MUTATOR PHENOTYPE OF INDUCED CRYPTIC COLIPHAGE LAMBDA PROPHAGE

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Thesis Abstract

These studies are based on the isolation of λ replication defective mutants that had acquired multiple point mutations within λ replication initiation genes *O* and *P* in a cryptic prophage (Hayes *et al*., 1998). Each mutant cell arose after shifting wild type cells with a *cI*[Ts] cryptic λ prophage deleted for *int-kil*, and from *ren* into *E. coli*, from 30°C to 42°C. Derepression of the trapped cryptic prophage kills the host cells (designated as RK^+). Rare colony forming units survive and were designated as RK^- mutants. This led to a hypothesis that λ replication-triggered cell stress provokes mutator activity, i.e., increases the frequency of replication errors within the simultaneously replicating chromosome of the host *E. coli* cells. We tested this hypothesis by asking three questions: (1) Do unselected, untargeted (with no link to λ fragment) auxotrophic mutations appear within the RK^- mutant population selected from RK^+ culture cells? (2) Is replication initiation from the cryptic λ fragment, or, alternatively, just expression of one or more λ genes required for the appearance of the unselected auxotrophic mutations? (3) Do *E. coli* functions participate in the appearance of unselected auxotrophic mutations within the RK^- mutant population? Our results indicate that auxotrophic mutations unlinked to the λ fragment appeared at high frequency within RK^- mutants. RK^- auxotrophs arising on rich medium were identified by screening the survivor clones for growth on minimal medium. The appearance of RK^- auxotrophic colonies at high frequency (>1 per 100 RK^- mutants) leads us to conclude that auxotrophic mutations arise during the independent selection for RK^- mutants. Conditions that inhibited λ fragment induction fully suppressed the mutator phenotype. Mutation of host *dnaB* such that the helicase does not support replication initiation from the induced λ fragment completely

suppressed host cell killing, but not the appearance of auxotrophic mutations. We asked if *E. coli* error-prone polymerases IV and V, or gene functions regulated as part of the host SOS response contributed to the provoked mutator phenotype and observed no close correlation. We demonstrated that the RK^+ starting cells did not have a distinct intrinsic mutator activity in several ways, including moving the cryptic λ fragment to different *E. coli* host cells, blocking λ fragment induction by the addition of a cI^+ plasmid to eliminate λ gene expression at high temperatures, and independent assays for spontaneous rifampicin resistance. We found that the induced mutator phenotype associated with the appearance of untargeted auxotrophs was linked to the expression of lambda gene *P*, and did not require replication initiation from the cryptic λ prophage. We also found that the mutator phenotype of the induced cryptic λ fragment increased the frequency of rifampicin resistant colonies among the RK^- mutant population.

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List of Abbreviations and Terms

Ts	temperature sensitive
WT	wild-type
phage	bacteriophage
ori	origin of replication
λ	bacteriophage λ
oriC	origin of replication for *E. coli*
oriλ	origin of replication for λ
cI	λ gene encoding the λ CI repressor required for maintaining lysogeny
cIII	λ gene encoding CIII participating in establishment of lysogeny
O	λ gene encoding protein O required for replication initiation
P	λ gene encoding protein P required for replication initiation
ren	λ gene that provides resistance to the λ encoded Rex exclusion system
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*
t_R	rightward termination site
t_L	leftward termination site
E. coli	*Escherichia coli*
dnaB	*E. coli* gene encoding DnaB helicase required for λ and *E. coli* replication

grpD55	An allelic mutation of *dnaB* conferring a conditional [Ts] phenotype to λ replication at 42°C
AFI	Auxotroph Formation Index = frequency of plating (colony forming units at 42°C per colony forming units at 30°C) on rich tryptone agar per frequency of plating on minimal medium agar. For cells in which λ replication initiation arises upon shifting them from 30°C to 42°C the AFI represents the RK^- mutants arising on rich tryptone agar per frequency of RK^- mutants arising on minimal medium agar. For cells in which λ replication initiation is blocked upon shifting them from 30°C to 42°C this represents the efficiency of colony formation (colony forming units at 42°C per colony forming units at 30°C) on rich tryptone agar per efficiency of colony formation (colony forming units at 42°C per colony forming units at 30°C) on minimal medium agar.
PDS	Pereira da Silva selection for measuring survivor colonies arising at 42°C from lysogenic cells with a λ*N*⁻ *cI*[Ts] prophage grown at 30°C.
cfu	colony forming units
Replicative killing	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 devective cells that plate with equal efficiency at 30°C and 42°C and are defective for λ replication initiation.

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 *ori*λ.

Transcriptional activation The CI repressor bound at o_R prevents rightward transcription from the major rightward promoter, p_R, and inhibits the expression of downstream genes *O* and *P*. The *O* and *P* gene products are required for the initiation of λ replication. The binding of repressor at o_L prevents transcription from p_L. This action inhibits the expression of *N*, required for the antitermination of p_R-initiated transcription at the downstream site, t_{R1}, and other sites.

The product of gene *N* is required for maximal derepression of *O* and *P* transcription. By inhibiting transcription from p_R, the repressor also blocks a *cis* requirement for replication initiation described as transcriptional activation. This requirement is understood because it can be suppressed by ri^c (replication-inhibition-constitutive) mutations which lie outside and downstream from the assigned λ replicator region (Furth *et al*, 1977). The need for transcriptional activation apparently represents a requirement for transcription across the origin, which may serve to denature the transcribed DNA strands (Hayes, 1988b).

1. Introduction

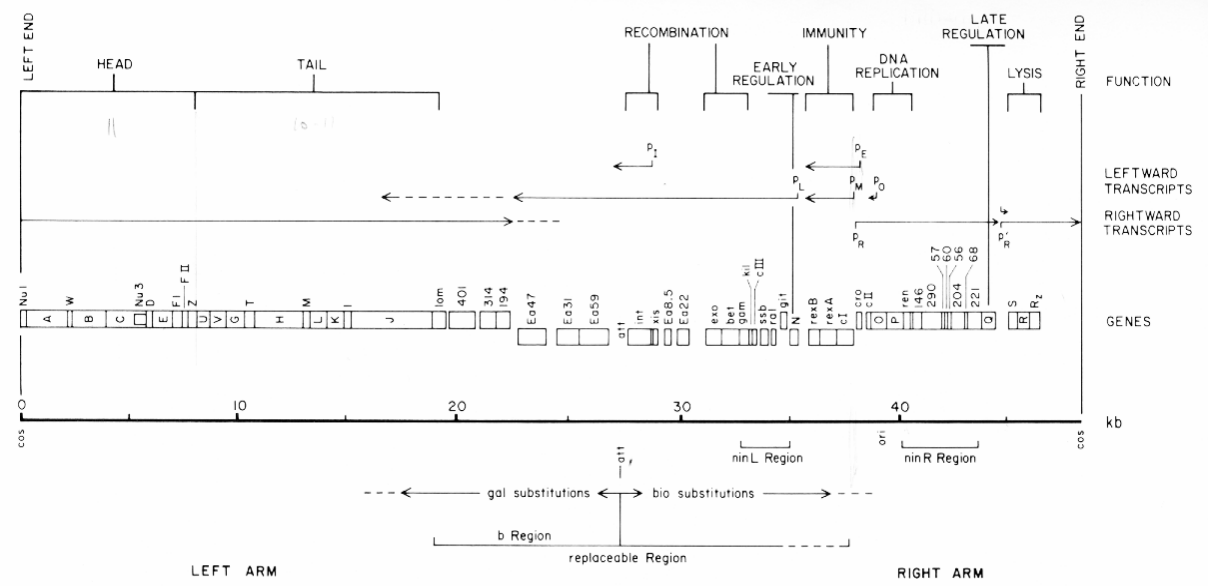
1.1. Bacteriophage Lambda Overview of Genes and Regulations

Bacteriophages (phages) are viruses that infect bacterial hosts. Phages that infect *Escherichia coli (E. coli*) vary in size, shape and lifestyle (Campbell, 1971). The group of lambdoid phages are categorized by their ability to exchange modules of genes when intercrossed (Wrobel and Wegrzyn, 2002), make the choice between lysis and lysogeny of a cell upon infection, integrate prophage DNA into a host chromosome, use positive control of operons by antitermination, have their early lytic functions organized into divergently transcribed operons, and regulate lysogeny by a single repressor specified by a gene lying between two operons. Lambdoid phages include λ, φ80, 434, HK022, HK97, P22, and LP-7 to name a few (Campbell, 1971).

Lambda was identified in 1951 (Juhala, 2000). It has a linear double stranded DNA genome comprising of 48,502 nucleotides with 12-base pair single stranded complementary 5' ends (Daniels *et al*, 1982) with an isometric head. The head capsid is approximately 50 nm in diameter and is comprised of two major capsid proteins, gpD and gpE (Campbell, 1971). Lambda has a flexible non-contractile tail about 150 nm long which recognizes the cellular receptor maltose transport protein, LamB. The phage particle itself is metabolically inert and phage vegetative growth can only begin once phage DNA is injected into its host. This is accomplished through contact between the λ tail tip and the *E. coli* LamB protein. Lambda DNA is released from the head through the tail core and is immediately circularized via the complementary cohesive 5'ends.

Figure 1. Lambda Map

Map of phage λ DNA (Daniels *et al* 1983)



There are seven genes (*AWBCDE* and *F*) required for the formation of an active isometric phage head. All seven genes are clustered together at the left end of the phage map. Eleven tail genes (*ZUVGTHMLKI* and *J)* are grouped to the right of the head genes and are required for the formation of the tail structure (Campbell, 1971).

Lambda possesses five recombination genes located to the right of the tail genes. Two of these are required for site-specific recombination (*int* and *xis*) of the viral chromosome into the bacterial chromosome. The other three (*exo, beta, gam*) are needed for general recombination.

Lambda is a temperate phage which makes a choice when its genome enters a cell between lysis and lysogeny of the host. In the lytic pathway there is replication of the injected genome, packaging of the phage DNA into the phage particles and lysis of the host cell. Alternatively, in the lysogenic pathway the λ chromosome can insert within

the bacterial genome, shut down the expression of its genes and be regulated by the host. (Hershey and Dove, 1971). Three λ genes determine lysogeny, *cI, cII*, and *cIII*. The *cI* gene product codes for the repressor responsible for maintaining the repression of the lytic λ genes while the prophage is integrated within the host chromosome as a viral prophage during lysogeny. The CI protein is autoregulatory and is able to stimulate its own synthesis and thus promote lysogeny. The CI repressor is the major protein required for maintenance of the lysogenic state. Functional CI is a homodimer composed of two identical polypeptides that consist of two domains (Ptashne *et al*, 1980). CI binds to the same operator sites as Cro but in reverse affinity and thus with very different consequences. When the concentration of the repressor is low, only o_{R1} and o_{L1} are occupied by CI. Transcription from both p_R and p_L is inhibited and essentially phage replication and λ gene expression from the prophage is inhibited (Ptashne *et al*, 1980). As well, when repressor concentrations increase in the cell, CI can stimulate its own synthesis from p_M by binding to o_{R2} thus producing more protein required for the maintenance of the lysogenic state.

The *cII* and *cIII* gene products serve auxiliary roles to *cI* in lysogenization. CII protein, which is 97 amino acids long, acts as a transcriptional activator of three promoters p_E, p_I, and p_{antiQ} whose expression favors the establishment of lysogeny (Kobiler *et al*, 2002). CIII stimulates the lysogenic pathway by stabilizing the CII protein. In the establishment of the lysogenic pathway, the *cII* gene, part of the rightward transcript from p_R immediately downstream from *cro* is very important. The CII protein promotes lysogeny by acting as a transcriptional activator at two promoters, p_E and p_I. Expression of genes from these two critical promoters is needed for two essential aspects

of lysogeny, phage gene repression and prophage integration. The p_E promoter allows transcription of the *cI* gene. And the p_I promoter allows transcription of the integrase gene whose gene product integrates the λ DNA into the bacterial chromosome to form the lysogen.

