essential for the apparent mutator activity?

GrpD55 mutations are 2 missense mutations in host DnaB helicase protein. *E. coli* is able to use DnaBgrpD55 for its replication. But, phage λ does not grow on a grpD55 cell. Mutations in λ gene *P* allow the phage to grow on groP$^-$ mutants. These compensatory mutations in *P* are referred to as π mutations (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977; Georgopoulos, 1977; Sunshine *et al.*, 1977) (Figure 12 and Supplemental Table 8). When wild-type gene *P* was replaced by a deletion in *P* (Δ*P*) or a π mutation in *P* (P_π) in a 594 or a 594*dnaB*grpD55 cell, the increase in rifampicin resistance frequency from 25°C to 37°C was greatly reduced. This suggested that P-DnaB interaction was necessary for the potential mutator activity observed.

Figure 12. Induction assay of P, ΔP and P_π-expression plasmid transformants of wild-type 594 and 594*dnaB*grpD55 cells for determining rifampicin resistance frequency at 25°C, 30°C and 37°C



Fold cell killing at 37°C	624	0	21	3	22

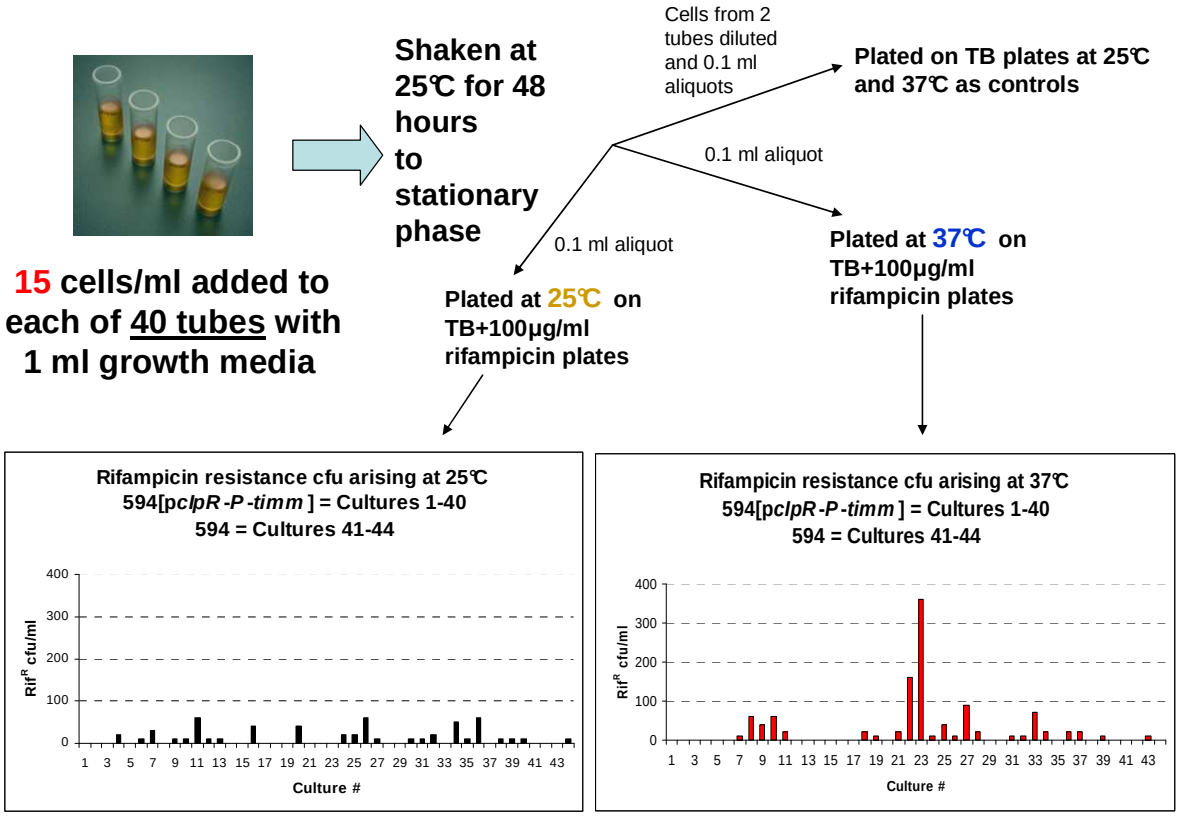
Figure 12. Induction assay of P, ΔP and P_π-expression plasmid transformants of wild-type 594 and 594*dnaB*grpD55 cells for determining rifampicin resistance frequency at 25°C, 30°C and 37°C. A volume of late-log to stationary phase cells were washed once and the pellet was resuspended in Φ80 buffer. The diluted cells were plated on rich tryptone agar plates and tryptone agar plates supplemented with rifampicin at a final concentration of 100μg/ml at 25°C, 30°C and 37°C for 48-72 hours. The 37°C plates were prewarmed for ~3½-4 hours. Fold changes in frequency of rifampicin resistance at 37°C w.r.t. 25°C = Frequency of rif^R at 37°C / Frequency of rif^R at 25°C. [See actual data in Supplemental Tables 8, 9 and 10.] When shifted from 25°C to 37°C, 594[pcIpR-P-timm] cells underwent 624-fold cell killing, 594[pcIpR-P_π-timm] cells underwent 21-fold cell killing, 594[pcIpR-ΔP-timm] cells underwent 22-fold cell killing, 594*dnaB*grpD55[pcIpR-P_π-timm] cells underwent 3-fold cell killing and no cell killing was observed in 594*dnaB*grpD55[pcIpR-P-timm] cells on rich TB plates.

3.8. Studying the variation in the appearance of rif^R mutants in presence and absence of P expression via Low Dose Inoculum Assay (LDIA)

The data in Figure 11 suggests that the increase in rifampicin resistant frequency from 25°C to 37°C in 594[pcIpR-P-timm] cells is linked to λP expression. Since 594[pcIpR-P-timm] cells were killed at 37°C, it was asked whether the appearance of rifampicin resistant mutants was stimulated by P or whether it was just an enrichment of pre-existing mutations. To test this, it had to be determined whether the actual number of rif^R cfu that arose at 37°C was higher than those that arose at 25°C. We used Low Dose Inoculum Assay (LDIA) which involved the assay of many individual cultures prepared from a few cells. In order to study the variation in the appearance of rifampicin resistant mutants in presence of P, 15 cells of 594[pcIpR-P-timm] were added to each of 40 tubes containing 1 ml TB and all 40 inoculated cultures were grown at 25°C for 48 hours to stationary phase. 0.1 ml cell aliquots from the 40 individual tubes were plated on two rich TB agar supplemented with rifampicin at a final concentration of 100μg/ml. One plate was incubated at 25°C and the other at 37°C. With only 15 cells added per 1 ml of TB, the chance of 1 cell out of 15 being a rif^R mutant is very low, about 1:10-100 million, as the spontaneous rif^R frequency is to the order of 10^{-8} to 10^{-9}. At 25°C (graph on top left of Figure 13), no rif^R cfu arose on TB+Rif selection plates when cells from culture # 8, 18, 19, 21, 22, 23,

28, 33 and 37 were plated. Whereas at 37°C (graph on top right of Figure 10), rif^R cfu arose from the same cultures when plated on TB+Rif selection plates. This proved that the rif^R cfu that grew on TB+Rif plates at 37°C were not present in the initial culture in the tubes. If the rif^R mutants had arisen in the culture, they would have formed colonies on the TB+Rif selection plates at 25°C which was not the case. The only difference between 25°C and 37°C is that there is P expression at 37°C unlike at 25°C. The same inference can be made from the data from culture # 9, 10, 25 and 27 which showed a higher number of rif^R mutants at 37°C when compared to 25°C. The above data suggested that the rif^R mutants that arose at 37°C upon P expression were not pre-existing mutants. The mutations must have been generated during colony formation. However, in case of majority of the cell cultures (culture # 1-7, 11, 12-17, 20, 24, 26, 29, 30, 32, 34-36, 38 and 40), the number of rif^R cfu dropped at 37°C when compared to 25°C which questioned the idea that the increase in the number of rifampicin resistant mutants (i.e., the apparent mutator phenotype) is linked to λP expression.

Figure 13. Quantitation of rif^R mutants via Low Dose Inoculum Assay (LDIA) approach (individual cultures exposed to P expression)



A B

Figure 13. Quantitation of rif^R mutants via Low Dose Inoculum Assay (LDIA) approach (individual cultures exposed to P expression). 15 cells of 594[pcIpR-P-timm] were added to each of 40 tubes containing 1 ml rich TB and all 40 inoculated cultures were grown at 25°C for 48 hours to stationary phase. 0.1 ml cell aliquots from each of the 40 tubes were plated on two rich TB plates supplemented with rifampicin at a final concentration of 100µg/ml. One plate was incubated at 25°C and the other was incubated at 37°C. The graphs show rif^R cfu/ml selected at 25°C (A) and 37°C (B). [See actual counts in Supplemental Tables 11 and 12.] It is to be noted that the control for Graph B is Graph A. There was an approximately 83-fold toxicity in 594[pcIpR-P-timm] cells at 37°C in comparison to 25°C on rich TB plates.

3.9. Isolation of independent rifampicin resistant mutants from Low Dose Inoculum Assay for sequence analysis

In order to study the *rpoB* gene of the rif^R mutants, one rif^R mutant was isolated from each tube of the Low Dose Inoculum Assay (Figure 13). 22 spontaneous rifampicin resistant mutants (that arose at 25°C) and 12 rifampicin resistant mutants that arose in the presence of P expression (at 37°C) were isolated (after confirmation for rif^R on TB+100μg/ml Rif plates) from starting rif^S 594[pcIpR-P-timm] cells (Table 5). Also, 4 independent rif^R mutants (that have never seen P) were isolated from wild-type 594 cells for control experiments. DNA sequence analysis (not shown) performed by S. Hayes and C. Hayes, showed that mutations in *rpoB* gene conferred rifampicin resistant phenotype to the mutants. Plasmid retention analysis of the isolated *rpoB* rif^R mutants revealed that 21 out of 22 spontaneous rif^R *rpoB* mutants (that arose at 25°C in the absence of P expression) retained the P-expression plasmid and 11 out of 12 *rpoB* rif^R mutants (that arose at 37°C in the presence of P expression) had lost the plasmid.