Lambda possesses two genes, *N* and *Q*, whose products serve to permit (antiterminate) the transcription of other λ genes and are therefore referred to as positive regulators (Campbell, 1971). The N protein is required for maximal transcription of the two viral replication genes *O* and *P*. The *Q* gene product increases transcription of the head and tail genes mentioned previously as well as the lysis genes, *S* and *R*.

1.1.1. Lysis Versus Lysogeny Decision

In the establishment of the lytic pathway, transcription is initiated from three major promoters, p_L, p_R, and p_R'. The N protein is the first gene product transcribed from p_L and is the antitermination factor that permits the RNA polymerase to continue along the DNA past critical transcription termination sequences. These include t_{L1} mapping immediately to the left of *N* and t_{R1} mapping between *cro* and *cII* and $t_{R2\text{-}4}$ mapping to the left of *Q* between *P* and *Q*. This allows transcription of critical genes required for the lytic pathway. The N protein will bind to the RNA polymerase once a specific sequence (N utilization or nut) appears in the mRNA (Kameyama *et al*. 1999). An RNA loop is created that connects the nut site to the RNA polymerase and this interaction reprograms the polymerase so that it no longer recognizes the ordinary signals for transcription termination. The RNA polymerase with the N protein does not stop at leftward (t_L) or rightward (t_{R1}, $t_{R2\text{-}4}$) termination sites it encounters, permitting efficient delayed-early transcription control of the λ genes (Kameyama *et al*. 1999). For example, transcription

from p_L continues through the termination site t_{L1} through the recombination genes from p_R through t_{R1} through the replication genes *O* and *P*.

The first gene transcribed from the p_R promoter is *cro*. Cro is a regulatory protein critical for the development of the lytic pathway. The *cro* gene product prevents the synthesis of the λ repressor CI. As Cro accumulates in the infected cell, Cro protein binds to the same operator sites as bound by CI (in the reverse order) to prevent CI from stimulating its own synthesis from the p_M promoter.

Transcription from p_R' results in the expression of the late genes controlling lysis and virion assembly. p_R' maps to the right of *Q* and must be antiterminated by protein Q interacting with a *qut* site in the DNA.

1.1.2. λ Replication

Transcription initiated at the p_R promoter proceeds past t_{R1} into the λ replication genes *O* and *P* via the anti-termination activity of the λ immediate-early gene product N. Lambda replication initiation requires phage *trans*-acting initiator functions (products of λ genes O and P), a *cis*-acting target site termed the origin (*oriλ*), transcriptional activation in vicinity of *oriλ*, and host gene functions, that interact with P, with λ DNA near *oriλ,* and with other host functions. Bi-directional replication of the λ DNA requires the complexing of several phage and host proteins at *oriλ* comprising of four 18 base pair iteron sequences and a rightward high A-T rich DNA sequence. The first step to building the replication complex is the binding of the initiator O proteins to the iteron sequences to wrap and bend the DNA around themselves, producing a structure termed an O-some. This initial complex of *oriλ*-O, attracts λP bound to the host DnaB helicase. λP and DnaB bind to the replication complex through an interaction between O and P proteins

forming a preprisomal *ori*λ-O-P-DnaB complex (Taylor and Wegrzyn, 1995). Since P protein inhibits the DNA helicase activity of DnaB, λP must be removed from the complex to allow DnaB helicase activity (Taylor and Wegrzyn, 1995). The release of P requires the activity of three host chaperone proteins DnaK, DnaJ and GrpE so that DnaB regains activity and is able to interact with the bacterial DnaG primase and so PolIII holoenzyme can initiate DNA synthesis (Taylor and Wegrzyn, 1995). It is believed that DnaJ first binds to the preprisomal complex followed by the binding of DnaK, GrpE which stimulates the ATPase activity of DnaK.

The bidirectional replication of the λ DNA resembles the Greek θ (theta). Hence this early circle-to-circle replication is known as θ replication. After a few rounds of θ replication, there is a switch to another mode of replication which results in structures resembling the Greek letter σ (sigma) which is unidirectional and produces linear concatemeric DNA several λ DNA units long. Linear λ DNA is cut from the concatemers between the *cos* sites (cohesive ends) and packaged into proheads of progeny phages (Taylor and Wegrzyn, 1995).

1.2. Genetics of Host Cell Survival Following λ Infection or λ Prophage Induction

1.2.1. Replicative Killing Selection

In 1968, Pereira da Silva *et al*. examined cell survival following the induction of a nonexcisable *N*-defective λ*cI*[Ts] prophage. The product of *N* is required for antitermination of p_L transcription distal to *N* and the t_{L1} termination site through genes *ral-ssb-cIII-kil-gam-bet-exo-xis-int*. The *N* product is also partially required for p_R transcription beyond t_{R1} through *cII-O-P* and is essential for transcription through terminators $t_{R2\text{-}4}$ to gene *Q*. Induction of the λ prophage was accomplished by the

thermal inactivation of the *cI*[Ts]857 encoded repressor protein by physically shifting cells grown at 30°C to 42°C. The up-regulation of λ gene expression from the induced λ*N*⁻ prophage was believed to permit λ replication initiation, but no researcher has ever reported actually measuring the extent of λ replication initiation from a thermally induced *N*-defective λ prophage. Derepression of the trapped λ*N*⁻ prophage kills the host cells. PDS *et al*. found that rare survivor clones that formed at 42°C were defective in the expression of λ genes *O* and *P* required for prophage initiation. This has been termed the PDS selection (Hayes 1988b). Dove *et al.* 1971 used the term "replicative killing" to describe the loss of cell viability upon the thermal induction of a *N*⁻*cI*[Ts] λ prophage. Mutations that suppress replicative killing arise spontaneously and permit the RK⁻ (replicative killing defective) mutant cells to form colonies on agar plates incubated at 42°C. Among 50 RK⁻ mutants isolated in 1968 by the PDS selection, 5 carried mutations within λ p_R region and the remainder carried mutations that inactivated λ genes *O* and *P* (Hayes, 1988a). Brachet *et al.* (1970) used the PDS selection, to isolate a population of mutants. One in particular, r32, had an IS*2* insertion between t_{R1} and gene *O* in the λ chromosome which conferred a polar effect on the expression of distal genes, for example, *O* and *P*. Rambach (1973) used the same PDS selection to isolate mutations within *ori*λ. Dove *et al.* (1971) using the PDS selection reported RK⁻ survivor cells at a frequency of approximately 10^{-6}. Among the mutant population four were recessive *O* mutations, six were recessive *P* mutations, 10 would not recombine with *imm*434, 15 were *N*-suppressible polars, and 5 remained unclassified (Hayes, 1988a). All of the above studies permitted the localization of sites in the λ genome for initiation of replication and contributed to an understanding of the requirements for λ replication

initiation. Tentative conclusions were drawn suggesting that replicative killing of an induced λ*N*⁻ defective prophage was suppressed by null mutations in *O* and *P,* inactivation of p_R, polar IS*2* insertions between t_{R1} and gene *O*, and mutations in *ori*λ (Hayes, 1988a).

Hayes *et al*, (1983) modified the PDS selection system in the development of a simple forward selection scheme for independently determining the toxicity and mutagenic effect of environmental chemicals. They measured the replicative killing of *E. coli* by an integrated cryptic prophage fragment of λ and described their assay as a RK bacterial test or RK assay (Hayes *et al*, 1984,1991; Hayes and Gordon, 1984). The λ*N*⁻ *cI*[Ts]857 prophage was replaced by a N^+ *cI*[Ts]857 cryptic λ prophage to avoid the isolation of *N*-suppressible polar blocks (predominately isolated by Dove *et al,* 1971), and to ensure that transcriptional activation occurred during the selection so that *O* and *P* expression was not limiting. It was never clear in the PDS selection if transcriptional activation was occurring in induced *N*⁻*cI*[Ts] prophages. In addition, the defective cryptic prophage was made nonexcisable and incapable of cell lysis because most of the initial prophage (λ*cI*[Ts]857) was removed by a large $(ren\text{-}Q\text{-}S\text{-}R\text{-}A\text{-}J\text{-}b)^{\lambda}$-*bioA-D-uvrB-chlA* (renamed *moaA*) deletion, termed Δ431. This deletion removed all late prophage functions as *Q*, lysis functions, *S* and *R*, and the head and tail genes as well as *E. coli* DNA rightward from the *attR* attachment site through the *bio* operon. Other strains were constructed (Hayes *et al*, 1983; Hayes and Hayes, 1986) that retained Δ431 and also removed the *int-xis-exo-bet-gam-kil-cIII-ral* or *int-kil* regions of the cryptic prophage as described in the following section.

1.2.2. Construction of Strain Y836

Strain SA431 (Stevens *et al*, 1971) containing the cryptic prophage (λ*cI*857Δ*Q-R-A-Jb*) is deleted for the right part of the λ prophage and the contiguous *E. coli bio A-D*, *uvrB* through *chlA* (renamed *moaA*) genes by the Δ431 deletion. The SA431 prophage retains all the λ early control genes including an intact immunity region (p_L through *cro*) and a functional p_R-*cro-cII-O-P* operon, which encodes the replication proteins O and P and the *oriλ* origin site within *O* necessary for the initiation of λ bidirectional replication. Strain SA431 was shown to be competent for the initiation of λ replication (Stevens *et al*, 1971; Hayes, 1979). It was modified in order to delete the λ prophage recombination genes *int-xis-exo-bet-gam-kil* (i.e. the λ genes rightward from *att*L). These prophage genes were substituted with *E. coli* DNA by lysogenization of SA431with λ*bio*275*imm*434*cI*ts. The substitution of λ prophage genes *int-ral* or *int-kil* also permits N^+ expression without the expression of λ gene *kil* (Greer, 1975) whose expression can cause cell death, cell filamentation and reduced host DNA synthesis. Clones lysogenic for *imm*434[Ts] and *imm*λ[Ts] were selected. Next, the double lysogens created were transiently induced and cured of the λ*imm*434*cI*ts. Strain SA431*bio*275 (*imm*λ[Ts] lysogens) were isolated that no longer released phage upon thermal induction and the strain was designated Y836.

Figure 2a. Replicative Killing Scheme

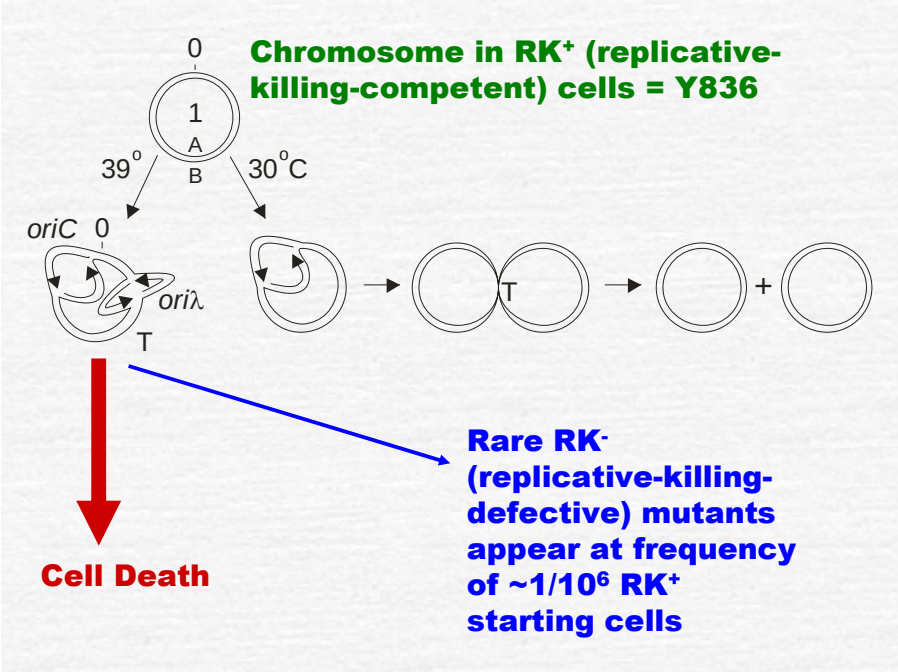


Figure 2a.