Table 5. Rif^R mutants isolated from rif^S 594[pcIpR-P-timm] cells via Low Dose Inoculum Assay (LDIA)

Spontaneous rif^R mutants isolated at 25°C	**Rif^R mutants isolated at 37°C in the presence of P expression**
sc325A4 [a] – from culture #4 in Figure 9A	sc337A10 [b] – from culture #10 in Figure 9B
sc325A6 – from culture #6 in Figure 9A	sc337B1 – from culture #11 in Figure 9B
sc325A7 – from culture #7 in Figure 9A	sc337B8 – from culture #11 in Figure 9B
sc325A9 – from culture #9 in Figure 9A	sc337C1 – from culture #21 in Figure 9B
sc325A10 – from culture #10 in Figure 9A	sc337C4 – from culture #24 in Figure 9B
sc325B1 – from culture #11 in Figure 9A	sc337C5 – from culture #25 in Figure 9B
sc325B2 – from culture #12 in Figure 9A	sc337C6 – from culture #26 in Figure 9B
sc325B3 (P-expression plasmid was lost) – From culture #13 in Figure 9A	sc337C7 (P-expression plasmid was retained) – from culture #27 in Figure 9B
sc325B6 – from culture #16 in Figure 9A	sc337D1 – from culture #31 in Figure 9B
sc325B10 – from culture #20 in Figure 9A	sc337D2 – from culture #32 in Figure 9B
sc325C4 – from culture #24 in Figure 9A	sc337D3 – from culture #33 in Figure 9B
sc325C5 – from culture #25 in Figure 9A	sc337D6 – from culture #36 in Figure 9B
sc325C6 – from culture #26 in Figure 9A	
sc325C10 – from culture #30 in Figure 9A	
sc325D1 – from culture #31 in Figure 9A	
sc325D2 – from culture #32 in Figure 9A	
sc325D4 – from culture #34 in Figure 9A	
sc325D5 – from culture #35 in Figure 9A	

sc325D6 – from culture #36 in Figure 9A	
sc325D8 – from culture #38 in Figure 9A	
sc325D9 – from culture #39 in Figure 9A	
sc325D10 – from culture #40 in Figure 9A	

[a] sc325A4 denotes that the rif^R mutant arose from starting rif^S 594[pcIpR-P-timm] sc 3 cells at 25°C from tube/culture # A4.

[b] sc337A10 denotes that the rif^R mutant arose from starting rif^S 594[pcIpR-P-timm] sc 3 cells at 37°C from tube/culture # A10.

Table 6. Rif^R mutants isolated from rif^S 594 cells

Rif^R mutants isolated at 25°C	**Rif^R mutants isolated at 37°C**
594sc125A2 – from another assay (data not shown)	594sc137A2 – from another assay (data not shown)
594sc325E [a] – from culture #44 in Figure 9A	594sc337D [b] – from culture #43 in Figure 9B

[a] 594sc325E denotes that the rif^R mutant arose from starting rif^S 594 sc 3 cells at 25°C from tube/culture # E.

[b] 594sc337D denotes that the rif^R mutant arose from starting rif^S 594 sc 3 cells at 37°C from tube/culture # D.

3.10. Does rifampicin resistance confer cellular resistance to P-toxicity?

In the Low Dose Inoculum Assay (LDIA), the 37°C rifampicin resistant mutants survived P-toxicity. So, it was asked whether the rif^R cfu that arose at 37°C in LDIA were resistant to P. To address this, the cured [a] or plasmid-less rif^R mutants (isolated from rif^S 594[pcIpR-P-timm] cells at 37°C (column on the right of Table 5)) that survived the thermal induction of P expression during selection, were transformed with the pcIpR-P-timm plasmid and were selected on TB+50μg/ml Amp plates at 37°C and 25°C. To find out whether rifampicin resistance is linked to P-resistance, 4 rif^R mutants (that have never seen P – Table 6), 594sc125A2, 594sc137A2, 594sc325E and 594sc337D, isolated from wild-type 594 cells, were transformed with pcIpR-P-timm plasmid and were selected on TB+50μg/ml Amp plates at 37°C and 25°C. It was found that all the rifampicin resistant mutants transformed with the P-expression plasmid exhibited some degree of resistance to P-toxicity. It is to be noted that the drop in transformation frequency of the rifampicin resistant mutants at 37°C (w.r.t. 25°C) were at least 10-fold lower than that of the 594 wild-type control strain. Moreover, many of the rif^R mutants allowed some transformants to arise at 37°C whereas the control 594 cells did not (Table 7).

[a] In the Low Dose Inoculum Assay (LDIA), one rif^R colony was picked from each of the 37°C TB+Rif plates (which corresponded to an individual culture tube) that had rif^R colonies on them. By examining the colonies, it was found that 11 out of 12 of the rif^R mutants that arose at 37°C in the presence of P expression had lost the P-expression plasmid. So, these rif^R cells had been spontaneously cured of the plasmid during selection.

Table 7. Rif^R mutants that survived the thermal induction of P expression during selection and 594 rif^R mutants (that have never been exposed to P) are sensitive to P-toxicity

Strains transformed with pcIpR-P-timm plasmid	Experiment #	Frequency of appearance of Amp^R transformants at: (transformants/5 µl of plasmid DNA)		Fold drop in the frequency of appearance of Amp^R transformants at 37°C w.r.t. ~25°C [e]	Average fold drop ± standard error	Mutation bp in *rpoB* [h]
		~25°C [c]	37°C [d]			
594 wt	1	1.24×10^{-4}	$< 7.4 \times 10^{-8}$	> 1680 [g]	1710 ± 340 [f]	-
	2	4.64×10^{-5}	$< 2 \times 10^{-8}$	> 2320		
	3	4×10^{-5}	$< 3.5 \times 10^{-8}$	> 1143		
sc337B8 [a]	1	1.65×10^{-6}	4.6×10^{-8}	35.9 [g]	21 ± 14	-
	2	2.53×10^{-7}	3.6×10^{-8}	7.03		
sc337D6 [a]	1	2.28×10^{-6}	$< 1.2 \times 10^{-8}$	> 190 [g]	100 ± 90 [f]	1609
	2	1.98×10^{-6}	5.45×10^{-7}	3.63		
sc337C1 [a]	1	9.81×10^{-7}	$< 1.2 \times 10^{-8}$	> 81.8 [g]	124 ± 42 [f]	-
	2	2.32×10^{-6}	$< 1.4 \times 10^{-8}$	> 166		
sc337C6 [a]	1	4.53×10^{-6}	1.7×10^{-8}	266 [g]	150 ± 110 [f]	-
	2	1.68×10^{-5}	4×10^{-7}	42		
sc337D2 [a]	1	2.02×10^{-5}	9.53×10^{-7}	21.2 [g]	12 ± 9	-
	2	3.3×10^{-6}	1.46×10^{-6}	2.26		
sc337C5 [a]	1	2×10^{-6}	2×10^{-8}	100 [g]	108 ± 8 [f]	1691
	2	1.05×10^{-6}	$< 9 \times 10^{-9}$	> 117		
sc337A10 [a]	1	7.44×10^{-6}	2.3×10^{-8}	323 [g]	325.1 ± 1.6 [f]	1547
	2	2.94×10^{-6}	$< 9 \times 10^{-9}$	> 327		

sc337D1 [a]	1	1.25×10^{-6}	$< 9 \times 10^{-9}$	> 139 [g]	144.5 ± 5.5 [f]	1714
	2	1.5×10^{-6}	$< 1 \times 10^{-8}$	> 150		
594sc125A2 [b]	1	3.88×10^{-6}	2×10^{-8}	194	194	1535
594sc137A2 [b]	1	2.41×10^{-6}	$< 8 \times 10^{-9}$	> 301	301 [f]	1586
594sc325E [b]	1	1.57×10^{-6}	$< 6 \times 10^{-9}$	> 262	262 [f]	1592
594sc337D [b]	1	2.67×10^{-6}	1.7×10^{-8}	157	157	1714

[a] These rifampicin resistant mutants have been isolated at 37°C. During selection, these mutants lost the P-expression plasmid (Table 5 – coloumn on the right).

[b] These rifampicin resistant mutants have been isolated from wild-type 594 cells in the absence of P (Table 6).

[c] The P-expression plasmid, pcIpR-P-timm was transformed into sc337C5 rif^R mutant cell and the transformants were selected on TB plates supplemented with ampicillin at a final concentration of 50μg/ml at ~25°C. Frequency of transformation at ~25°C = Transformant cfu/ml on TB+Amp plates at ~25°C / Cell titer (cfu/ml) on control TB plates at ~25°C.

[d] The P-expression plasmid, pcIpR-P-timm was transformed into sc337C5 rif^R mutant cell and the transformants were selected on TB plates supplemented with ampicillin at a final concentration of 50μg/ml at 37°C. Frequency of transformation at 37°C = Transformant cfu/ml on TB+Amp plates at 37°C / Cell titer (cfu/ml) on control TB plates at ~25°C.

[e] Fold drop in transformation frequency at 37°C w.r.t. ~25°C = Frequency of transformation at 37°C / Frequency of transformation at ~25°C.

[f] Minimum value possible.

[g] These assays were done with the same pcIpR-P-timm plasmid DNA preparation and hence, the concentration of the plasmid DNA should be the same. The unit for these is transformants/5 μl of plasmid DNA.

[h] DNA sequencing done by S. Hayes and C. Hayes.

Note: Other than 594 wt, 594sc125A2, 594sc137A2, 594sc325E and 594sc337D cells, standard error has been calculated by taking 2 sets of data into consideration.

3.11. Phenotypic characterization of isolated *rpoB* rifampicin resistant mutants

In order to characterize the isolated *rpoB* rif^R mutants, the different viability and plasmid retention phenotypes of the mutants upon P expression from the plasmid was studied. We asked whether any of the isolated rifampicin resistant mutants that retained the P-expression plasmid (25°C isolates and one 37°C isolate) were resistant to P-toxicity. To test this, the rif^R mutants containing the P-expression plasmid were induced for P expression at 37°C. Upon P expression, the cell viability and plasmid retention phenotypes were assessed. It was found that a subset of *rpoB* selected rif^R mutants were resistant to P. Some mutants were either resistant to P-lethality like sc325D6, sc325C5, sc337C7, sc325D10 and sc325B10 or resistant to plasmid loss like sc325C10, sc325D6, sc325D2, sc325B1, sc325C5, sc325D10, sc325B10, sc337C7, sc325C6, sc325D8, sc325A6, sc325D9 and sc325A9 (Table 8). This suggested that a subset of *rpoB* selected rifampicin resistant mutants confer resistance to P.

It was also asked whether the 594 rif^R mutants that were isolated from wild-type 594 cells in the absence of P expression, were resistant to P. After transforming the 594 rif^R mutants with the wild-type P-expression plasmid, the same assay as above was employed to study viability and plasmid retention upon P expression. 594sc137A2 and 594sc337D were found to have some degree of resistance to P (Table 8) which suggested that certain *rpoB* mutants can confer cellular resistance to P.