At 30°C, the CI[Ts] repressor protein is bound to *oriλ* inhibiting replication initiation of the cryptic λ prophage. Normal replication of the host *E. coli* chromosome occurs from *oriC*. At 42°C, the CI[Ts] repressor is inactivated and replication initiation from *oriλ* is induced. The majority of the time, the induction of λ replication from a cryptic prophage kills the host cell. Rare mutant colony forming units appear at a frequency of ~$1/10^6$ starting cells.

Figure 2b. Cryptic λ Prophage Fragment in Strain Y836

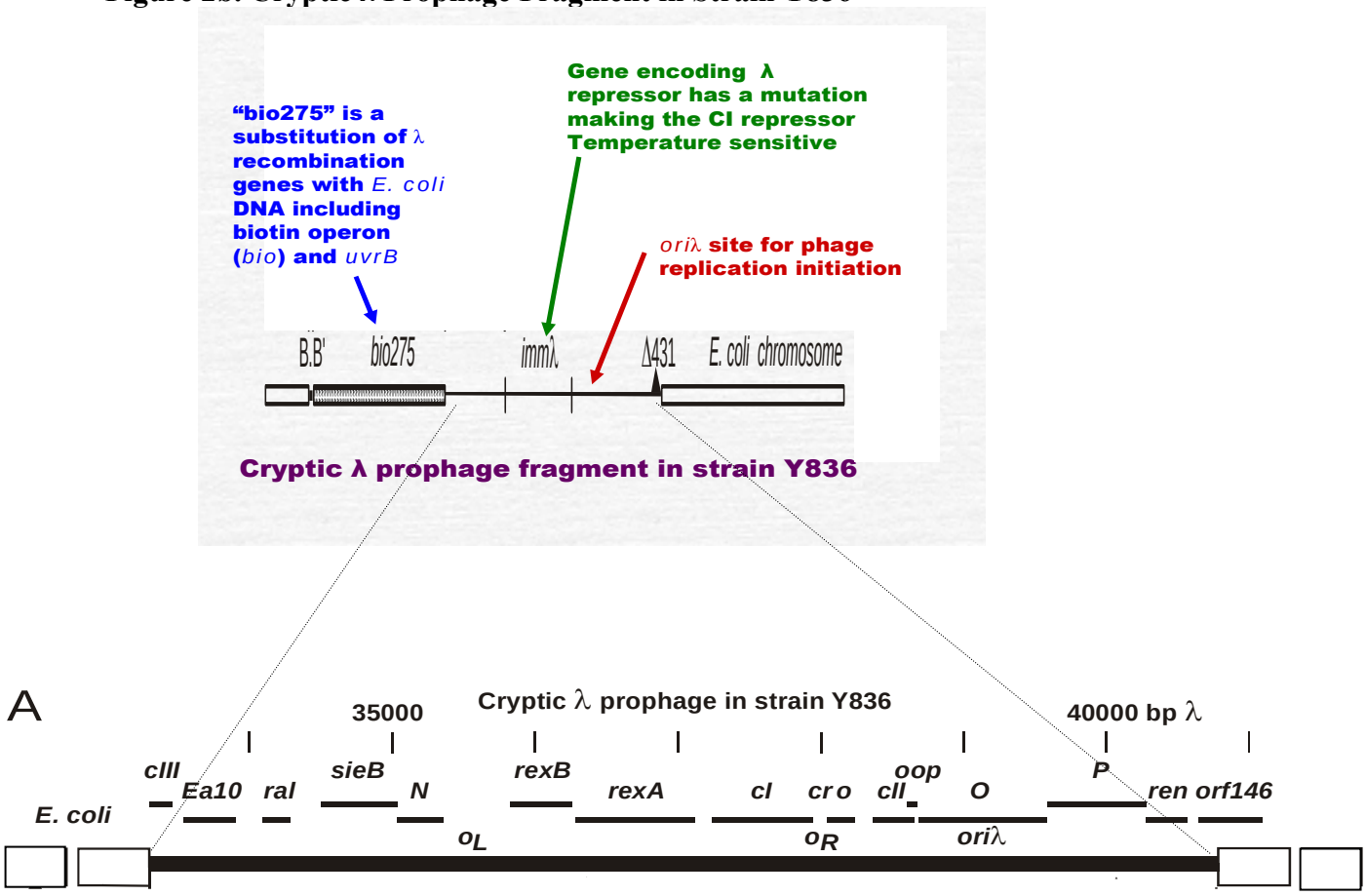


Figure 2b.

Cryptic λ fragment (dark line) integrated at the *att*$^{\lambda}$ site of the *E. coli* chromosome (open boxes) in strain Y836. Mutation *cI*857 confers temperature sensitivity at ≥39°C. 15.4 % of the λ genome is present in the cryptic prophage in strain Y836. The λ genes *int-kil* were substituted with *E. coli* DNA through ~ base 33,303 of λ. Deletion Δ431 is within a 46 base interval between λ base 40764 and 40810 (Hayes, 1991) and removes λ genes *nin-Q-R-A-J* and *E. coli* genes *uvrB* and *bio*. CI[Ts] binds at o_L and o_R blocking rightward and leftward transcription at 30°C. *Oriλ* is the site for prophage replication initiation when the repressor is thermally inactivated at 42°C.

1.2.3. The Nonimmune Exclusion Phenotype (Nie)

In 1986, Hayes and Hayes isolated RK^- survivor clones that exhibited normal λ immunity at 30°C but when placed at 42°C, the survivor clones excluded the plating of both λ wild type and the heteroimmune λ*imm*434. This phenotype was designated nonimmune exclusion (Nie). Among the RK^- Nie mutants isolated, classes A and B were identified. The class B survivors were distinguished because they permitted the formation of rare plaques at a somewhat higher frequency than did type A survivors. Each of seven rare λ wild type phage mutants that were able to plate on the class B Nie mutant cells at 42°C had acquired a single point mutation within o_{R1} or o_{R2} (Hayes and Hayes, 1986).

Seven independently arising class B mutant cells were chosen for characterization of the mutations conferring the RK^- and the Nie phenotypes and were subjected to a detailed genetic and physical analysis (Hayes *et al*. 1998). Six of the seven mutants had acquired a mutation that mapped to the right of the marker *Oam205* situated at the extreme C-terminal end of λ gene *O*. The other mutation in RK^- mutant designated 141e mapped to the left of *Oam205*. Further analysis revealed IS*2* insertions about 1300 base pairs within the λ prophage fragment in five of the seven RK^- class B Nie mutants. Four of the IS*2* insertions were at different sites within λ gene *P*. Mutant 141e had two IS*2* insertions. One was just left of *rexB* within the immunity region. The other was just to the left of the *oriλ* iteron sequences in gene *O*. Two other RK^- Nie mutants that had no apparent insertions were sequenced through genes *O* and *P*. These two mutants had 12 base pair substitutions in common within *O* and *P* and one additional mutation (either a point mutation or a single base pair deletion) within *P*. (Figure 3)

Figure 3 RK⁻ mutants isolated by Hayes, 1998: 145d and 101b

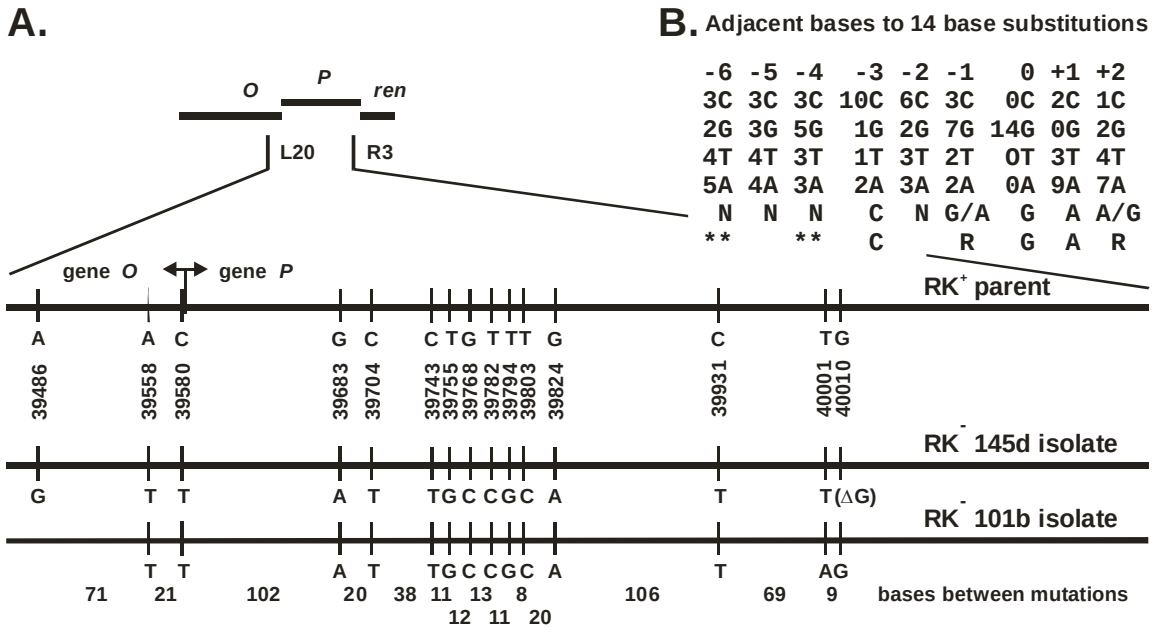


Figure 3. The two RK⁻ mutants (denoted as 145d and 101b) were isolated by Hayes *et al* (1998). The RK⁻ mutants had acquired 13 and 14 point mutations within the λ genes *O* and *P* which incapacitated λ replication and thus allowed cell survival and colony formation at 42°C.

Only two of the 12 substitutions were missense mutations, and the remainder was silent. The common 12 substitutions were symmetrically distributed, suggesting that they might localize to an unrecognized *cis*-acting target site for λ replication initiation.

It appeared that these common 12 point mutations in the two independently arising RK^- Nie mutants arose through replication errors occurring upon induction of the cryptic λ prophage, or they were rescued by prophage recombination with partially homologous DNA sequence within the *E. coli* chromosome. The former possibility suggested that the mutations arose adaptively during the selection for RK^- clones.

1.3. Bacterial and Phage Systems Developed for Measuring Mutation.

High-fidelity replicative DNA polymerases generate spontaneous errors when copying DNA, with mutation rates ranging from 10^{-4} to 10^{-5} per base pair (Tippin *et al*, 2004). Proofreading exonucleases associated with the DNA polymerase reduce spontaneous polymerase error rates to within the range of 10^{-5} to 10^{-7} (Tippin *et al*, 2004. The rate parameters/calculation were not given). Earlier estimates of the normal mutation frequencies (which we employ and will define herein) in bacteria, viruses or animal cells were stated as 10^{-7} to 10^{-9} per base pair per round of replication (Sinha and Goodman, 1983). Assay systems have been developed that detect increases in mutation rates. In 1984, Miller and Low used a *lacI-lacZ* fusion system within *E. coli* to detect SOS-induced mutations. The SOS response is responsible for inducing more than 20 genes enabling *E. coli* to cope with UV or chemically induced chromosomal damage. Miller *et al* used an *E. coli* strain they called *tif-1*. This strain was repressed for SOS genes at 30°C but becomes constitutive for their expression at 42°C because the RecA co-protease activity is more easily activated. Miller *et al* looked for nonsense mutations arising

within about 80 nonsense sites in *lacI* to monitor the occurrence of base substitutions. The *lacI* nonsense mutation assay allowed Miller *et al* to identify base substitutions generated by the SOS system. There was an increased level of spontaneous mutagenesis in the *tif-1* strain. In addition, rifampicin resistant mutants were as much as 80-fold more frequent in the *tif-1* strain than in a tif^{+} strain. Miller found that $lacI^{-}$ nonsense mutations formed by base substitutions were stimulated about 25-fold following the induction of the SOS response. The nonsense mutations were exclusively G:C to T:A and A:T to T:A transversions with G:C to T:A predominating. The appearance of these mutations would be explained some five years later by the finding that PolB and mutator polymerases DinB and UmuCD are part of the SOS regulon and are negatively regulated by the LexA repressor.

Standard high fidelity DNA polymerases are often hindered in fork progression when confronted by damaged template bases, causing replication to stall (Tippin *et al,* 2004). However, low-fidelity error-prone polymerases are able to copy damaged DNA templates permissively and efficiently alleviating cell death, but at the cost of an increase in cellular mutation. Typical error-prone polymerase error rates on undamaged DNA templates range from 10^{-1} to 10^{-3} per base pair (Tippin *et al*, 2004). Thus, it is imperative that cells regulate both when and where error-prone polymerases are used to reduce the chance that mutations occur when copying DNA. The SOS-induced polymerases are primarily involved in error-prone translesion synthesis pathways, allowing replication beyond the block to PolIII (Tippin *et al,* 2004). The error-prone polymerases IV and V exhibit broad specificity translesion synthesis and copy undamaged DNA with extremely poor fidelity. *In vivo* translesion synthesis by polymerase V includes base substitutions

that are generated in a two step mechanism. First, a nucleotide is misincorporated directly opposite a damaged DNA nucleotide followed by accurate DNA synthesis downstream from the lesion. DNA polymerase IV replaces polymerase III holoenzyme at the replication fork and continues DNA synthesis (Wagner & Nohmi, 2000). Polymerase IV adds one nucleotide onto the primer end that is mismatched (Goodman & Tippin, 2000). This process has the potential to be very mutagenic due to the error prone activity of polymerase IV. Once polymerase IV dissociates, polymerase III can re-associate with the DNA template and continue DNA synthesis, this is called a DNA polymerase switch. The errors induced by polymerase IV can be repaired by a mismatch repair system (Fuchs *et al*, 2004).

In 1985, Skopek *et al.* developed a lambda phage/*E. coli* system that could be used to determine changes in base sequence induced by various mutagenic agents. The genetic target in the system was the *cI* repressor gene. λ mutants defective for *cI* cannot form stable lysogens and are identified by their clear-plaque morphology. The use of a lambda system for studying mutagenesis in *E. coli* has several advantages. Skopek states that whether lambda is growing lytically or as a lysogen, host *E. coli* replication enzymes are used. Therefore the response seen should be characteristic of that occurring in the *E. coli* genome. Also, lambda can be exposed while in the *E. coli* genome (lysogen) or can be exposed separately as either intact phage or naked DNA. This allows separate exposure of the host cells to mutagenic treatment in order to induce the SOS response required for mutagenic expression of many compounds. This provides greater flexibility in the experimental design. These researchers studied base alterations induced in the *cI* gene when lambda was irradiated with 30 J/m^2 UV. Their results showed that

approximately two-thirds of the mutations were transitions and the remaining one-third was almost equally divided among transversions, frameshifts, and double mutation events.

In 1994, Santos and Drake used a T4 mutagenesis assay system to investigate whether various *E. coli* gene products influenced the fidelity of bacteriophage T4 DNA replication dependent on T4 DNA polymerase. T4 strains consisted of wild type and various *rII* mutants chosen for their ability to revert by the pathways expected to be promoted if a host mutator acted on T4 with the same specificity it displays on its own genome. They screened host genes for their influence on spontaneous mutation rates in T4 including: *mutT* (enhances A:T to G:C transversions), *polA*, *polB*, and *polC* (DNA polymerases II and III respectively), *dnaQ* (exonucleolytic proofreading for Pol III), *mutH, mutS, mutL*, and *uvrD* (methyl-directed mismatch repair), *mutM* and *mutY* (excision repair of oxygen-damaged DNA), *mutA* (unknown function), and *topB* and *osmZ* (affecting DNA topology). They concluded that the proteins involved in *E. coli* replication fidelity did not increase the T4 spontaneous mutation rates within a resolving power of about twofold. This would suggest that factors influencing host replication fidelity cannot be assumed to influence phage replication fidelity and presumably vice versa.

1.4. Hypothesis for Current Study.

Based upon the high frequency of spontaneous RK^- Nie base substitutions in the λ genes *O* and *P*, we hypothesize that cell stress, resulting from λ replication initiation from the trapped cryptic prophage in assay strain Y836 will increase the frequency of

replication errors within the simultaneously replicating chromosome of the host *E. coli* cells.

We predict that there will be an increased frequency of untargeted mutations within the host *E. coli* chromosome of survivor RK^- mutants. We define an "untargeted" mutation as being unlinked to the DNA damage responsible for the RK^- mutation that incapacitated replication initiation from the cryptic λ fragment.

We assume that some of the untargeted DNA lesions will arise within nutritional genes and contribute to the formation of auxotrophic mutations which can be detected within a population of RK^- mutant cells. The RK^- auxotrophic cells will be incapable of forming a colony on minimal medium.

2. Materials, Methods and Reagents

2.1. Bacterial and bacteriophage strains, and plasmids.

2.1.1. *E. coli* strains Employed.

Table 1 *E. coli* Strains

Strain	Relevant Genotype	Source	Strain #
Y836 (18)	SA500 λ*bio*275 *cI*[Ts]857 Δ431 *his*$^-$	Hayes *et al*,1986	NY1049
Y836*ΔrecA*	*recA* (Δ*srlR-recA*306::Tn*10*)	K. Asai this lab	NY1048
Y836 *dnaB*grpD55	*dnaB*grpD55*malF*::Tn*10*	K. Asai this lab	NY1050
594	F$^-$ *lac*-3350 *galK*2 *galT*22	B. Bauchmann	B10-c
594::(*cIII-ren*)$^{\lambda}$	Tn*10* [*zbh29* at 17 min] *bio*$^+$ transductant: assumed 594 *bio*275 (λ*cIII-cI[*Ts*]857-ren*) Δ431 = **	This study	NY1057
Y836::Tn*10* [*zbh29*]		This study	NY1047
594::(*cIII-ren*)$^{\lambda}$ [pCI]	**	This study	NY1055
CAG12147=*nad*A57::Tn*10* at 16.85 minutes	λ$^-$, *nadA57*::Tn*10*, *rph*-1	C.A.Gross	NY1053
Y836*his*$^+$	*his*$^+$	This study	NY1046
Y836*his*$^+$[pCI]	Amp^R *imm*λ CI$^+$ mid copy	This study	NY1056
Y836 *dnaB*grpD55[pCI]		This study	NY1054
W3101	*gal*T22 IN(rrnD-rrnE)	B.Bauchmann	B25-b
W3101::(*cIII-ren*)$^{\lambda}$	**	This study	NY1051
W3101::(*cIII-ren*)$^{\lambda}$[pCI]	**	This study	NY1059
SF2006	*dinB*	M. Goodman	NY1045
DE407	DE258 but is *lexA*-3, *malB* ::Tn*9* ; *sul*A211, *recA*$^+$,*src*$^+$	Don Ennis	NY1062
GW3200	AB1157*umuD*$_{44}$	Grahan Walker	NY1063
Y836 ilr566a [$N^+O^+P^-$ RK$^-$ mutant]		Hayes lab	NY10
Y836 ilr544c [$N^+O^+P^+$ RK$^-$ mutant]		Hayes lab	NY10
Y836 ilr534c [$N^+O^-P^-$ RK$^-$		Hayes lab	NY10

mutant]			
594 :(*cIII-ren* ilr566a)$^{\lambda}$	**	This study	NY1065
594::(*cIII-ren* ilr544c)$^{\lambda}$	**	This study	NY1061
594 :(*cIII-ren* ilr534c)$^{\lambda}$	**	This study	NY1064

2.1.2. Bacteriophage.

Table 2 Phage Strains Used

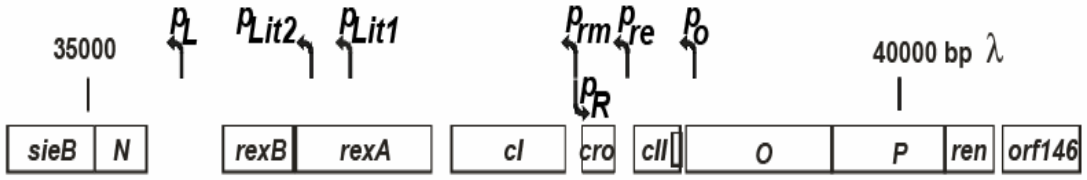
Phage	**Lysate Collection #**	**Source**
λ*cI*857	121	Hayes lab
λ*vir*	556	Hayes lab
λ*cI*72	135	Hayes lab
λ*cI*857(*18,12*)P22	395	Hayes lab
λ*imm*434*cI*ts *P*3	521	Hayes lab
λ*imm*434 *cI*ts	524	Hayes lab
λ*imm*434*cI*ts *O8*	518	Hayes lab

2.1.3. Plasmids.

Table 3 Plasmids Used

Plasmid	Vector Contents	Source
pHB30nl-42	pBR322[375-4286] containing bp λ inserted at 34499-34696; 36965-38103; 38814-40806. (See below map.)	Isolated and sequenced by M. Horbay this lab. Is a *cI*[Ts]857 to cI^{+} revertant. Derived from pHB30 (Bull, 1995)

Plasmid Map of [pHB30nl-42]



λ Genes in Plasmid

34499-34696 36965-38103 38814-40806

2.2. Reagents, Growth Media and Culture Incubation.

2.2.1. Solid support media.

i) Tryptone broth plates include 10 grams per liter of Bacto tryptone, 5 grams per liter of NaCl, 11 grams per liter of Bacto-agar, and 0.67 mg/ml thiamine (Arber *et al*, 1983).

ii) Minimal medium plates include 0.001 M $MgSO_4$, 1% glucose, 100 ml of 10X M9 salts, and 13.2 grams per liter of Bacto-agar (Arber *et al*, 1983).

iii) Minimal medium plates plus casamino acids include 0.001 M $MgSO_4$, 1% glucose, 100 ml of 10X M9 salts, 13.2 grams per liter of Bacto-agar, and 0.3% casamino acids supplement (Arber *et al*, 1983).

iv) Minimal medium plates plus one amino acid supplement includes 0.001M $MgSO_4$, 1% glucose, 100 ml of 10X M9 salts, and 13.2 grams per liter of Bacto-agar and an amino acid at a final concentration of 0.1 mg/ml.

Table 4 Concentration of Amino acid used in the Minimal Medium plates

Nutrient	Final Concentration per ml of agar
Alanine	0.1 mg/ml
Arginine	0.1 mg/ml
Aspartic acid	0.1 mg/ml
Cysteine	0.1 mg/ml
Glutamic acid	0.1 mg/ml
Glutamine	0.1 mg/ml
Glycine	0.1 mg/ml
Histidine	0.1 mg/ml
Isoleucine	0.1 mg/ml
Leucine	0.1 mg/ml
Lysine	0.1 mg/ml
Methionine	0.1 mg/ml
Phenylalanine	0.1 mg/ml
Proline	0.1 mg/ml
Serine	0.1 mg/ml
Threonine	0.1 mg/ml
Tyrosine	0.1 mg/ml
Tryptophan	0.1 mg/ml
Valine	0.1 mg/ml

2.2.2. Liquid Growth Media.

i) Tryptone broth (TB) includes 10 grams of bacto tryptone and 5 grams of NaCl per liter. The broth is stirred until the ingredients dissolve, dispensed into 8oz bottles, and autoclaved for 30 minutes.

ii) Lauria broth (LB) includes 10 grams of bacto tryptone, 5 grams of bacto yeast extract, and 5 grams of NaCl per liter. The broth is stirred until the ingredients dissolve, dispensed into 8oz bottles, and autoclaved for 30 minutes.