Table 8. Induction of pcIpR-P-timm plasmid-containing *rpoB* rif^R mutants

Rif^R mutants (containing P-expression plasmid) [e]	**Viability at 37°C w.r.t. 25°C titer** [f] **(Mean ± Standard error)**	**Plasmid retention among the 37°C survivors (no. of cfu that have the plasmid/total no. of cfu stabbed)** [g]	**%-plasmid retention among the 37°C survivors**	**Mutation bp in *rpoB*** [i]
sc325C10 [a]	<0.001 & 0.000007	26/26	100	1586
sc325D1 [a]	0.0012 & 0.0008 (0.001 ± 0.0002 [h])	3/25	12	1574
sc325D6 [a]	0.1517 & 0.9048 (0.53 ± 0.38 [h])	25/25	100	1601
sc325A10 [a]	<0.0007 & 0.2093	2/25	8	1592
sc325D2 [a]	<0.001 & 0.0368	23/25	92	1576
sc325D5 [a]	0.0125 & 0.3881 (0.20 ± 0.19 [h])	3/25	12	1527
sc325A7 [a]	0.0087 & 0.1144 (0.062 ± 0.053 [h])	0/26	0	1527
sc325B2 [a]	0.012	0/28 & later, same 28 cells stabbed again – 6/28	10.71 (average)	No mutation in region 1391-2140
sc325B1 [a]	0.0034 & 0.172 (0.088 ± 0.084 [h])	21/27	77.78	1605-13
sc325C5 [a]	1.0803	20/26	76.92	1691
sc325B6 [a]	0.0024 & 0.0303 (0.0164 ± 0.014 [h])	16/28	57.14	No data
sc337C7 [b]	1.0336 & 0.9753 (1.005 ± 0.029 [h])	26/26 & 65/65	100	1601
sc325C6 [a]	1.0476; 0.1964 & 0.0385	26/26 & 3/22	100 & 13.64	1592

	(0.43 ± 0.31)		(57 ± 43)	
sc325B10 [a]	0.8155	28/28	100	1600
sc325D10 [a]	1.1039 & 0.9389 (1.021 ± 0.083 [h])	45/45 & 40/40	100	1604-12
sc325D4 [a]	0.0196	4/26	15.38	No data
sc325D8 [a]	0.0665	27/30	90	1691
sc325A6 [a]	0.1222	29/29; 25/25 & 38/38	100	1595
sc325D9 [a]	0.025	29/29; 28/28 & 36/36	100	1565
sc325A9 [a]	0.0261	32/46	69.57	1687
sc325C4 [a]	0.1639	0/36	0	1687
sc325A4 [a]	< 0.00096	No data	-	1585
594sc125A2 [c]	0.0000164 & $< 4 \times 10^{-9}$	15/22	68.18	1535
594sc137A2 [c]	0.5586 & 4.8×10^{-4} (0.28 ± 0.28 [h])	23/26	88.46	1586
594sc325E [c]	0.0898 & 0.0023 (0.046 ± 0.044 [h])	0/28 & 0/4	0	1592
594sc337D [c]	0.6115 & 0.029 (0.32 ± 0.29 [h])	20/25 & 0/89	80 & 0 (40 ± 40)	1714
sc337D6 [d]	0.027	95/102	93.14	1609
Control *rpoB*$^+$ 594[pcIpR-P-timm]	0.0043 ± 0.0016	0/30	0	

[a] These rifampicin resistant mutants have been isolated at 25°C. These mutants retained the P-expression plasmid (Table 5 – coloumn on the left).

[b] This rifampicin resistant mutant arose at 37°C in the presence of P expression. But, this is the only 37°C isolated mutant that retained the P-expression plasmid. This mutant complemented for P at 37°C which suggests that the *P* gene in the plasmid was wild-type.

[c] These rifampicin resistant mutants have been isolated from wild-type 594 cells in the absence of P expression (Table 6).

[d] This rifampicin resistant mutant arose at 37°C in presence of P. The P-expression plasmid was lost from this mutant strain during selection.

[e] Except sc325D4 (P complementation assay not done), all the rif^R mutants (that contain a P-expression plasmid, pcIpR-P-timm) complemented for P at 37°C. [See data in Supplemental Table 1.]

[f] Rif^R mutant cells containing the P-expression plasmid, pcIpR-P-timm were diluted and plated on two rich TB agar plates that were incubated at 25°C and 37°C. Viability at 37°C w.r.t. 25°C titer = Titer on TB at 37°C / Titer on TB at 25°C.

[g] Rif^R mutant cells containing the P-expression plasmid, pcIpR-P-timm were diluted and plated on two rich TB agar plates that were incubated at 25°C and 37°C. Survivor cfu at 37°C were picked with a sterile toothpick and stabbed onto a TB plate supplemented with ampicillin at a final concentration of 50μg/ml and a control TB plate. Ability of a cfu to grow on TB+Amp plate suggests that the cfu has the plasmid in it.

[h] Standard error has been calculated by taking 2 sets of data into consideration.

[i] DNA sequencing done by S. Hayes and C. Hayes.

Note: The 37°C plates were prewarmed for approximately 2 to 3 hours before spreading cells.

3.12. Determining if a 9 base pair deletion in host *rpoB* gene that affected 4 amino acids confers cellular resistance to P-toxicity

S. Hayes and C. Hayes determined by DNA sequence analysis that sc325D10 had a 9 bp deletion in *rpoB* that affected 4 amino acids. The spontaneous rif^R mutant, sc325D10 was isolated at 25°C (in the absence of P expression) from the Low Dose Inoculum Assay (LDIA) and it retained the P-expression plasmid. Upon thermal induction of P expression at 37°C, sc325D10 was found to be resistant to P-lethality and the 37°C cfu were found to retain the P-expression plasmid (Table 8). As sc325D10 mutant was found to be completely resistant to P, it was asked whether the 9 bp deletion in *rpoB* that affected 4 amino acids was responsible for P-resistance. To test this, the sc325D10 *rpoB* DNA (actual sequenced PCR product) containing the deletion was electroporated into 594[pSIM6] cells containing the λ Red recombination system. Rif^R recombinants were selected on TB+100μg/ml Rif plates at 30°C. Sequencing of the recombinants revealed that the 9 bp deletion (that affected 4 amino acids) successfully recombined with the wild-type *rpoB* DNA. The P-expression plasmid was transformed into the resulting recombinant, 594*rpoB*$^-$(D10). Upon thermal induction of P expression in the resulting transformant (594*rpoB*$^-$(D10)[pcIpR-P-timm]), it was found that 594*rpoB*$^-$(D10) was sensitive to P which suggested that the deletion in *rpoB* alone did not contribute to P-resistance in the parent strain, sc325D10 (Table 9).

Table 9. 9 base pair deletion in *rpoB* that affected 4 amino acids does not confer cellular resistance to λP

Rif^R recombinant constructed by recombineering [a]	**Viability at 37°C w.r.t. ~25°C titer** [c]	**Plasmid retention among the 37°C survivors (no. of cfu that have the plasmid/total no. of cfu stabbed)** [d]	**%-plasmid retention among the 37°C survivors**	**Mutation bp in *rpoB***	**AA change**
594*rpoB*$^-$(D10) [b] [pcIpR-P-timm] or Tsc325D10 [b] [pcIpR-P-timm]	0.0015; 0.0073 and 0.0053 (0.0047 ± 0.0017)	8/121; 2/50 and 0/20	6.61; 4 and 0 (3.54 ± 1.92)	1604-12	4AAΔ

[a] sc325D10 rif^R mutant (that retained the P-expression plasmid) was isolated at 25°C (in the absence of P) from rif^S 594[pcIpR-P-timm] cells. sc325D10 mutant was resistant to P-killing at 37°C and the 37°C cfu retained the P-expression plasmid. DNA sequencing of sc325D10 revealed that the isolated rif^R mutant had a 9 bp deletion in *rpoB* (S. Hayes and C. Hayes) that affected 4 amino acids. To find out whether the *rpoB* deletion was responsible for the P-resistant phenotype, the PCR fragment sequenced was electroporated into a wild-type 594[pSIM6] cell. pSIM6 plasmid contains λ Red recombination genes that will allow the electroporated fragment to recombine with the wild-type recipient chromosome. Rif^R recombinants were selected at 30°C on rich TB plates supplemented with rifampicin at a final concentration of 100μg/ml. After rif^R confirmation, the 594*rpoB*$^-$(D10) or Tsc325D10 recombinants were transformed with the pcIpR-P-timm plasmid. The transformants were selected at ~25°C on rich TB plates supplemented with ampicillin at a final concentration of 50μg/ml. Then, these resulting transformants were induced for P-expression to study the viability and plasmid retention in the recombinant mutant strain.

[b] Cells were very slow growing. They took ~4 days to reach mid-log phase.

[c] Rif^R mutant cells containing the P-expression plasmid, pcIpR-P-timm were diluted and plated on two rich TB agar plates that were incubated at 25°C and 37°C. Viability at 37°C w.r.t. ~25°C titer = Titer on TB at 37°C / Titer on TB at ~25°C.

[d] Rif^R mutant cells containing the P-expression plasmid, pcIpR-P-timm were diluted and plated on two rich TB agar plates that were incubated at ~25°C and 37°C. Survivor cfu at 37°C were picked with a sterile toothpick and stabbed onto a TB plate supplemented with ampicillin at a final concentration of 50μg/ml and a control TB plate. Ability of a cfu to grow on TB+Amp plate suggests that the cfu has the plasmid in it.

4. Discussion

4.1. λP-lethality

E. coli replication licensing factor, DnaC (Stillman, 1994) is known to bind DnaB helicase (Wickner and Hurwitz, 1975) and targets it to the bacterial origin of replication, *oriC*. Bacteriophage λ gene product P (λ replication licensing factor) outcompetes host DnaC protein for binding to host DnaB helicase (Mallory *et al.*, 1990; Konieczny and Marszalek, 1995) and directs it to λ origin of replication, *oriλ*. The sequestration of host DnaB helicase by P deprives host DnaC protein the opportunity to bind DnaB and direct it to *oriC*. This is probably the simplest explanation of why host cell viability is lowered upon P expression. Section 3.1 shows that λP is lethal to host cells which agrees with the concept of 'P-lethality' established by Klinkert and Klein (Klinkert and Klein, 1979). Low levels of P protein do not kill the host cells but elevated levels of P are lethal (Maiti *et al.*, 1991b). The re-repression (of P) assay in Section 3.2 suggested that host cell viability is significantly lowered upon P expression. Prolonged cellular exposure to P kills the cells. One explanation for the reduced cell viability upon P expression can be the effect of P on cell replication.

A class of *dnaB* mutations called *groP* prevented wild-type phage λ growth at permissive temperatures for bacterial DNA synthesis, but allowed the growth of phage λ that possessed π mutation in gene *P* (Dari *et al.*, 1975). Georgopoulos and Herskowitz mapped *groP* mutations in the host *dnaB* gene (Georgopoulos and Herskowitz, 1971). Maiti *et al.* reported that although *dnaBgroP* mutants do not allow λ DNA replication, they remain susceptible to P-lethality (Maiti *et al.*, 1991b). They inferred that wild-type DnaB was not required for P-lethality. However, Hayes (Hayes, 1979) proved that DnaB was required for λ replication initiation. Later, Hayes *et al.* showed that grpD55 allele of *dnaB* blocked λ replication initiation which suggested that P needs wild-type DnaB to initiate replication from *oriλ* (Hayes *et al.*, 2005). Bull mapped the grpD55 mutations to the *dnaB* gene (Bull, 1995; Bull and Hayes, 1996). Later, the grpD55 mutations were sequenced by Horbay (Horbay, 2005). Bull suggested that the grpD55 mutations in *dnaB* (that do not allow wild-type phage λ growth) might suppress P-lethality (Bull, 1995)

which was confirmed in Section 3.1 of this work. This proved that P-DnaB interaction was essential for P-lethality which refuted Maiti *et al.*'s findings (Maiti *et al.*, 1991b) stated above.

4.2. ColE1 Plasmid Loss among P-survivors

Section 3.9 showed that 11 out of 12 of the rif^R mutants that were isolated at 37°C upon P expression (from Low Dose Inoculum Assay) had lost the P-expression plasmid. Section 3.2 showed similar results related to plasmid loss upon P exposure. My data suggests that the majority of the cells that survive P-lethality lose the P-expression ColE1 plasmid. It also shows that the cells that lose the P-expression plasmid survive. In our laboratory, experiments have been done by other researchers to isolate plasmid DNA from P-exposed 594[pcIpR-P-timm] cultures at 37°C and control 594[pcIpR-P-timm] cells at 25°C (when there is no P expression). pUC19 plasmid was used as a control for agarose gel analysis. Conclusions from gel data from the above experiments were similar to those from my stab assays. This supports the idea that P probably interferes with ColE1 plasmid replication. Unpublished data from our laboratory showed that plasmid loss does not occur upon P expression in a DnaBgrpD55 host. This suggests that since P cannot interact with the grpD55 conformation of DnaB helicase, ColE1 replication is not inhibited. In ColE1 plasmid replication, RNaseH cleaves the RNA-DNA hybrid to form primers. DnaB is reported to promote replication fork advancement in vivo (Lebowitz and McMacken, 1986; McMacken *et al.*, 1977). For the ColE1 replication fork to advance, DnaB is required to unwind the DNA double strand. If P is interacting with DnaB during this step or if P is sequestering DnaB and making DnaB unavailable for this step, ColE1 replication will be inhibited. ColE1 plasmids do not have a partitioning system. So, there is unequal distribution of plasmid copies among daughter cells. If P inhibits ColE1 replication, plasmid copy number decreases. This increases the chances for plasmid loss. Also, PriA is known to restart stalled replication forks by loading DnaB helicase and is required for DnaB loading on the ColE1 plasmid. So, if P is interacting with DnaB or sequestering DnaB, PriA may not be able to restart replication forks, and may be inhibited for ColE1 replication (Minden and Marians, 1985; Lee and Kornberg, 1991). These are possible models which explain ColE1 plasmid loss among P-survivors.

4.3. DNA replication and causes of mutation

DNA polymerase enzymes are very particular about their choice of nucleotides during DNA synthesis. They ensure that the bases added to a growing strand are correctly paired with their complements on the template strand (i.e., A's with T's, and C's with G's). But, they also make mistakes at a rate of about 1 per 100,000 nucleotides. Cells have highly sophisticated ways of fixing most, but not all, mistakes made by DNA polymerases. Proofreading is a process which corrects most of the errors immediately during replication. Some errors are corrected after replication by a process called mismatch repair. During proofreading, DNA polymerases recognize the error and replace the wrongly inserted nucleotide, thus, allowing replication to continue. Proofreading fixes about 99% of these types of errors. But, this is still not good enough for cell functioning. After replication, the final error rate is further reduced by mismatch repair where enzymes recognize and fix the deformities, caused by the incorrectly paired nucleotides in the secondary structure of the final DNA molecule, by replacing the incorrectly paired nucleotide with the correct one. Incorrectly paired nucleotides that persist even after mismatch repair, become permanent mutations after the next cell division. This is because once the mistakes are established; the cell no longer recognizes them as errors. The DNA strand with established mistakes, serve as the template strand for future replication events causing all base pairings thereafter to be wrong (Pray, 2008).

Mutations can also be caused by various environmentally induced and spontaneous changes to DNA that occur prior to replication but are perpetuated in the same way as unfixed replication errors. As with replication errors, most environmentally induced DNA damage is repaired, resulting in fewer than 1 out of every 1,000 chemically induced lesions actually becoming permanent mutations. The same is true of so-called spontaneous mutations. "Spontaneous" refers to the fact that the changes occur in the absence of chemical, radiation, or other environmental damage. Rather, they are usually caused by normal chemical reactions that go on in cells, such as hydrolysis. Most of these spontaneous errors are repaired by DNA repair processes. But, if they are not repaired, a nucleotide that is added to the newly synthesized strand can become a permanent mutation (Pray, 2008).

In this work, we studied λ gene product P which is a toxic agent. *E. coli* cells are killed upon P expression (Section 3.1). My data suggests that the mutator phenotype observed among the P-survivor cells is linked to P expression (Sections 3.4, 3.5, 3.6, 3.7 and 3.8). One possibility is that P is stimulating the mutations in the host chromosome by interacting with DnaB helicase during the replication propagation step, thus, perturbing replication fidelity. If this is true, P will be a toxic and a mutagenic agent. Upon P expression, most of the *E. coli* cells will be killed. Among the P-survivors, some will have a genome that has not been damaged (no lesions) and some will have DNA lesions (in this case, a mismatch) in their chromosome. If this mismatch is repaired, it will restore the pristine DNA sequence. Otherwise, the next round of replication will convert the mismatch into a permanent mutation (base alteration).

4.4. Assessment of the apparent mutator phenotype by Low Dose Inoculum Assay (LDIA)

In Luria-Delbrück Fluctuation Test, bacteriophage T1 was used as the selective agent (Luria and Delbrück, 1943). In the Low Dose Inoculum Assay (LDIA) used in this work, λP is the selective agent which means that any cell that has a pre-existing mutation (that make the cell resistant to P) in any of the genes with which P interacts (like *dnaB* or *dnaA*), will be resistant to P-toxicity. The other possibility is that the cells are killed or mutated upon expression of P, the selective agent. Our initial hypothesis was that P expression causes random mutations in the host *E. coli* chromosome. I tested this hypothesis by selecting for rifampicin resistant mutants from each of the LDIA parallel cultures at 25°C (not P-induced) and 37°C (P-induced) as shown in Section 3.8. I obtained more rif^R mutants at 37°C than 25°C for some of the cultures, but not others. This was initially interpreted to suggest P-expression induced rif^R mutants to arise after induction of P at 37°C. However, for this to be proven true, a number of controls need to be considered. For example, rifampicin antibiotic is known to stop transcription by inhibiting RNA polymerase. How rapidly does rifampicin inhibit RNA polymerase? We show that for some culture tubes in the Low Dose Inoculum Assay, rifampicin resistant mutants arose on the plates in the presence of P. We also argue that these mutants are not pre-existing mutants as they did not show up on the plates at 25°C when there was no P, and thus appear to support the hypothesis that P induction increases the frequency of rifampicin resistance.

However, a number of additional factors need to be taken into consideration before accepting this finding as proof that P expression is increasing the mutation frequency. For example, is it even possible that the cells plated on TB+100μg/ml Rif plates would have sufficient time to induce P (or, transcribe P), allow P to interact with some unknown protein and cause mutations in the host DNA, allow mutant DNA to be transcribed with the rifampicin-sensitive RNA polymerase to express the rifampicin resistant version that then accumulates to allow the cell to grow in the presence of rifampicin? This calls into question the idea that "when wild-type cells are exposed to P at 37°C on TB+100μg/ml Rif plates, P stimulates chromosomal mutations that confer rifampicin resistance phenotype to the cells".

It has been reported that the minimum inhibitory concentrations (MICs) of rifampicin for gram-negative bacteria are much higher (8-32 μg/ml) than those of gram-positive bacteria such as staphylococci (0.01 μg/ml). Hence, in gram-negative bacteria (in our case, *E. coli*), penetration of rifampicin through the outer membrane is the rate-limiting step and any kind of reduction in the outer membrane permeability may result in mutants with a significant degree of resistance to the antibiotic (Wehrli, 1983). But, in my experiments, 100 μg/ml rifampicin was used which is much higher than the highest MIC value for gram-negative bacteria reported by Wehrli.

Hayes (Hayes, 1979) compared lambda transcription from induced rifampicin sensitive and rifampicin resistant lysogenic cultures. For a rif^S W3350(λ*cI*857) cell, RNA synthesis from both the lambda and host DNA was reduced by 59- and 7-fold, respectively, by either 15.5- or 1.5-min pretreatment with 200 μg/ml of rifampicin (Hayes, 1979). Therefore, transcription from the p_R promoter is probably not completely shut off, though significantly reduced, which might result in the transcription of gene *P* and eventual synthesis of the P protein. The residual expression of P may be sufficient to generate a “P-induced” mutator effect, if any.

Rifampicin resistance used in this work is not an ideal target to determine whether P is stimulating rifampicin resistant mutations in the host chromosome by altering the *E. coli rpoB* gene. This is because some rif^R mutants were found to be resistant to P-toxicity (Sections 3.10 and 3.11) and thus, selection for rif^R mutants in presence of P expression could be a result of enrichment for pre-existing mutants. Also, selecting for rifampicin resistant mutants upon P expression involves a double selection – seletion of P-resistant colonies and seletion of rif^R colonies, i.e., upon thermal induction of P expression from 594[pcIpR-P-timm] cells at 37°C, the

rif^R colonies that arise on TB+100μg/ml Rif plates at 37°C have to first survive P-lethality. It has been reported that stationary phase or adaptive mutations occur more often when selected than when not (Wright *et al.*, 1999). So, double seletion might result in the increase in the rate of occurrence of mutations. Therefore, screening is a better way to look for mutations than selection. It is always a better way to screen, i.e., to do a survey among the P-survivors to look for possible mutants than to select for a mutation as this forces the mutation to arise. Hence, screening for auxotrophs among P-survivors was a good assay to determine whether P is involved in stimulating the nutrient gene mutations among the P-survivors. An important control that could have been done was to test whether the auxotrophic mutants were resistant to P-toxicity. This could have been done by transforming the wild-type P-expression plasmid into few of the isolated auxotrophs and thermally inducing the plasmid for P expression and looking for P-killing. Screening for Mal^- and Xyl^- fermentation-defective mutants among P-survivors should have been used to show whether P was responsible for triggering the mutator phenotype in the host. A P^- strain will serve as a control. Bull *et al.* used these targets for their study of hypermutation in chromosomal genes in association with adaptive Lac^+ mutation (Bull *et al.*, 2000). Screening for auxotrophs or nutrient gene mutations, Mal^- and Xyl^- fermentation-defective mutants are better assays for studying mutagenesis as these genes are silent during cellular growth phase on a rich medium at permissive temperatures.

The *E. coli rpoB* gene is a relatively small target and mutations in the *rpoB* gene represent a very small "window". In other words, only a select few base changes might be allowed which may result in certain amino acid changes in RNA polymerase. Too many mutations in *rpoB* will probably have a lethal effect on the cell as the mutated RNA polymerase might not be able to function at all, thus shutting down transcription. I have reported the appearance of a very high number of rifampicin resistant mutants upon P expression at 37°C for some cultures (in Low Dose Inoculum Assay) and my data for those cultures suggest that P might be causing it as no mutants arose at 25°C for those cultures. If P is stimulating the mutator phenotype, i.e., if the appearance of high numbers of rifampicin resistant mutants at 37°C are triggered by P, it could be expected that there should be other mutations accumulating in the essential host genes too. During a burst of "P-induced" mutagenesis, how high must the level of mutations in other essential genes in the host chromosome be to support the very high number of rifampicin resistant mutants seen in Cultures 22 and 23 in Figure 13B. This is analogous to

'Error Catastrophe' (Crotty *et al.*, 2001). A burst of random P-induced mutations in the host chromosome should decrease the chances of cell survival, thus decreasing mutant yield. While some of my data in Figure 13B show elevated rif^R mutants to arise upon P induction, there are multiple cultures which did not yield increased rifampicin resistant mutants following P induction. It is difficult to envisage a P-dependent mutator mechanism that appears to be "hit or miss".