Both the liquid and plate cultures were grown up at 30^{o}C unless otherwise indicated. 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. Buffers

i) ϕ80 buffer includes 5.8 grams of NaCl per liter and 1.2 grams per liter of Tris-HCl. The pH was adjusted to 7.6 with 12 N HCl and the buffer was dispensed into 8oz bottles and autoclaved for 30 minutes.

2.2.4. Molten Top Agar

i) Tryptone top agar is used for overlaying plates and includes 10 grams of bacto-tryptone, 5 grams of NaCl, and 6.5 grams of bacto agar per liter. The agar solution was stirred and the agar melted by placing the solution in an autoclave for 10 minutes. The resulting solution was stirred to distribute the melted agar and then dispensed into 8oz bottles and autoclaved for 30 minutes.

2.3. Protocols for Experimentation in Microbial Genetics.

2.3.1. Plasmid Isolation.

The Wizard *Plus* SV Miniprep DNA Purification System from Promega was used to isolate plasmid DNA. The centrifugation protocol was performed as outlined: A 10 ml overnight culture was centrifuged at 8000 rpm for 5 minutes. The pellet was resuspended with 250 ul of cell resuspension solution. 250 ul of cell lysis solution was added and the sample was inverted ~ 4 times. 10 ul of alkaline protease solution was added and the solution was inverted ~ 4 times and allowed to incubate at room temperature for 5 minutes. 350 ul of neutralization solution was added. The solution was centrifuged at top speed (~13000 rpm) in an Eppendorf microfuge tube for 10 minutes at room temperature. Cleared lysate was decanted into a spin column and centrifuged at top speed for 1 minute at room temperature. Flowthrough was discarded. 750 ul of wash solution was added and the solution centrifuged at top speed for 1 minute. Flowthrough was discarded. 250 ul of wash solution was added and the solution centrifuged at top speed for 1 minute. Flowthrough was discarded. The spin column was transferred to a sterile 1.5 ml microcentrifuge tube. 100 ul of nuclease free water was added and the solution centrifuged at top speed for 1 minute at room temperature. The DNA was stored at -20^{o}C

2.3.2. Transformation.

2.3.2.1. Preparing Competent Cells:

Competent cells were prepared by the calcium shock method. Tryptone broth was inoculated with an aliquot of overnight culture of the desired strain. The cells were allowed to grow at 30^{o}C to A575= 0.4. The cells were collected, chilled on ice, and centrifuged at 6K for 5 minutes. The pellet was resuspended to 0.5 X the initial volume

in cold 0.01 M NaCl and recentrifuged. The pellet was resuspended in 0.5 X the initial volume in 0.03 M $CaCl_2$ and held on ice for 30 minutes. After centrifugation, the cell pellet was again resuspended in 0.03 M $CaCl_2$ at 0.1 X the initial culture volume and held on ice for immediate use.

2.3.2.2. Transformation:

0.2 ml of calcium shocked cells were placed in a thin walled plastic tube. 0.2 μg of plasmid DNA was added and the mixture was left on ice for 60 minutes. The mixture was heat pulsed at 42°C for 2 minutes, then plunged into ice to chill. The entire mixture was then diluted into 1.0 ml tryptone broth, prewarmed to the transformation temperature and incubated for 60-90 minutes with very gentle shaking in a water bath set to the transformation temperature.

2.3.2.3. Selection for Transformants:

The mixture was spread on tryptone plates at dilutions of 10^{-7} and 10^{-8}, and on tryptone plus ampicillin plates (or some other antibiotic-containing plates to which the plasmid encoded resistance) at dilutions of 10^{-1}, 10^{-2}, and 10^{-3} to determine the efficiency of transformation. The tryptone agar plates were incubated at the transformation temperature overnight. The tryptone plus ampicillin agar plates were incubated up to 48 hours at the same temperature.

2.3.3. Replica Plating Assay.

A single colony of Y836*his*$^-$ was inoculated into tryptone broth and allowed to grow for ~20 hours. A 10-fold dilution series was used to dilute cells from which 0.1 ml aliquots were spread on TB agar plates. The TB plates were incubated until small colonies

appeared on the plates (~18 hours) after which the small colonies were replica plated onto MM+his and then to a TB control using the same velvet to ensure transfer of cells. The replica plates were incubated at 30°C overnight (~18 hours).

2.3.4. Stab Assay.

A single colony of Y836*his*$^-$ was inoculated into tryptone broth and allowed to grow for ~20 hours. A 10-fold dilution series was used to dilute out the cells so that ~100 cells ultimately were spread to a TB plate. TB plates were incubated overnight and every well isolated colony forming unit was picked with a toothpick and stabbed into a MM+his plate and then to a TB control to ensure transfer of cells. The plates were incubated at 30°C overnight.

2.3.5. UV Sensitivity Assay.

Strains defective in SOS regulated genes were tested for UV sensitivity in order to confirm the loss of the SOS gene function. The UV lamp was set at a height of 30 cm and warmed up for approximately 10 minutes on the short wave setting (254 nm). Fresh single colonies of the strains to be tested were streaked across TB plates. Regions of the plate were then exposed to UV light for 10 seconds, wrapped in tinfoil to keep dark and incubated overnight at 30°C.

2.3.6. EMS Spot Assay.

A fresh overnight culture of the desired strain was centrifuged at 8000 rpm and resuspended in φ80 buffer in the initial culture volume. Cells were centrifuged and resuspended a second time to ensure culture media was washed away. 0.1 ml aliquots of the washed culture cells were spread onto MM (+his) plates in quadruplet. Two of the

plates were then spotted in the middle with 5 ul of concentrated EMS. The four plates were incubated after allowing the EMS to be absorbed into the agar plate for at least 48 hours at 30^{o}C. We expected to see an increase in auxotroph to prototroph cells in the presence of the mutator polymerases as the concentration of EMS increased.

2.3.7. Replicative Killing Assay.

An isolated colony of assay strain was grown in TB to stationary phase (~20 hours) in a shaking water bath at 30^{o}C. The culture was centrifuged for 10 minutes at 8000 rpm. The supernatant was decanted and the cell pellet was resuspended to original culture volume in ϕ80 buffer. A 10-fold dilution series in φ80 buffer was used to dilute cells for plating. Minimal medium and tryptone broth plates were preheated to 42^{o}C for ~2 hours after which the resuspended RK^{+} cells were spread at 10^{-1}, 10^{-2}, and 10^{-3} dilutions. The RK^{-} survivor colony forming units were counted after 48 hours of incubation at 42^{o}C. The 30^{o}C cell titers were also determined on both minimal medium and TB plates and these plates were incubated inverted for 48 hours and the cell number determined. The extent of cell killing is shown by the cell titer at 42^{o}C / cell titer at 30^{o}C.

2.3.8. Auxotroph Typing.

Table 5 Amino acid, vitamins, and purine/pyrimidine pools for determination of auxotrophic requirements.

Pool	1	2	3	4	5	6
7	adenine	biotin	phenylalanine	alanine	arginine	Leucine
8	hypoxanthine	folic acid	serine	cysteine	ornithine	Glycine
9	cytosine	pantothenic acid	tryptophan	threonine	aspartic acid	Isoleucine
10	guanine	pyridoxin	tyrosine	sodium thiosulfate	proline	Histidine
11	thymine	thiamin	p-amino-benzoic acid	methionine	glutamic acid	Lysine
12	uracil	riboflavin	nicotinic acid	choline	inositol	Valine

The final concentration of each amino acid was 0.1 mg/per ml of agar.

The final concentration of each vitamin and purine/pyrimidine were as follows:

Table 6 Nutrient final concentrations for vitamins, and purine/pyrimidine pools.

Nutrient	Final Concentration
Adenine	0.05 mg/ml
Hypoxanthine	0.05 mg/ml
Folic acid	0.5 ug/ml
Ornithine	0.1 mg/ml
Pantothenic acid	0.5 ug/ml
Guanine	0.05 mg/ml
Pyridoxin	0.5 ug/ml
Sodium thiosulfate	0.05 mg/ml
Thymine	0.05 mg/ml
Thiamin	0.01 ug/ml
p-amino benzoic acid	0.5 ug/ml
Uracil	0.05 mg/ml

Riboflavin	2.5 ug/ml
Nicotinic acid	0.5 ug/ml
Choline	10 ug/ml
Inositol	5 ug/ml
Biotin	0.01 ug/ml

2.3.9. Preparation of P1vir Lysate.

An overnight culture of *E. coli* carrying the marker to be donated was streaked for single colonies on a TB agar plate containing the appropriate antibiotic. A single colony from this incubated plate was inoculated in TB broth and shaken for 16-20 hours at 30^{o}C. 0.1 ml of the culture was subcultured into 5 ml TB broth + 0.1% glucose and allowed to grow until the culture reached ~2x10^{8} cells (~2 hours). 5 ul of 0.5 M $CaCl_2$ was added to the 5 ml culture. 3 ml were removed to a new flask where 0.1 ml infecting P1*vir* was added and was incubated with the cells for 20 minutes at 30^{o}C in a shaking water bath. 7.5 ml of molten top agar was added to the 3.1 ml cell-phage mixture and the solution was layered onto a TB agar plate. The plate with the 10.6 ml hardened overlay was incubated overnight at 37^{o}C. The overlay agar was then scrapped off into a 30 ml corex centrifuge tube. 6 ml of TB broth was added to the tube along with 100 ul of chloroform and the slurry was mixed. This primary (1^{o}) lysate was centrifuged for 10 minutes at 8000 rpm and the supernatant decanted and transferred to a sterile capped bottle which was stored 4^{o}C. Every P1*vir* lysate was serially passaged twice on the host strain, and only the secondary (2^{o}) lysate was used for the P1 transduction assay to avoid any carry-over of markers from the original P1*vir* lysate into the current transduction assay.

2.3.10. P1 Transduction for Strain Construction.

The recipient *E. coli* strain was grown in 20 ml TB broth overnight. 0.5 ml 15 mM $CaCl_2$ 30 mM $MgSO_4$ and 0.1 ml of the twice passaged P1*vir* lysate were mixed with 0.1 ml of the overnight culture of recipient cells. A cell control was made in parallel to above using 0.1 ml TB broth in place of the 0.1 ml of the twice passaged P1*vir* lysate. The phage and cell mixture was incubated for 20 minutes at 30°C and then centrifuged for 10 minutes at 7K. The supernatant was decanted and the tiny pellet was resuspended in 0.5 ml TB broth containing 20 mM sodium citrate. The resuspended cells were incubated for 60 minutes at 30°C. Aliquots (0.1 ml) of this transducing mixture were spread to minimal medium plates containing 15 ug/ml of the selection antibiotic plus 20 mM sodium citrate. The spread plates were incubated overnight at 30°C. A P1-only control (without cells) was also plated in order to ensure the lysate contained no contaminating donor cells.

2.3.11. Moving Cryptic λ Prophage Between *E. coli* strains.

Strain CAG12147 possesses a tetracycline resistant (Tet^R) marker (i.e. Tn*10* insertion) at 17.0 min on the *E. coli* genetic map. Using P1 transduction, the Tet^R marker was moved into the Y836his^+ strain and tetracycline resistant colonies were isolated. The second step was to co-transduce the Tet^R marker plus the cryptic λ prophage genes $(cIII\text{-}ren)^{\lambda}$ present in strain Y836 into two prototrophic strains 594 and W3101. First, 594 and W3101 tetracycline resistant colonies were isolated. Then the tetracycline resistant colonies were screened for their acquisition of the λ fragment phenotype. This was accomplished by stabbing the single colonies into minimal medium + 15 ug/ml

tetracycline plates at 30°C and 42°C. Colonies that did not grow at 42°C were assumed to have acquired both the Tet^R marker and the cryptic λ prophage since replication initiation from the integrated λ fragment kills the host cells that are shifted to 42°C. These cfu (from master plate) were crossed streaked against λ*vir* and λ*cI*857 to confirm that they acquired the new *imm*λ phenotype, which identifies the presence of a cryptic λ prophage fragment. The resulting constructs were designated 594::(*cIII-ren*)$^{\lambda}$ and W3101::(*cIII-ren*)$^{\lambda}$.

2.3.12. P1 Transduction used for moving the *lexA3*[Ind] mutation to Y836.

Strain CAG12164 has a *malF*::Tn*10* Tet^R marker at ~ 91.5 minutes on the *E. coli* linkage map. Using P1 transduction, the Tet^R marker was moved into strain DE407*lexA3*[Ind] and tetracycline resistant colonies were isolated. The Tet^R colonies were examined for increased sensitivity to UV to confirm that they retained the *lexA3*[Ind] allele. These cells were used as donors to co-transduce the Tet^R marker plus the *lexA3*[Ind] allele from DE407*malF*::Tn*10* into the recipient strain Y836. First, Y836 tetracycline resistant colonies were isolated. Then the tetracycline resistant colonies were screened for their acquisition of the *lexA3*[Ind] phenotype by assaying for increased UV sensitivity. Note that the *bio*275 addition confers $UvrB^+$ and Bio^+ phenotypes to cells with a Δ431 deletion. Colonies that were both tetracycline resistant and very UV sensitive were assumed have acquired both the Tet^R marker and the *lexA3*[Ind] mutation. These cfu (from 30°C master plate) were crossed streaked against λ*vir* and λ*cI*72 to verify the presence of the cryptic prophage and were shown to be lysed by λ*vir* and resistant to λ*cI* when assay plates were incubated at 30°C.

2.3.13. P1 Transduction Protocol used for moving the *dinB* mutation into Y836.

Strain SF2006 has a *dinB*::Tn*5* allele located at 5 minutes on the *E. coli* linkage map and is able to form colonies on agar plates containing kanamycin. Using P1 transduction, the Kan^R marker was moved into Y836 assay strain and kanamycin resistant colonies were isolated. The EMS spot assay was performed on the Kan^R transductants to further verify the *dinB* allele.

2.3.14. P1 Transduction Protocol used for moving the RK^- prophage from Y836 ilr (Initiation of lambda replication defective mutants of Y836) strains into 594.

Strain CAG12147*nad*A57::Tn*10* has a Tn*10* insertion conferring Tet^R phenotype at 16.85 min on the *E. coli* linkage map. Using P1 transduction, the Tet^R marker was moved into the Y836ilr strains and tetracycline resistant colonies were isolated. (Note that ilr = initiation of lambda replication defective, and these strains carry λ mutations preventing λ replication.) The second step was to co-transduce the Tet^R marker plus the cryptic ilr $(cIII\text{-}ren)^{\lambda}$ prophage into strain 594. First, 594 Tet^R colonies were isolated. Then the 594 Tet^R colonies were screened for their co-acquisition of the $(cIII\text{-}ren)^{\lambda}$ fragment phenotype. This was accomplished by cross streaking against λ*vir* and λ*cI*72 to assay for the acquisition of an *imm*λ phenotype, which identifies the presence of a cryptic λ prophage fragment in Y836 cells. Colony growth at both 30^oC and 42^oC was also checked to confirm the ilr phenotype property of the $(cIII\text{-}ren)^{\lambda}$ transductants.

2.3.15. Functional Immunity Assay.

Deletions within the *imm-rep*$^{\lambda}$ region of a cryptic λ prophage can be detected using the Functional Immunity (FI) spot assay. The assay is based upon rescue of the *imm*λ region of the cryptic prophage by a heteroimmune, λ*imm*434 infecting phage. Cryptic λ fragments deleted for the *imm*λ region, which includes promoter p_R for *O-P* transcription will not support *imm*λ marker rescue. RK$^-$ clones were stabbed into agar overlays containing W3350(λ*imm*434T) lysogen plus λ*imm*434*cI*$^-$ and incubated overnight at 39^{o}C. A zone of lysis in the cell lawn surrounding a stab indicated phage λ*imm*434*cI*$^-$ could infect and exchange by a double recombination event its *imm*434 region with the *imm*λ segment within the chromosome of the stabbed clone, producing a viable phage recombinant capable of growing lytically in the W3350(λ*imm*434) indicator lawn. Clones in which *imm*λ marker rescue was possible (yielding a lysis spot) were designated as "functionally immune" (i.e. FI$^+$) and those without signs of lysis were designated FI$^-$. The FI$^-$ phenotype implied that the mutation responsible for the RK$^-$ phenotype occurred within the *imm*λ region of the λ fragment, or within a contiguous DNA interval, and prevented a double recombinational exchange (*imm*434 for *imm*λ).

3. Results.

3.1. Preliminary Results, Predictions and Hypotheses.

Derepression of replication initiation from a trapped cryptic λ prophage kills the host cells. Cells in which λ replication can be induced are termed RK^+, i.e. replicative killing competent cells. Rare mutant cells survive at a frequency of $\sim 10^{-6}$ that are able to form a colony at 42°C. These survivor clones are designated as RK^- (replicative-killing defective) mutants (Hayes 1988b). RK^- mutants are subdivided as follows: (i) those unable to support λ*vir* plating at 42°C or (ii) those that support efficient plating by λ*vir* at 30°C - 42°C.

We hypothesize that cell stress, resulting from λ replication initiation from the trapped cryptic prophage will increase the frequency of replication errors within a simultaneously replicating *E. coli* chromosome. Previous results have shown that upon the induction of λ replication initiation from the trapped cryptic prophage, λ replication-initiation defective mutants arose that had acquired 13 and14 point mutations within the λ replication initiation genes *O* and *P (*Hayes *et al*, 1998), as shown in Figure 3. We predict that the same process that produces these λ mutations will also induce untargeted mutations within the host cell chromosome. To assay for untargeted host mutations, the assay can be specific for a unique target such as inactivation of LacZ activity or the reversion of an existing mutation as in a nutritional gene or antibiotic resistance marker. The assay target size could be increased by assaying for example, for the appearance of auxotrophic mutations. We decided to assay for the co-acquisition of auxotrophic mutations within the RK^- mutants arising on rich medium from prototrophic RK^+ cell populations. We assumed that the appearance of auxotrophs within the RK^- mutants

could initially be estimated by comparison of the RK^- mutation frequencies determined on rich TB agar and minimal medium agar at 42°C. We then confirmed that some of the RK^- mutants isolated on rich TB agar had acquired an auxotrophic mutation that prevented their growth when stabbed to minimal agar. We define untargeted auxotrophic mutations as being unlinked to the spontaneous DNA damage responsible for the parallel RK^- mutation that actually incapacitated replication initiation from the cryptic λ fragment.

3.2. Plating Efficiencies on Rich and Minimal Medium of E. coli Strains.

Prototrophic RK^+ assay cells were tested for their ability to plate at equal efficiency on rich TB agar and on minimal medium (MM) agar at 30°C. For cells incubated at 30°C the prophage genes remain repressed and therefore they are not subjected to replicative killing.

A single colony of each strain to be compared was inoculated into rich TB broth and grown up overnight and then subjected to 10-fold serial dilutions in φ80 buffer. Aliquots of the serial dilutions were plated simultaneously on rich TB and minimal medium agar plates and allowed to incubate for 48 hours at 30°C. Each titer shown in Table 7 represents the average of two duplicate experiments. We show that the plating efficiencies for each of the various RK^+ *E. coli* strains used in this study were equivalent on MM and on TB at 30°C.

Table 7 Plating Efficiencies on *E. coli* Strains on Rich and Minimal Medium at 30°C

Strain	Cfu # on TB		Titer on TB	Cfu # on MM		Titer on MM (+his)
	10^{-7}	10^{-8}		10^{-7}	10^{-8}	
Y836	118,114 131,128	11,12 15,16	$1.2x10^{9}$	113,101 122,118	11,10 12,11	$1.1x10^{9}$
Y836*his*$^{+}$	127,121 123,131	10,13 12,15	$1.2x10^{9}$	131,128 125,130	12,15 11,10	$1.3x10^{9}$
594	61,45 100,94	5,5 11,12	$5.2x10^{8}$	69,70 92,90	5,8 9,10	$6.7x10^{8}$
594::(*cIII-ren*)$^{\lambda}$	68,66 83,77	5,10 6,9	$7.1x10^{8}$	72,61 87,91	9,8 10,11	$7.6x10^{8}$
W3101	26,28 32,31	3,2 3,3	$7.1x10^{8}$	25,23 29,31	4,1 3,1	$7.6x10^{8}$
W3101::(*cIII-ren*)$^{\lambda}$	20,16 29,26	2,1 3,5	$1.7x10^{8}$	14,15 17,22	2,1 2,3	$1.5x10^{8}$

3.3. Comparison of RK⁻ Frequencies on Rich TB Agar plates and M.M. Agar Plates at 42°C.

We examined whether any unselected auxotrophic mutations arose during the selection for RK^- mutants from Y836 RK^+ starting culture cells by comparing the frequency of appearance of RK^- mutants on rich TB agar and minimal medium at 42°C (Table 8). This screen for auxotrophs was the broadest possible target for random mutations. The frequency for appearance of RK^- mutants was 13-90 - fold less when selected on MM than on TB in four parallel assays. Since the starting RK^+ cells plated on both TB and MM with equal efficiency (Table 7), we tentatively propose that DNA lesions may arise in host nutritional genes during the selection for RK^- mutants.

In order to eliminate the possibility that the *his* mutation in Y836 influenced the appearance of RK^- mutants on MM, we transduced a Y836*his*$^+$ marker from strain 594 into Y836 *his* and selected for *his*$^+$ transductants on MM. A comparison of the RK^- mutation frequencies on rich TB and minimal agar at 42°C was determined using Y836 *his*$^+$, Table 9.

The RK^- mutants selected from Y836 *his*$^+$ arose at 31-fold higher frequency on TB than on MM at 42°C. Clearly, the *his* mutation in the original Y836 RK^+ strain was not responsible for the difference in RK^- plating frequencies found on rich TB and MM.

The experiments in Tables 8 and 9 showed that RK^- mutants arise at a 13-90-fold higher frequency on rich versus minimal medium. We hypothesize from this result that DNA lesions with the potential to be converted into auxotrophic mutations occur during the selection for RK^- mutants at 42°C.

Table 8 Comparison of RK^- mutant frequency determined on rich and minimal media for Y836

Strain	RK^- Titer at 42°C		RK^- frequency at 42°C		Freq. on TB/ Freq. on M.M.+his
	TB	MM+his	TB	MM+his	
Y836*his*$^-$	$4.7x10^4$	$2.4x10^3$	$2.0x10^{-6}$	$1.2x10^{-7}$	17
Y836*his*$^-$	$8.0x10^4$	$6.9x10^3$	$3.5x10^{-6}$	$2.8x10^{-7}$	13
Y836*his*$^-$	$3.2x10^4$	$3.5x10^2$	$1.1x10^{-6}$	$1.2x10^{-8}$	90*
Y836*his*$^-$	$1.9x10^5$	$5.4x10^3$	$5.6x10^{-6}$	$2.1x10^{-7}$	27

The values for titer and frequency were rounded off. The calculations for Frequency on TB / Frequency on MM+his are based on the actual frequencies obtained.

* For example, the raw data is $1.08x10^{-6} / 1.2x10^{-8} = 90$ whereas the table data calculation is $1.1x10^{-6} / 1.2x10^{-8} = 91.6$.