4.5. Rifampicin resistance and P-resistance

One possible explanation for the observed apparent high rif^R mutation rate among P expression surviving colonies could be that pre-existing rif^R mutations somehow make the cell less sensitive to P-induced killing. If this were the case, selection for survivor of P induction would result in an enrichment of rif^R colony forming units among the survivors and generate an apparent increase in rif^R cfu among P-expression survivors.

To determine whether the plasmid-less or cured rifampicin resistant mutants that survived the thermal induction of P expression at 37°C, and 594 rifampicin resistant mutants (that were never exposed to P expression) were resistant to P-toxicity, a transformation assay was performed where the wild-type P-expression plasmid was transformed and ampicillin resistant transformants were selected at 25°C and 37°C on TB+Amp plates. My results (Section 3.10) show that the wild-type 594 control cells did not yield any P-expression plasmid transformants at 37°C; whereas 2 out of 4 594 rif^R mutants showed some transformants at 37°C suggesting that the presence of rif^R mutations in *rpoB* can influence susceptibility to P expression. In support of this finding, almost all (6 out of 8) of the rif^R mutants obtained from P-induced cultures were able to be transformed with the P-expression plasmid at 37°C. Significantly, many of these mutants were transformed at relatively high efficiency at 37°C in comparison to 25°C. Moreover, it has to be noted that the transformation frequency at 25°C for all of the rif^R mutants was significantly lower than that of wild-type 594 cells possibly due to the altered RNA polymerase. The possibility that the altered RNA polymerase is interfering with the efficiency of ColE1 replication of the transformed plasmid remains to be tested and if true, would also explain the

observed enrichment for rif^R mutants among 37°C survivors of P expression, as any increase in the rate of plasmid loss will increase the number of apparent surviving cells.

In Section 3.8, an induction assay was performed by thermally inducing P from the P-expression plasmid that was retained in the 25°C isolated rif^R mutants obtained from Low Dose Inoculum Assay. For one 37°C rif^R mutant which lost the P-expression plasmid (sc337D6) and for the 594 rif^R mutants, the wild-type P-expression plasmid was transformed into the cells. My data (Table 8) indicated that many of the rifampicin resistant mutants (that arose at 25°C from LDIA) and 2 of the 4 rifampicin resistant mutants (that have never been exposed to P) that were isolated from wild-type 594 cells were resistant to P-toxicity. The only difference between Tables 7 and 8 was that Table 7 is a transformation assay and Table 8 is an induction assay.

A few control experiments can be done to help establish whether rifampicin resistant mutants are resistant to P-toxicity. For example, a wild-type pBR322 plasmid without *P* can be transformed into a wild-type 594 cell and a 594 rif^R cell and the relative frequencies of appearance of transformants from 25°C to 37°C for both transformant strains can be determined. This would have determined whether rifampicin resistance influences the fidelity of plasmid maintenance. In essence, further experiments are needed to test this possibility.

4.6. Debate about λP and RNA polymerase interaction

There are no reports describing a physical interaction between λP and host RNA polymerase in the literature. However, McKinney and Wechsler demonstrated a genetic interaction between λP and host RNA polymerase in that specific combinations of λP$_\pi$ mutants and *rpoB* rif^R mutants increased or decreased λ's plaque forming ability in an allele specific manner (McKinney and Wechsler, 1983) suggesting that λP, host DnaB helicase and RNA polymerase form a functional complex that is required for λ replication. The λ replication initiation protein P is known to interact with lambda O protein (Tomizawa, 1971) that binds to *oriλ* sites (Tsurimoto and Matsubara, 1981), and with host proteins DnaB (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977; Klein *et al.*, 1980) required as a helicase for chromosomal replication, and with heat shock proteins DnaJ (Yochem *et al.*, 1978), DnaK

(Yochem *et al.*, 1978), GrpE (Zylicz *et al.*, 1987), and with DnaA (Wegrzyn *et al.*, 1996; Datta *et al.*, 2005b; Datta *et al.*, 2005a), required for *E. coli* replication initiation, and possibly with RNA polymerase (McKinney and Wechsler, 1983). In λ replication initiation, P brings DnaB to an O-*oriλ* complex to form the preprimosomal *oriλ*-O-P-DnaB complex (Dodson *et al.*, 1989) involved in the θ mode of λ bidirectional replication initiation. The C-terminal region of λO contains a P-binding domain (Furth *et al.*, 1977; Furth and Yates, 1978; Wickner and Zahn, 1986). Recently, Szambowska *et al.* published an article showing that λO protein physically interacts with host RNA polymerase (Szambowska *et al.*, 2011). Therefore, it will be of no surprise if P is found to be a part of such a complex.

My data do not show any evidence of a direct physical interaction between λP and *E. coli* RNA polymerase. However, the fact that a lot of the isolated rifampicin resistant mutants are resistant to P-toxicity suggest that P and RNA polymerase might be interacting with each other. It is known that the *E. coli rpoB* gene encodes the β-subunit of RNA polymerase. Mutations in *rpoB* confer rifampicin resistance to the cells. I found that a 9 base pair deletion in *rpoB* that affected 4 amino acids did not make the cell resistant to P. Hence, I do not have any data showing that P interacts with RNA polymerase. Further studies are required to resolve this debate.

4.7. Future Experiments

Our introductory hypothesis was that λP expression stimulates random mutations in the host *E. coli* chromosome. We tested this hypothesis my using auxotrophy (mutation(s) in nutrient gene) and rifampicin resistance (mutation(s) in *rpoB* gene) as chromosomal targets. As some of the rifampicin resistant mutants showed some degree of resistance to P-toxicity, the observed apparent increase in the rif^R frequency can be explained as an enrichment of pre-existing *rpoB* mutations. Hence, rifampicin resistance is not a good target for investigating whether P stimulates random mutations in the host chromosome. Similarly, it is important to find out whether auxotrophic mutations were making the host cells resistant to P-toxicity. To test this, a P-defective RK^- auxotroph containing the *cI* immunity region, can be transformed with a P-expression plasmid with a 434 immunity region. A P-expression plasmid with a cI immunity

region (pcIpR-P-timm) can be used to transform λ^- RK^- auxotrophs and auxotrophs obtained among the plasmid-cured P survivors (obtained upon thermal induction of P expression in 594[pcIpR-P-timm] cells) and the resulting transformants should be thermally induced for P expression. Then, it has to be seen whether these cells survive P-toxicity.

Upon thermal induction of P-expression from the ColE1 plasmid in 594[pcIpR-P-timm] cells, the P-survivors were found to be cured of the plasmid. One hypothesis is that P is stimulating ColE1 plasmid loss from host cells. Another explanation is that the cells which lose the plasmid survive. This is a more probable explanation as the toxic agent, P is expressed from the plasmid itself which when lost, allows the cell to grow without inhibition. To test whether P stimulates ColE1 plasmid loss, a chromosomal P-expression system should be used instead of a plasmid P-expression system. P should be expressed from a prophage in a cell containing a pBR322 plasmid and then, plasmid loss should be studied among the P-survivors.

My data in Section 3.10 shows that the rifampicin resistant mutants were resistant to P-toxicity. A few additional control experiments are needed to confirm this observation. A wild-type pBR322 plasmid without *P* can be transformed into a wild-type 594 cell and into a 594 rif^R cell and the relative frequencies of appearance of transformants from 25°C to 37°C for both transformant strains can be determined. These important controls will substantiate the data in Table 7. Since some rif^R mutants are resistant to P-toxicity, it indicates that P might be interacting with RNA polymerase. Assays have to done to show whether λP and *E. coli* RNA polymerase physically interact with each other.

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APPENDED SUPPORTING CONTROL EXPERIMENT:

P complementation assay was employed to study P expression from assay cells as a control experiment. A *P* amber mutant phage grew on host cells which expressed P at a particular temperature. The *P* amber mutant phage also grew on a suppressor TC600 bacterial strain [a] (control).

[a] Nonsense suppressor strain: A bacterial strain / cell which has a mutation within the anticodon of a normal tRNA, enabling the mutated tRNA to recognize and insert an amino acid at a stop codon.

Supplemental Table 1. P complementation assay using λimm434*P*am3 phage

Host strain	Temperature (°C)	Experiment #	% Efficiency of plating [a]	Mean ± Standard error
Y836	39	1	58.18	64.6 ± 6.4 [b]
		2	70.93	
	30	1	0.0524	0.0524
Y836 ilr 534c O^+P^+	39	1	46.92	41 ± 12
		2	57.64	
		3	17.5	
	30	1	0.0178	0.0178
Y836 ilr *O*208b P^+	39	1	56.92	67 ± 18
		2	100.91	
		3	41.67	

	30	1	0.0044	0.0070 ± 0.0026 [b]
		2	0.0095	
Y836 ilr *O*223a P^+	39	1	33.08	34.5 ± 1.4 [b]
		2	35.83	
	30	1	0.0081	0.0081
Y836 ilr O^+P::IS2	39	1	0.00007	0.00022 ± 7.54 x 10^{-5}
		2	0.00026	
		3	0.00032	
	30	1	0.00008	0.00008
594[pcIpR-P-timm]	37	1	140.74	93 ± 24 [c]
		2	74.93	
		3	63.41	
	30	1	82.35	82.35
	25	1	0.0762	0.0762 [b]
594[pcIpR-ΔP-timm]	37	1	< 0.000024	< 0.000024
		2	< 0.000024	
sc325A6	37	1	157.69	157.69
sc325A7	37	1	103.53	103.53
sc325A9	37	1	126.92	126.92
sc325A10	37	1	80	80
sc325B1	37	1	98.82	98.82
sc325B2	37	1	130.6	130.6
sc325B6	37	1	87.06	87.06

sc325B10	37	1	97.01	97.01
sc325C4	37	1	165.38	165.38
sc325C5	37	1	85.82	85.82
sc325C6	37	1	138.06	138.06
sc337C7	37	1	126.87	126.87
sc325C10	37	1	35.29	35.29
sc325D1	37	1	84.71	84.71
sc325D2	37	1	82.35	82.35
sc325D5	37	1	98.82	98.82
sc325D6	37	1	101.18	101.18
sc325D8	37	1	130.77	130.77
sc325D9	37	1	126.92	126.92
sc325D10	37	1	88.46	88.46
594sc125A2 [pcIpR-P-timm]	37	1	65.4	84.9 ± 6.8
		2	88.56	
		3	88.66	
		4	97.01	
594sc137A2 [pcIpR-P-timm]	37	1	108.99	107.6 ± 9.7
		2	95.37	
		3	91.64	
		4	134.33	
594sc325E [pcIpR-P-timm]	37	1	111.72	113.1 ± 8.7
		2	89.92	

		3	120.3	
		4	130.6	
594sc337D [pcIpR-P-timm]	37	1	79.02	97.5 ± 12.4
		2	73.57	
		3	114.33	
		4	123.13	
594*rpoB*$^{-}$(D10) [pcIpR-P-timm]	37	1	49.47	49.47