Table 9 RK^- mutant frequencies determined on rich and minimal media for Y836*his*$^+$ Transductant

Strain	RK^- Titer at 42°C		RK^- frequency at 42°C		Freq. on TB/ Freq. on M.M.
	TB	MM	TB	MM	
Y836*his*$^+$	$4.5x10^3$	$1.6x10^2$	$9.5x10^{-8}$	$3.1x10^{-9}$	31

Table 10 RK$^-$ mutant frequencies determined on rich and minimal media supplemented with Casamino Acids Supplement (not vitamin free)

Strain	RK$^-$Titer at 42^{o}C		RK$^-$ frequency at 42^{o}C		Freq. on TB/ Freq. on M.M.+CA
	TB	MM+CA	TB	MM+CA	
Y836*his*$^-$	$4.3x10^4$	$5.4x10^4$	$2.0x10^{-6}$	$2.9x10^{-6}$	0.7
Y836*his*$^-$	$8.4x10^4$	$6.6x10^4$	$4.4x10^{-6}$	$4.5x10^{-6}$	1.0

We examined if the addition of a Casamino acids (not vitamin free) supplement to MM agar plates would suppress the drop in RK^- frequency on MM. Table 10 shows that the RK^- frequencies on MM supplemented with Casamino acids were $2.9x10^{-6}$ and $4.5x10^{-6}$ which are equivalent to the RK^- frequencies obtained on TB which were $2.0x10^{-6}$ and $4.4x10^{-6}$. This resulted in AFI's of 0.7 and 1.0. This result supports the above hypothesis. The appearance of lesions or actual mutations within host nutritional genes during the RK^- selection would not be inhibitory to cell growth (appearance of an RK^- colony) on rich medium.

3.4. Determining if Unselected Auxotrophic Mutations Actually Appear Within Colonies Arising on Rich Agar Plates at 42°C.

We asked whether RK^- mutants selected on TB from prototrophic RK^+ culture cells had acquired auxotrophic mutations. Individual RK^- mutant colonies arising on TB after 48 hours incubation at 42°C were tested for acquisition of an auxotrophic mutation by stabbing them to minimal medium plates and TB plates incubated at either 30°C or 42°C, Table 11.

It is seen that auxotrophic mutations are co-isolated during the selection for RK^- mutant cells. We discuss elsewhere a model to explain the difference in RK^- frequencies on TB and MM. The model suggests that DNA lesions accumulate in nutritional genes during the RK^- selection and prevent growth of the RK^- colonies at 42°C on MM, although this remains to be tested.

Table 11 Stab Assay of Y836 RK$^-$ Clones Isolated on Rich TB Agar

Strain	Colonies picked from 42^{o}C TB plates	Stabbed Colonies able to Grow Up on		Auxotrophs per colonies picked from TB plates
		MM at 30^{o}C	MM at 42^{o}C	
Y836	99	84	56	43/99
Y836	98	93	76	*22/98

***** These 22 mutant colony forming units were screened for amino acid auxotrophy and 5/22 were shown to be bio$^-$ and the remainder 17/22 are described in Section 3.12. ~5% of the auxotrophic mutants isolated in this experiment were bio$^-$ and therefore, all the RK$^-$ mutants isolated are not bio$^-$.

3.5. Assessing if the Unselected Auxotrophic Mutations are Linked to the Cryptic λ Prophage.

Strains with the Δ431 deletion are deleted for the biotin operon. The *bio*275 substitution in Y836 replaces the λ recombination genes with *E. coli* DNA includes the biotin operon that was deleted by Δ431 (Fig. 2b) conferring back a Bio^+ phenotype to Y836 (Hayes lab). We asked if the auxotrophic mutations arising in parallel with the RK^- selection were all biotin auxotrophs. For example a large deletion could remove part or all of the λ fragment and the adjacent biotin operon. Such a mutant would be RK^- but would also be a biotin auxotroph requiring biotin for growth. Therefore, we asked whether all of the RK^- auxotrophic mutants we isolated were biotin requiring? This required adding biotin to the minimal medium and then determining the RK^- frequencies on both rich TB agar and MM plus biotin agar.

Adding biotin to the minimal medium did not yield equivalent plating frequencies on rich TB agar and minimal medium plus biotin at 42°C (Table 12). This result shows that the addition of biotin to the media does not correct the difference in plating efficiencies at 42°C on MM and TB. In the second experiment in Table 11, we show that 5 of 98 RK^- mutants would not grow on MM at either 30°C or 42°C. Further analysis of these 5 RK^- mutants using the auxotroph typing plates referred to in Table 5, revealed that they were biotin requiring. We conclude that auxotrophic mutations arise during the selection for RK^- mutants, which are not biotin requiring, i.e., are Bio^+. We propose that these auxotrophic mutations are unlinked to the DNA damage responsible for the RK^- mutation. We suggest that these mutations arise within nutritional gene(s) in the *E. coli* chromosome during the selection for RK^- mutants.

Table 12 RK^- frequency of Y836 determined on rich and minimal medium supplemented with biotin

Strain	**RK^-Titer at 42°C**		**RK^- frequency at 42°C**		**Freq. on TB/ Freq. on M.M.+biotin**
	TB	MM+his+bio	TB	MM+his+bio	
Y836	$9.9x10^4$	$5.7x10^2$	$5.0x10^{-6}$	$3.3x10^{-8}$	150

3.6. Assessing if Strain Y836 Has An Intrinsic Mutator Activity.

To test if Y836 had an intrinsic mutator activity we transduced the cryptic λ fragment from Y836 into *E. coli* strains 594 and W3101, each of which are prototrophs and form colonies on minimal medium. The first step involved moving a Tet^R marker near to the cryptic λ fragment in Y836. The second step involved moving Tet^R plus the $(cIII\text{-}ren)^{\lambda}$ fragment into the recipient hosts. The results are shown in Table 13. The Tet^R $(cIII\text{-}ren)^{\lambda}$ transductants of 594 and W3101 were used to determine the RK^- mutant frequencies on rich TB agar and minimal medium. We wanted to learn whether prototrophic cells 594 and W3101 with an inserted cryptic λ fragment also yielded fewer RK^- mutant cells on MM at 42°C.

We found that the RK^- frequencies for 594$(cIII\text{-}ren)^{\lambda}$ and W3101$(cIII\text{-}ren)^{\lambda}$ did not differ significantly from those seen with Y836, showing a similar disparity of 42°C growth on rich versus MM agar. The RK^- mutant frequencies for these three strains were between 3- and 150-fold higher on TB than on MM at 42°C (Table 14). These results show that cells 594 and W3101 with an inserted cryptic λ prophage fragment also yield fewer RK^- mutant cells on MM at 42°C. Thus the reduction in RK^- colony frequency on MM compared to TB was not an intrinsic property of strain Y836, and the effect moved with the cryptic λ fragment to 594 and W3101. Therefore, Y836 does not have an intrinsic mutator activity. We define the difference in RK^- frequencies on TB versus MM as the AFI (auxotroph formation index) and assume that mutations conferring an auxotrophic phenotype to a cell will be recessive and that auxotrophic mutants will

Table 13 P1 cotransduction of cryptic λ prophage with selectable marker from the 17.0 min interval of the *E. coli* linkage map.

Strain	Selectable Phenotype	Tet^R Transductants	Tet^R λ+ Cotransductants	Linkage %
594	Tetracycline	89	30	34
W3101	Tetracycline	95	70	74

* The values for titer and frequency were rounded off. The calculations for Frequency on TB / Frequency on MM+his are based on the actual frequencies obtained.

Table 14 RK^- mutant frequencies determined on rich and minimal media for 594::*(cIII-ren)*$^{\lambda}$ and W3101::*(cIII-ren)*$^{\lambda}$.

Strain	RK^- Titer at 42°C		RK^- frequency at 42°C		Freq. on TB/ Freq. on M.M.
	TB	MM	TB	MM	
594::(*cIII-ren*)$^{\lambda}$	$1.3x10^4$	$6.6x10^2$	$1.1x10^{-6}$	$3.4x10^{-8}$	31
594::(*cIII-ren*)$^{\lambda}$	$4.5x10^4$	$2.5x10^2$	$3.0x10^{-6}$	$2.1x10^{-8}$	147
594::(*cIII-ren*)$^{\lambda}$	$1.1x10^4$	$5.0x10^2$	$5.2x10^{-7}$	$2.5x10^{-8}$	21
594::(*cIII-ren*)$^{\lambda}$	$5.2x10^3$	$7.1x10^2$	$4.2x10^{-6}$	$6.3x10^{-7}$	7.0
*W3101::(*cIII-ren*)$^{\lambda}$	$2.5x10^3$	$2.5x10^2$	$2.0x10^{-5}$	$1.7x10^{-6}$	9.0
*W3101::(*cIII-ren*)$^{\lambda}$	$2.0x10^3$	$7.5x10^2$	$3.8x10^{-6}$	$1.3x10^{-6}$	3.0

* Strain gives lower Frequency on TB / Frequency on MM for unknown reason. Nothing further was done with this strain since it was found harder to transduce (Table 13).

appear after a phenotypic lag (requiring at least two rounds of replication). The simplest hypothesis for the difference in RK^- frequencies on TB and MM is that DNA chromosomal perturbations (DNA lesions) accumulate (e.g., in nutritional genes) during the RK^- selection. Such lesions will prevent cell growth of the RK^- cells arising at 42°C on MM, resulting in a positive AFI value (= AFI >1). Therefore, if the above hypothesis is true, the AFI value would account for cells with DNA lesions and those with fixed mutations, each conferring an auxotrophic phenotype. (We expect that only some of the DNA lesions that arise will be fixed into mutations within a nutritional gene; whereas, others will be correctly repaired. In this work, we have not investigated the formation, nature or frequency of the proposed lesions.)

3.7. Testing the Influence of Various SOS Gene Products On The Appearance Of Auxotrophic Mutations Among The RK^- Population Isolated On Rich Media.

The LexA repressor binds to the operator regions of the genes regulated by the SOS response. Upon DNA damage, the LexA repressor is cleaved by an activated RecA gene product leading to the expression of the SOS regulon. A *lexA3*[Ind] mutant allele whose gene product is not cleavable was transduced into Y836 making the variant SOS induction deficient. This construct is referred to as Y836 *lexA3*[Ind] and was used to determine if induction of the SOS response is required for observing AFI's of >1. The AFI, possibly paralleling the co-formation of auxotrophic mutations within the population of RK^- mutant cells arising on rich medium, does not require induction of the SOS response (Table 15).

Table 15 Influence of the SOS Response on AFI

Strain	**RK^+ cell titer at 30^oC**		**RK^- 42^oC Titer**		**RK^- frequency at 42^oC**		**Freq. on TB/ Freq. on M.M.**
	TB	**MM+his**	**TB**	**MM+his**	**TB**	**MM+his**	
Y836*lexA3*[Ind]	$2.3x10^9$	$1.8x10^9$	$6.0x10^2$	$2.0x10^1$	$2.6x10^{-7}$	$1.1x10^{-8}$	24

* The values for titer and frequency were rounded off. The calculations for Frequency on TB / Frequency on MM+his are based on the actual frequencies obtained.

A deletion of *recA*, whose product is required for induction of the SOS response was introduced into Y836. The deleted strain was used to determine if a host function which is induced as part of an SOS response participates in the appearance of unselected auxotrophic mutations. The RK^- frequencies for Y836 Δ*recA* on MM+his are between 4- and 30-fold lower when compared to the RK^- frequencies on TB at 42°C, suggesting that the observed AFI is not dependent on the RecA protein (Table 16).

SOS genes *umuC umuD* and *dinB* encode mutator polymerases PolV and PolIV. Insertionally inactivated alleles of these genes (Table 1) were used to determine if the mutator polymerases were essential for whatever cellular effect accounts for achieving AFI's of >1, and for the appearance of auxotrophic mutations that were documented to arise within the RK^- survivors (Table 11). We show in Tables 15-17, that achieving AFI's of > 1 (defined previously) does not require induction of the SOS regulon, genes *dinB* (polymerase IV) or *umuD umuC* (mutator polymerase polV). (The influence of these genes on the appearance of auxotrophic mutations, is discussed in Section 3.11 and Table 22.)

3.8. Determining if Blocking λ Prophage Induction/Gene Expression Suppresses Auxotroph Formation and Eliminates AFIs >1..