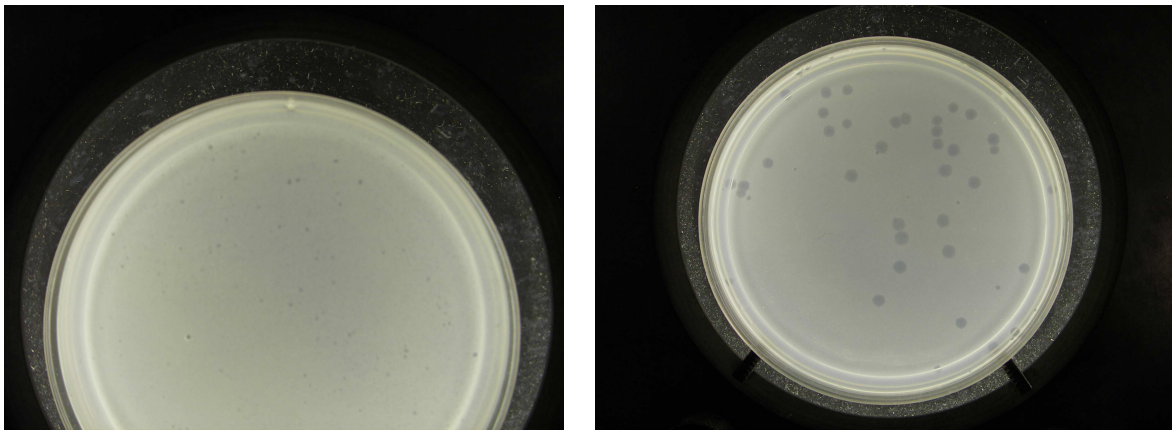
[a] 0.1 ml of the diluted *P* amber mutant phage was added to 0.3 ml of host cells. Then, 3 ml of tryptone top agar was added to the cell-phage mixture and the whole mixture was immediately poured onto a rich TB plate. % efficiency of plating of *P* amber mutant phage on all host strains was calculated w.r.t. *P* amber mutant phage titer on suppressor strain, TC600. % efficiency of plating of *P* amber mutant phage = (Titer (pfu/ml) of *P* amber mutant phage on a host cell / Titer (pfu/ml) of *P* amber mutant phage on TC600) x 100.

[b] Standard error has been calculated by taking 2 sets of data into consideration.

[c] Refer to Supplemental Figure 1 for pictures of plaques.

Supplemental Figure 1. λimm434*P*am3 plaques on 594[pcIpR-P-timm] cell lawn

A **B**



Supplemental Figure 1. λimm434*P*am3 plaques on 594[pcIpR-P-timm] cell lawn. (A) Marker rescue resulting in very small plaque formation on 594[pcIpR-P-timm] cell lawn at 25°C (plating dilution: 10^{-3}). 594[pcIpR-P-timm] did not complement for P at 25°C. (B) As 594[pcIpR-P-timm] cells complemented for P at 37°C, λimm434*P*am3 plaques grew on the cell lawn (plating dilution: 10^{-7}). P expression from the plasmid complemented for the nonsense mutation in *P* gene in λimm434*P*am3 phage allowing plaque formation. The cell lawn was weaker at 37°C (in comparison to 25°C) because 594[pcIpR-P-timm] cells were killed upon P expression at 37°C.

APPENDED SUPPORTING EXPERIMENTAL DATA TABLES:

Supplemental Table 2. P expression at 42°C (from a cryptic λ prophage) kills host *E. coli* cells and P-DnaB interaction is required for P-killing (Data for Figure 5 in Section 3.1a)

Strain	Experiment #	Viability at 42°C w.r.t. 30°C [a]	Mean ± Standard error
Y836	1	3.0×10^{-6}	$7.0 \times 10^{-6} \pm 1.7 \times 10^{-6}$
	2	1.5×10^{-6}	
	3	4.8×10^{-6}	
	4	1.7×10^{-5}	
	5	1.1×10^{-5}	
	6	1.3×10^{-5}	
	7	1.7×10^{-5}	
	8	8.7×10^{-7}	
	9	1.3×10^{-6}	
	10	3.9×10^{-6}	
	11	6.3×10^{-6}	
	12	4.5×10^{-6}	
Y836*his*$^{+}$	1	1.3×10^{-5}	$1.45 \times 10^{-5} \pm 9.6 \times 10^{-7}$
	2	1.7×10^{-5}	
	3	1.5×10^{-5}	
	4	1.3×10^{-5}	
Y836*dnaB*grpD55	1	0.39	0.255 ± 0.055
	2	0.13	

	3	0.28	
	4	0.22	
594::(*cIII-ren*)$^{\lambda}$	1	2.6×10^{-5}	$3.2 \times 10^{-5} \pm 1.6 \times 10^{-5}$
	2	1.8×10^{-5}	
	3	6.5×10^{-6}	
	4	7.9×10^{-5}	
Y836 *O*$^{+}$*P*::*Kan*	1	0.21	0.26 ± 0.12
	2	0.93	
	3	0.0073	
	4	0.0017	
	5	0.041	
	6	0.22	
	7	0.59	
	8	0.069	
Y836 ilr *O*223a*P*$^{+}$	1	6.2×10^{-5}	$3.88 \times 10^{-4} \pm 0.00012$
	2	3.7×10^{-4}	
	3	1.2×10^{-3}	
	4	3.7×10^{-4}	
	5	7.0×10^{-4}	
	6	2.0×10^{-4}	
	7	2.4×10^{-4}	
	8	1.4×10^{-5}	
	9	3.4×10^{-4}	

Y836 ilr *O*$^{+}$*P*::*IS2*	1	0.87	0.61 ± 0.11
	2	0.90	
	3	0.65	
	4	0.59	
	5	0.47	
	6	0.15	
594*dnaB*grpD55	1	0.15	0.268 ± 0.082
	2	0.64	
	3	0.28	
	4	0.31	
	5	0.41	
	6	0.073	
	7	9.5×10^{-3}	
594	1	0.16	0.223 ± 0.099
	2	0.20	
	3	0.50	
	4	0.033	

[a] Assay cells were washed and the cell pellets were resuspended in Φ80 buffer and were diluted. The diluted cells were plated on rich TB plates in duplicates that were incubated at 30°C and 42°C. Viability at 42°C w.r.t. 30°C = Cell titer on rich TB plates at 42°C / Cell titer on rich TB plates at 30°C.

Supplemental Table 3. P expression at 37°C (from a ColE1 plasmid) kills host *E. coli* cells and P-DnaB interaction is required for P-killing (Data for Figure 6 in Section 3.1b)

Strain	Experiment #	Viability at 30°C w.r.t. 25°C [a]	Mean viability at 30°C w.r.t. 25°C ± Standard error	Viability at 37°C w.r.t. 25°C [b]	Mean viability at 37°C w.r.t. 25°C ± Standard error
594[pcIpR-P-timm]	1	1.2	0.951 ± 0.058	0.013	0.0043 ± 0.0016 (less than value not taken into account)
	2	0.79		2.0×10^{-3}	
	3	1.1		9.4×10^{-3}	
	4	1.1		9.1×10^{-4}	
	5	0.73		1.1×10^{-3}	
	6	0.90 [c]		2.7×10^{-3}	
	7	0.64		$< 7.1 \times 10^{-4}$	
	8	1.1		1.7×10^{-3}	
	9	0.95		3.6×10^{-3}	
	10	1.0			
594*dnaB*grpD55 [pcIpR-P-timm]	1	0.95	1.001 ± 0.068	1.1	0.81 ± 0.11
	2	1.3		1.0	
	3	0.78		0.50	
	4	0.96		0.69	
	5	0.97		0.74	
	6	1.3			

	7	0.71			
	8	1.1			
	9	0.94			

[a] Assay cells were washed and the cell pellets were resuspended in Φ80 buffer and were diluted. The diluted cells were plated on rich TB plates that were incubated at 25°C, 30°C and 37°C. Viability at 30°C w.r.t. 25°C = Cell titer on rich TB plates at 30°C / Cell titer on rich TB plates at 25°C.

[b] Assay cells were washed and the cell pellets were resuspended in Φ80 buffer and were diluted. The diluted cells were plated on rich TB plates that were incubated at 25°C, 30°C and 37°C. Viability at 37°C w.r.t. 25°C = Cell titer on rich TB plates at 37°C / Cell titer on rich TB plates at 25°C.

[c] Instead of a TB plate, an LB plate was used in this experiment.

Supplemental Table 4. Re-repression (of P) assay (Data for Figures 7 and 8 in Section 3.2)

Temp. (°C)	**Set # / exposure time at 37°C for plates to be shifted** [a]	**CFU/plating dilution**				**Average titer (cells/ml)** [b]	**% plasmid retention among cfu** [c]
		10^{-4}	**10^{-5}**	**10^{-6}**	**10^{-7}**		
25	#1				193 [e]	1.93×10^9	100
30	#1				138 [e]	1.59×10^9	100
	#2				179 [e]		
37	#1		48 [d]	9 [d]		6.9×10^6	0
37→30	1 hour	TNTC		TNTC [e]	162	1.62×10^9	100
	2 hours	TNTC	TNTC [e]	difficult to count as a lot of colonies are fused [e]	72 [e]	7.2×10^8	100
	6 hours	TNTC (big and small)	123 (98 big and ~25 small)	13 (9 big and 4 small)		1.27×10^7	63 [f]

[a] A volume of stationary phase cells of 594[pcIpR-P-timm] were washed and the cell pellet was resuspended in the same volume of Φ80 buffer. The diluted cells were plated on TB plates that were incubated at 25°C, 30°C and 37°C (controls). 3 sets of plates were also plated with the same cells at 37°C which after 1, 2 and 6 hours, respectively were cooled on ice for a while and were then shifted to 30°C.

[b] Average titer (cells/ml) is the cfu count on rich TB plates obtained at each temperature.

[c] Cfu obtained at different temperatures on rich TB plates were picked with a sterile toothpick and stabbed onto a rich TB plate supplemented with ampicillin at a final concentration of 50μg/ml and a TB control plate. The stabbed plates were incubated at 25°C. % plasmid retention among cfu that arose at a particular temperature = (Number of cfu that were able to grow on TB+Amp plates / Total number of cfu stabbed) x 100.

[d] Same sized colonies - quite big.

[e] Same or similar sized colonies.

[f] All of the small cfu and ~50% of the big cfu retained the P-expression plasmid.

Supplemental Table 5. Linkage between cryptic λ prophage induction and appearance of auxotrophs (Data for Figure 9 in Section 3.4)

Assay strains	No. of colonies picked up from 30°C rich TB plates [a]	No. of auxotrophs obtained at 30°C	No. of colonies picked up from 42°C rich TB plates [b]	No. of auxotrophs obtained at 42°C
Y836	520	0	707	34
Y836 *O*$^{+}$*P*::*Kan*	324	0	716	0
594::(*cIII-ren*)$^{\lambda}$	347	0	295	47
Y836*dnaB*grpD55	179	0	337	11
Y836 ilr 534c *O*$^{+}$*P*$^{+}$	167	0	875	1
Y836 ilr *O*208b *P*$^{+}$	322	0	1065	1
Y836 ilr *O*223a *P*$^{+}$	223	0	654	11 [c]
Y836 ilr *O*$^{+}$*P*::IS2	140	0	503	0
594*dnaB*grpD55 [d]	413	0	919	1
594 [d]	551	0	260	0

[a] To determine whether spontaneous auxotrophs arise within assay cells at 30°C, the 30°C cfu were picked with a sterile toothpick and stabbed onto a MM agar plate and a rich TB control plate. All plates were incubated at 30°C.

[b] Survivor cfu at 42°C on TB plates were picked with a sterile toothpick and were stabbed to duplicate MM agar plates that were incubated at 30°C and 42°C and a TB control plate incubated at 30°C. Inability of a colony to grow on MM plate suggests that the stabbed colony is an auxotroph.

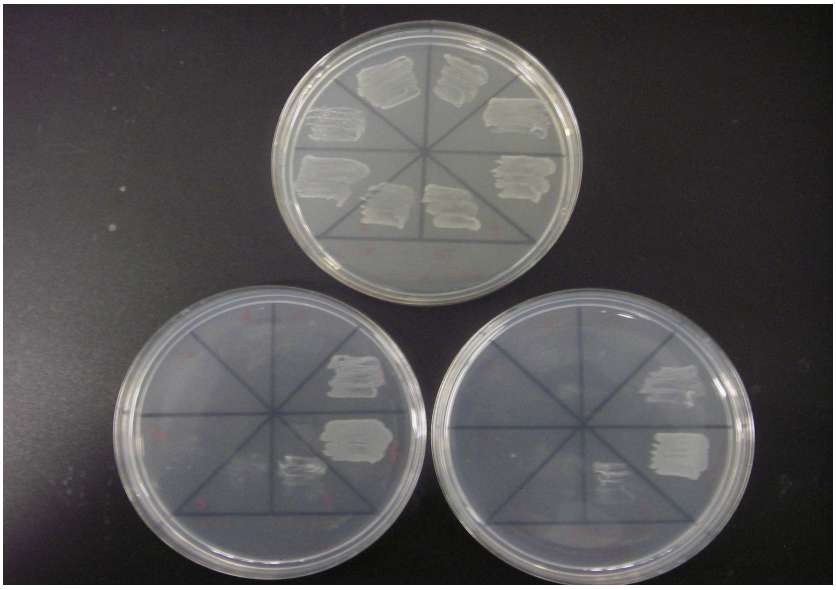
[c] Refer to Supplemental Figure 2 for auxotrophic phenotype.

[d] These assay strains do not have a cryptic λ fragment.

Note: Y836 is a *his*$^-$ strain. Any derivatives or mutants of Y836 are also *his*$^-$. Therefore, for plating these strains, MM agar plates supplemented with histidine at a final concentration of 0.1 mg/ml was used.

Supplemental Figure 2. Auxotrophy assessment of Y836 ilr *O*223a *P*$^+$ mutants

Rich TB at 30°C



MM+his at 30°C **MM+his+bio at 30°C**

Supplemental Figure 2. Auxotrophy assessment of Y836 ilr *O*223a *P*$^+$ mutants. 5 auxotrophic mutants did not grow on MM+his and MM+his+bio plates at 30°C which suggested that they were not biotin auxotrophs. The mutation was in some other nutrient gene. If by chance, they are *bio*$^-$, they are also defective in some other nutrient gene.

Supplemental Table 6. P-DnaB interaction is essential for the apparent mutator phenotype – P expression from cryptic λ prophage (Data for Figure 10 in Section 3.5)

Strains	Experiment #	Rif^R frequency at:		Mean ± Standard error	
		30°C [a]	**42°C** [b]	**30°C**	**42°C**
Y836	1	3×10^{-8}	Not finite [c]	$4.33 \times 10^{-8} \pm 6.8 \times 10^{-9}$	2.94×10^{-2}
	2	5.2×10^{-8}	2.94×10^{-2}		
	3	4.8×10^{-8}	Not finite [c]		
Y836 *O*$^+$*P*::*Kan*	1	9.6×10^{-8}	2.4×10^{-7}	$5.3 \times 10^{-8} \pm 2.2 \times 10^{-8}$	$3.1 \times 10^{-7} \pm 1.9 \times 10^{-7}$
	2	2.2×10^{-8}	2.5×10^{-8}		
	3	4×10^{-8}	6.66×10^{-7}		
Y836*dnaB*grpD55	1	2.25×10^{-7}	4.52×10^{-7}	$1.38 \times 10^{-7} \pm 3.8 \times 10^{-8}$	$3.9 \times 10^{-7} \pm 1.5 \times 10^{-7}$
	2	1.42×10^{-7}	Not finite [c]		
	3	1.44×10^{-7}	6.12×10^{-7}		
	4	4×10^{-8}	9.3×10^{-8}		
Y836 ilr *O*223a*P*$^+$	1	1.2×10^{-8}	Not finite [c]	$8.5 \times 10^{-8} \pm 4.3 \times 10^{-8}$	$1.85 \times 10^{-4} \pm 7.3 \times 10^{-5}$
	2	1.86×10^{-7}	2.67×10^{-4}		
	3	1.6×10^{-8}	2.5×10^{-4}		
	4	1.27×10^{-7}	3.91×10^{-5}		
Y836 ilr *O*$^+$*P*::IS2	1	3.9×10^{-8}	7.6×10^{-8}	$2.83 \times 10^{-8} \pm 8.3 \times 10^{-9}$	$9.1 \times 10^{-8} \pm 2.4 \times 10^{-8}$
	2	3.4×10^{-8}	5.8×10^{-8}		
	3	1.2×10^{-8}	1.38×10^{-7}		

[a] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100μg/ml and control TB plates at 30°C. Rif^R frequency at 30°C = Titer on TB+Rif at 30°C / Titer on TB at 30°C.

[b] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100μg/ml and control TB plates at 42°C. Rif^R frequency at 42°C = Titer on TB+Rif at 42°C / Titer on TB at 42°C.

[c] Frequency values are not finite because no cfu grew on the lowest dilution plate used.

Supplemental Table 7. P-DnaB interaction is essential for the apparent mutator phenotype – P expression from a ColE1 plasmid (Data for Figure 11 in Section 3.6)

Strains	**Experiment #**	**Rif^R frequency at:**			**Mean ± Standard error**		
		25°C [a]	**30°C** [b]	**37°C** [c]	**25°C**	**30°C**	**37°C**
594 [pcIpR-P-timm]	1	1.3×10^{-8}	1.2×10^{-8}	1.72×10^{-6}	$1.5 \times 10^{-8} \pm 4.0 \times 10^{-9}$	$3.0 \times 10^{-8} \pm 1.1 \times 10^{-8}$	$1.22 \times 10^{-5} \pm 5.2 \times 10^{-6}$
	2	1.3×10^{-8}	1.6×10^{-8}				
	3	6×10^{-9}	3.6×10^{-8}	5×10^{-6}			
	4	3×10^{-8}	8×10^{-8}	2×10^{-5}			
	5	3×10^{-8}					
	6	7×10^{-9}	1.1×10^{-8}				
	7	6×10^{-9}	$2.2.x\ 10^{-8}$	2.22×10^{-5}			
594*dnaB*grpD55 [pcIpR-P-timm]	1	2.7×10^{-8}	1.4×10^{-8}	2.5×10^{-8}	$1.87 \times 10^{-8} \pm 5.6 \times 10^{-9}$	$1.33 \times 10^{-8} \pm 2.1 \times 10^{-9}$	$1.5 \times 10^{-7} \pm 1.1 \times 10^{-7}$
	2	Not finite [d]	7×10^{-9}	5.82×10^{-7}			
	3	Not finite [d]	1.6×10^{-8}	7.7×10^{-8}			
	4	2.1×10^{-8}	Not finite [d]	5.2×10^{-8}			
	5	8×10^{-9}	1.6×10^{-8}	3.2×10^{-8}			

[a] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100μg/ml and control TB plates at 25°C, 30°C and 37°C. Rif^R frequency at 25°C = Titer on TB+Rif at 25°C / Titer on TB at 25°C.

[b] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100μg/ml and control TB plates at 25°C, 30°C and 37°C. Rif^R frequency at 30°C = Titer on TB+Rif at 30°C / Titer on TB at 30°C.

[c] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100μg/ml and control TB plates at 25°C, 30°C and 37°C. Rif^R frequency at 37°C = Titer on TB+Rif at 37°C / Titer on TB at 37°C.

[d] Frequency values are not finite because no cfu grew on the lowest dilution plate used.

Supplemental Table 8. P-DnaB interaction is essential for the apparent mutator phenotype – Assay of various plasmid-containing strains (Data for Figure 12 in Section 3.7)

In order to determine whether P requires DnaB for the potential mutator phenotype observed (Section 3.7), P, P_π or ΔP-expression plasmid-containing cells expressing wild-type DnaB or DnaBgrpD55 were assayed to obtain rif^R frequencies at 25°C, 30°C and 37°C. (Supplemental Table 8 is a combination of Supplemental Tables 9 and 10.)

Strains	**Mean rif^R frequency ± Standard error**			**Fold changes in rif^R frequencies at**			**Number of assays**
	25°C [a]	**30°C** [b]	**37°C** [c]	**25°C** [d]	**30°C** [e]	**37°C** [f]	
594[pcIpR-P-timm]	$1.5 \times 10^{-8} \pm 4.0 \times 10^{-9}$	$3.0 \times 10^{-8} \pm 1.1 \times 10^{-8}$	$1.22 \times 10^{-5} \pm 5.2 \times 10^{-6}$	1.0	2.0	810	7
594*dnaB*grpD55*malF3089*::Tn*10* [pcIpR-P-timm]	$1.87 \times 10^{-8} \pm 5.6 \times 10^{-9}$	$1.33 \times 10^{-8} \pm 2.1 \times 10^{-9}$	$1.5 \times 10^{-7} \pm 1.1 \times 10^{-7}$	1.0	0.71	8.0	5
594[pcIpR-P_π-timm]	8×10^{-9}	3.3×10^{-8}	2.0×10^{-7}	1.0	4.1	25	1
594*dnaB*grpD55*malF3089*::Tn*10* [pcIpR-P_π-timm]	3.5×10^{-8}	Not finite [g]	4.2×10^{-7}	1.0	Not finite [g]	12	1
594[pcIpR-ΔP-timm]	5.2×10^{-8}	$4.3 \times 10^{-8} \pm 1.4 \times 10^{-8}$	3.33×10^{-7}	1.0	0.83	6.4	3
594*dnaB*grpD55*malF3089*::Tn*10* [pcIpR-ΔP-timm]	Not finite [g]	2×10^{-9}	$9.5 \times 10^{-9} \pm 5.5 \times 10^{-9}$	1.0	Not finite [g]	Not finite [g]	2

[a] Mean rif^R frequency values and standard errors have been tabulated. Rif^R frequency at 25°C = Titer on TB+Rif at 25°C / Titer on TB at 25°C.

[b] Mean rif^R frequency values and standard errors have been tabulated. Rif^R frequency at 25°C = Titer on TB+Rif at 30°C / Titer on TB at 30°C.

[c] Mean rif^R frequency values and standard errors have been tabulated. Rif^R frequency at 25°C = Titer on TB+Rif at 37°C / Titer on TB at 37°C.

[d] Fold change in rif^R frequency at 25°C = Rif^R frequency at 25°C / Rif^R frequency at 25°C.

[e] Fold change in rif^R frequency at 30°C w.r.t. 25°C = Rif^R frequency at 30°C / Rif^R frequency at 25°C.

[f] Fold change in rif^R frequency at 37°C w.r.t. 25°C = Rif^R frequency at 37°C / Rif^R frequency at 25°C.

[g] Frequency and fold change values are not finite because no cfu grew on the lowest dilution plate used.

Note: Figure 12 contains fold change values at 25°C and 37°C. Data for 594*dnaB*grpD55*malF3089*::Tn*10* [pcIpR-ΔP-timm] strain has not been plotted in Figure 12.

Supplemental Table 9. P-DnaB interaction is essential for the apparent mutator phenotype – Assay of various plasmid-containing strains (Data for Figure 12 in Section 3.7)

Strains	Expt. #	Rif^R frequency at:			Mean ± Standard error		
		25°C [a]	30°C [b]	37°C [c]	25°C	30°C	37°C
594[pcIpR-P-timm]	1	1.3×10^{-8}	1.2×10^{-8}	1.72×10^{-6}	$1.5 \times 10^{-8} \pm 4.0 \times 10^{-9}$	$3.0 \times 10^{-8} \pm 1.1 \times 10^{-8}$	$1.22 \times 10^{-5} \pm 5.2 \times 10^{-6}$
	2	1.3×10^{-8}	1.6×10^{-8}				
	3	6×10^{-9}	3.6×10^{-8}	5×10^{-6}			
	4	3×10^{-8}	8×10^{-8}	2×10^{-5}			
	5	3×10^{-8}					
	6	7×10^{-9}	1.1×10^{-8}				
	7	6×10^{-9}	$2.2.x\ 10^{-8}$	2.22×10^{-5}			
594*dnaB*grpD55 *malF3089*::Tn*10* [pcIpR-P-timm]	1	2.7×10^{-8}	1.4×10^{-8}	2.5×10^{-8}	$1.87 \times 10^{-8} \pm 5.6 \times 10^{-9}$	$1.33 \times 10^{-8} \pm 2.1 \times 10^{-9}$	$1.5 \times 10^{-7} \pm 1.1 \times 10^{-7}$
	2	Not finite [d]	7×10^{-9}	5.82×10^{-7}			
	3	Not finite [d]	1.6×10^{-8}	7.7×10^{-8}			
	4	2.1×10^{-8}	Not finite [d]	5.2×10^{-8}			
	5	8×10^{-9}	1.6×10^{-8}	3.2×10^{-8}			
594[pcIpR-P_π-timm]	1	8×10^{-9}	3.3×10^{-8}	1.98×10^{-7}	8×10^{-9}	3.3×10^{-8}	2.0×10^{-7}
594*dnaB*grpD55 *malF3089*::Tn*10* [pcIpR-P_π-timm]	1	3.5×10^{-8}	$> 3.5 \times 10^{-8}$	4.2×10^{-7}	3.5×10^{-8}	Not finite [d]	4.2×10^{-7}

594[pcIpR-ΔP-timm]	1	Not finite [d]	5 x 10^{-8}	Not finite [d]	5.2 x 10^{-8}	4.3 x 10^{-8} ± 1.4 x 10^{-8}	3.3 x 10^{-7}
	2	Not finite [d]	1.6 x 10^{-8}	Not finite [d]			
	3	5.2 x 10^{-8}	6.2 x 10^{-8}	3.33 x 10^{-7}			
594*dnaB*grpD55 *malF3089*::Tn*10* [pcIpR-ΔP-timm]	1	Not finite [d]	Not finite [d]	1.5 x 10^{-8}	Not finite [d]	2 x 10^{-9}	9.5 x 10^{-9} ± 5.5 x 10^{-9} [e]
	2	Not finite [d]	2 x 10^{-9}	4 x 10^{-9}			

[a] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100µg/ml and control TB plates at 25°C, 30°C and 37°C. Rif^R frequency at 25°C = Titer on TB+Rif at 25°C / Titer on TB at 25°C.

[b] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100µg/ml and control TB plates at 25°C, 30°C and 37°C. Rif^R frequency at 30°C = Titer on TB+Rif at 30°C / Titer on TB at 30°C.

[c] Assay cells were diluted and plated on TB plates supplemented with rifampicin at a final concentration of 100µg/ml and control TB plates at 25°C, 30°C and 37°C. Rif^R frequency at 37°C = Titer on TB+Rif at 37°C / Titer on TB at 37°C.

[d] Frequency values are not finite because no cfu grew on the lowest dilution plate used.

[e] Standard error has been calculated by taking 2 sets of data into consideration.

Supplemental Table 10. P-DnaB interaction is essential for the apparent mutator phenotype – fold change values (Data for Figure 12 in Section 3.7)

Strains	Fold changes in rif^R frequencies at		
	25°C [a]	**30°C** [b]	**37°C** [c]
594[pcIpR-P-timm]	1.0	2.0	810
594*dnaB*grpD55*malF3089*::Tn*10* [pcIpR-P-timm]	1.0	0.71	8.0
594[pcIpR-P_π-timm]	1.0	4.1	25
594*dnaB*grpD55*malF3089*::Tn*10* [pcIpR-P_π-timm]	1.0	Not finite [d]	12
594[pcIpR-ΔP-timm]	1.0	0.82	6.4
594*dnaB*grpD55*malF3089*::Tn*10* [pcIpR-ΔP-timm]	1.0	Not finite [d]	Not finite [d]

[a] Fold change in rif^R frequency at 25°C = Rif^R frequency at 25°C / Rif^R frequency at 25°C.

[b] Fold change in rif^R frequency at 30°C w.r.t 25°C = Rif^R frequency at 30°C / Rif^R frequency at 25°C.

[c] Fold change in rif^R frequency at 37°C w.r.t 25°C = Rif^R frequency at 37°C / Rif^R frequency at 25°C.

[d] Fold change values are not finite because frequencies were not finite as no cfu grew on the lowest dilution plate used.

Supplemental Table 11. Rif^R cfu (at 10^{-1} plating dilution) from starting rif^S 594[pcIpR-P-timm] cells selected on TB+100µg/ml Rif at 25°C (Data for Figure 13A in Section 3.8)

In Low Dose Inoculum Assay (Section 3.8), the number of rif^R cfu that grew from each culture tube (A1-A10, B1-B10, C1-C10 and D1-D10) at 25°C has been tabulated in Supplemental Table 11.

	A	**B**	**C**	**D**
1	0	6 [a]	0	1
2	#1 – 0 #2 – 0 #3 – 0	1	0	2
3	0	1	0	0
4	#1 – 3 #2 – 0	0	#1 – 2 #2 – 2	5
5	#1 – 0 #2 – 0	0	#1 – 1 #2 – 2	1
6	1	4	#1 – 7 #2 – 4	6
7	#1 – 2 #2 – 3	0	1	0
8	0	0	0	#1 – 1

				#2 – 1
9	1	0	0	#1 – 0 #2 – 1
10	1	#1 – 0 #2 – 8	1	1

[a] Rif^R cfu obtained on TB+Rif plates at 25°C from Tube B1 or Culture # 11 (in Figure 13A). 15 cells of 594[pcIpR-P-timm] sc 3 were added to each of 40 tubes containing 1 ml rich TB broth and all 40 inoculated cultures were grown at 25°C for 48 hours to stationary phase. 0.1 ml cell aliquots from each of the 40 tubes were plated on two rich TB plates supplemented with rifampicin at a final concentration of 100μg/ml. One plate was incubated at 25°C and the other at 37°C. #1, #2 and #3 refers to plates in duplicates or triplicates.

Note: In graph (Figure 13A), tube A1 refers to Culture 1, tube A2 refers to Culture 2, tube A3 refers to Culture 3 and so on.

Supplemental Table 12. Rif^R cfu (at 10^{-1} plating dilution) from starting rif^S 594[pcIpR-P-timm] cells selected on TB+100µg/ml Rif at 37°C (Data for Figure 13B in Section 3.8)

In Low Dose Inoculum Assay (Section 3.8), the number of rif^R cfu that grew from each culture tube (A1-A10, B1-B10, C1-C10 and D1-D10) at 37°C has been tabulated in Supplemental Table 12.

	A	**B**	**C**	**D**
1	0	2	2 [a]	1
2	0	0	16	1
3	0	0	36	7
4	0	0	1	2
5	0	0	4 & other very tiny cfus	0
6	0	0	1	2
7	1	0	9	2
8	6	2	2	0
9	4	1	0	1
10	6	0	0	0

[a] Rif^R cfu obtained on TB+Rif plates at 37°C from Tube C1 or Culture # 21 (in Figure 13B). 15 cells of 594[pcIpR-P-timm] sc 3 were added to each of 40 tubes containing 1 ml rich TB broth

and all 40 inoculated cultures were grown at 25°C for 48 hours to stationary phase. 0.1 ml cell aliquots from each of the 40 tubes were plated on two rich TB plates supplemented with rifampicin at a final concentration of 100µg/ml. One plate was incubated at 25°C and the other at 37°C.

Note: In graph (Figure 13B), tube A1 refers to Culture 1, tube A2 refers to Culture 2, tube A3 refers to Culture 3 and so on.

Supplemental Table 13. Rif^R cfu (at 10^{-1} plating dilution) from starting rif^S 594 cells selected on TB+100µg/ml Rif at 25°C (Data for Figure 13A in Section 3.8)

In Low Dose Inoculum Assay (Section 3.8), the number of rif^R cfu that grew from each culture tube (B, C, D and E) at 25°C has been tabulated in Supplemental Table 13.

	B	**C**	**D**	**E**
Set 1	0	0	0	1 [a]
Set 2	0	0	0	0

[a] Rif^R cfu obtained on TB+Rif plates at 25°C from Tube E or Culture # 44 (in Figure 13A). 19 594 cells were added to each of 4 tubes containing 1 ml rich TB broth and all 4 inoculated cultures were grown at 25°C for 48 hours to stationary phase. 0.1 ml cell aliquots from each of the 4 tubes were plated on two rich TB plates supplemented with rifampicin at a final concentration of 100µg/ml. One plate was incubated at 25°C and the other at 37°C.

Note: In graph (Figure 13A), tube B refers to Culture 41, tube C refers to Culture 42, tube D refers to Culture 43 and tube E refers to Culture 44. Set 1 and Set 2 are duplicate platings of cells from the same tube.

Supplemental Table 14. Rif^R cfu (at 10^{-1} plating dilution) from starting rif^S 594 cells selected on TB+100µg/ml Rif at 37°C (Data for Figure 13B in Section 3.8)

In Low Dose Inoculum Assay (Section 3.8), the number of rif^R cfu that grew from each culture tube (B, C, D and E) at 37°C has been tabulated in Supplemental Table 14.

	B	**C**	**D**	**E**
Set 1	0	0	1 [a]	0
Set 2	0	0	0	0

[a] Rif^R cfu obtained on TB+Rif plates at 37°C from Tube D or Culture # 43 (in Figure 13B). 19 594 cells were added to each of 4 tubes containing 1 ml rich TB broth and all 4 inoculated cultures were grown at 25°C for 48 hours to stationary phase. 0.1 ml cell aliquots from each of the 4 tubes were plated on a rich TB plate supplemented with rifampicin at a final concentration of 100µg/ml at 25°C and 37°C.

Note: In graph (Figure 13B), tube B refers to Culture 41, tube C refers to Culture 42, tube D refers to Culture 43 and tube E refers to Culture 44. Set 1 and Set 2 are duplicate platings of cells from the same tube.