Culture shift from 30°C to 42°C results in the thermal derepression of gene expression from the cryptic $(cIII\text{-}ren)^{\lambda}$ Table 1, and heretofore designated as $(cIII\text{-}cI[Ts]857\text{-}ren)^{\lambda}$ fragment and the initiation of λ replication due to inactivation of the CI [Ts] repressor. We introduced a mid-copy plasmid (Table 3) that expresses the wild type

Table 16 Influence of *recA* gene product on AFI

Strain	RK^+ cell titer at 30°C		RK^- 42°C Titer		RK^- frequency at 42°C		Freq. on TB/ Freq. on M.M.+his
	TB	MM+his	TB	MM+his	TB	MM+his	
Y836Δ*recA*	$6.0x10^9$	$3.1x10^9$	$2.8x10^4$	$1.9x10^3$	$4.7x10^{-6}$	$6.3x10^{-7}$	7.0
Y836Δ*recA*	$4.1x10^8$	$2.0x10^8$	$8.8x10^2$	$1.1x10^2$	$2.2x10^{-6}$	$5.2x10^{-7}$	4.0
Y836Δ*recA*	$2.4x10^8$	$2.3x10^8$	$1.4x10^3$	$4.5x10^1$	$5.7x10^{-6}$	$1.2x10^{-7}$	30
Y836Δ*recA*	$3.8x10^8$	$1.9x10^8$	$1.9x10^3$	$6.6x10^1$	$5.1x10^{-6}$	$3.5x10^{-7}$	15

Table 17 Influence of *umuC* / *umuD* and *dinB* gene product on AFI

Strain	**RK^+ Cell Titer at 30^oC**		**RK^- 42^oC Titer**		**RK^- frequency at 42^oC**		**Freq. on TB/ Freq. on M.M.**
	TB	**MM+his**	**TB**	**MM+his**	**TB**	**MM+his**	
Y836*umuC*	$5.3x10^9$	$5.8x10^9$	$1.04x10^5$	$1.89x10^3$	$1.96x10^{-5}$	$3.25x10^{-7}$	60
Y836*umuC*	$1.4x10^9$	$1.4x10^9$	$3.3x10^4$	$2.9x10^2$	$2.3x10^{-5}$	$2.1x10^{-7}$	110
Y836*dinB*::*kan*	$2.0x10^9$	$2.1x10^9$	$1.3x10^4$	$1.1x10^2$	$6.3x10^{-4}$	$4.7x10^{-8}$	133

CI repressor at both 30°C and 42°C into the *E. coli* cells with a cryptic λ fragment. This plasmid prevents the induction of λ gene expression and λ replication initiation when the cells are raised to 42°C. We show that the pCI^+ plasmid expressing wild type CI repressor at 30°C and 42°C, completely prevented replicative killing in strains Y836, 594, and W3101 (Table 18). The loss of replicative killing was indicated by the near identical efficiency of plating of the cells with cryptic λ prophage at both 30°C and 42°C on TB or MM, i.e. the AFI was approximately unity. Without λ gene expression there was no difference in the plating frequencies at 30°C or 42°C on TB or MM indicating that both the RK phenotype (formation of RK^- mutants) and observing AFI's > 1 depend upon λ gene expression. We examined the cfu arising at 42°C for auxotrophic mutations by a stab assay (Section 3.11) and no auxotrophs were observed among the 298 cfu stabbed (Table 23). We conclude that blocking λ gene expression and replication initiation from the cryptic $(cIII\text{-}cI[Ts]857\text{-}ren)^{\lambda}$ fragment eliminated both the AFI and co-formation of auxotrophic mutations. This result suggests that the accelerated formation of auxotrophic mutations is dependent upon either the gene expression and/or replication initiation of the cryptic λ prophage when the cells are shifted to 42°C (Table 18).

3.9. Experiments to Assay Whether Blocking λ Replication Initiation Suppresses Auxotroph Formation and Eliminates AFI's >1.

Certain mutations within the *dnaB* gene of *E. coli* prevent replication of phage λ. One of these mutations is grpD55 (Saito and Uchida, 1977; Bull and Hayes, 1996; unpublished sequence data Horbay and Hayes). This mutation completely prevents λ replication initiation at 42°C from the cryptic prophage in strain Y836 but does not prevent *E. coli* cell replication (Hayes and Hayes, unpublished). The grpD55 allele was

Table 18 RK^+ and RK^- plating frequencies of Y836, 594::(*cIII-ren*)$^\lambda$, and W3101::(*cIII-ren*)$^\lambda$ on TB and minimal medium when λ replication initiation and λ Gene Expression has been blocked

Strain	**RK^+ Cell Titer at 30°C**		**RK^- 42°C Titer**		**Freq. Cfu 42°C/ 30°C**		**Freq. on TB/ Freq. on M.M.**
	TB	**MM**	**TB**	**MM**	**TB**	**MM**	
Y836*his*$^+$[pCI]	$1.6x10^9$	$1.7x10^9$	$1.7x10^9$	$1.5x10^9$	1.06	$9.2x10^{-1}$	1.2
Y836*his*$^+$[pCI]	$5.4x10^8$	$5.1x10^8$	$4.2x10^8$	$4.0x10^8$	0.79	0.79	1.0
W3101::(*cIII-ren*)$^\lambda$ [pCI]	$1.9x10^8$	$1.8x10^8$	$2.2x10^8$	$2.1x10^8$	1.2	1.2	1.0
594::(*cIII-ren*)$^\lambda$ [pCI]	$1.3x10^9$	$1.2x10^9$	$1.2x10^9$	$1.1x10^9$	$9.2x10^{-1}$	$9.0x10^{-1}$	1.0

transduced into Y836*his* and the RK^+ and RK^- plating frequencies on TB and MM+his were determined (Table 19).

Essentially equal plating efficiencies were observed for Y836 *dnaB*grpD55 cells at 30°C and 42°C on rich TB agar plates (Table 19). Thus the presence of the grpD55 allele of *dnaB* prevented replicative killing. This result supports the observation that the introduction of a grpD55 allele of *dnaB* into Y836 was able to block λ replication initiation without preventing the expression of cryptic $(cIII\text{-}cI[Ts]857\text{-}ren)^{\lambda}$ genes at 42°C. The results in Table 19 also shows that λ replication initiation is not required for achieving a high AFI. The efficiency for Y836 *dnaB*grpD55 cells plating on TB at 42°C was 3- to 73-fold higher than it was on MM. From these results we suggest that DNA lesions preventing colony formation at 42°C likely appear within the population of Y836 *dnaB*grpD55 cells induced for λ gene expression, but blocked for replication initiation from the cryptic λ fragment.

Therefore, we conclude that the effect accounting for the reduction in colony formation on MM plates at 42°C is linked to the induction of gene expression from the cryptic λ fragment, but does not require actual replication initiation from *ori*λ. We examined 96 well isolated cfu arising at 42°C on rich TB agar for auxotrophic mutations by a stab assay and observed 4 colonies that had acquired auxotrophic mutations that prevented their colony formation on MM (Table 24). These results are dealt with further in the Discussion.

Table 19 Plating of Y836 *dnaB*grpD55 on TB and MM+his

Strain	**RK^+ Cell Titer at 30^oC**		**RK^- 42^oC Titer**		**Freq. Cfu 42^oC/ 30^oC**		**Freq. on TB/ Freq. on M.M.**
	TB	**MM+his**	**TB**	**MM+his**	**TB**	**MM+his**	
Y836 *dnaB*grpD55	$8.7x10^8$	$8.0x10^8$	$9.2x10^8$	$1.0x10^7$	1.03	$1.3x10^{-2}$	73.0
Y836 *dnaB*grpD55	$1.4x10^9$	$1.3x10^9$	$8.6x10^8$	$4.2x10^7$	$6.1x10^{-1}$	$3.2x10^{-2}$	19.2
Y836 *dnaB*grpD55	$4.0x10^8$	$4.0x10^8$	$4.3x10^8$	$1.6x10^8$	1.08	$4.1x10^{-1}$	3.0
Y836 *dnaB*grpD55 [pCI]	1.6x109	1.5x109	1.3x109	1.2x109	0.77	0.82	0.9

3.10. Assessment of the Frequency for Spontaneous Auxotrophs Arising Within RK$^+$ Cultures at 30^{o}C.

Based on the above results, we examined the frequency of spontaneous auxotrophs arising within RK$^+$ cultures at 30^{o}C. We wanted to measure if auxotrophs are detectable within an RK$^+$ cell population that was not induced for λ replication initiation and λ gene expression. This was assayed using replica plating (experiment #1), and stab assays (experiments #2-4). No spontaneous auxotrophs were detected among 3000 RK$^+$ Y836 and Y836 *dnaB*grpD55 colonies forming at 30^{o}C on TB agar plates (Table 20). This suggests that the auxotrophic mutants spontaneously arise within these cells at a frequency of <3-5x10^{-4}.

3.11. Determining if Unselected Auxotrophic Mutations Actually Appear Within Colonies Arising On Rich Agar Plates Among Cells Harboring a Cryptic Prophage.

We asked if auxotrophic mutations could be demonstrated within a population of RK$^-$ mutants. All of the RK$^-$ colonies arising on a 42^{o}C rich TB agar plate(s) spread with either Y836 or 594::(*cIII-cI[*Ts*]*857-*ren*)$^\lambda$ cells were stabbed to duplicate minimal medium plates that were incubated at 30^{o}C or 42^{o}C and to a TB master plate incubated at 42^{o}C (Table 21). The results clearly demonstrate that auxotrophic mutations arose within the RK$^-$ colonies selected at 42^{o}C on TB agar plates (control). Most of the acquired auxotrophic mutations were temperature sensitive for growth on minimal medium. The same assay was used to determine if auxotrophic mutations arise in Δ*recA* or *dinB* variants of Y836 (Table 22). Temperature sensitive auxotrophs were observed among

Table 20 Assessing the frequency for spontaneous auxotrophs arising within Y836 and Y836 *dnaB*grpD55 RK^+ cultures at 30^oC

Experiment #	Strain	Colony forming units on TB	Colony forming units on MM+his
1	Y836	254	254
2	Y836	996	996
3	Y836	750	750
4	Y836 *dnaB*grpD55	1000	1000
Total		3000	3000

Table 21 Auxotrophic mutants arising within Y836 and 594(*cIII-ren*)$^{\lambda}$ cfu selected at 42^{o}C

Strain	Colonies picked from 42^{o}C TB plates	Stabbed colonies able to grow on		Auxotrophs per colonies picked from TB plates
		MM at 30^{o}C	MM at 42^{o}C	
Y836	99	84	56	43/99
Y836	98	93	76	22/98
594::(*cIII-ren*)$^{\lambda}$	132	132	122	10/132

Table 22 Auxotrophic mutants arising within Y836Δ*recA* and Y836*dinB* cfu selected at 42°C

Strain	Colonies picked from 42°C TB plates	Stabbed colonies able to grow Up on		Auxotrophs per colonies picked from TB plates
		MM at 30°C	MM at 42°C	
Y836Δ*recA*	259	259	223	36/259
Y836*dinB*	100	100	92	8/100

RK^- colony isolates from both strains. (We consider these results further within the Discussion). The possibility that spontaneous auxotrophs form independently of λ gene expression within the assay strains harboring the cryptic λ fragment was investigated through the addition of a wild type CI repressor (encoded by pCI^+) which remains active at both 30°C and 42°C. This involved examining for the appearance of auxotrophic mutations within Y836, 594(*cIII-cI*857-*ren*)$^{\lambda}$ and Y836 *dnaB*grpD55 cultures, where both λ replication initiation and λ gene expression at 42°C was blocked by the addition of the CI^+ plasmid. No auxotrophic mutations were observed within colonies arising at 42°C (Table 23) from any of the above cultures.

We asked if auxotrophic mutations appear within colonies arising at 42°C when gene expression from the cryptic (*cIII-cI*857-*ren*)$^{\lambda}$ fragment is induced, but *ori*λ replication initiation is blocked by the host *dnaB*grpD55 allele. Colonies arising on rich TB agar plates were stabbed to TB and to minimal medium plates that were incubated at 30°C and 42°C (Table 24). About four percent of the stabbed colonies acquired an auxotrophic mutation. The results suggest that the induced (*cIII-cI*857-*ren*)$^{\lambda}$ fragment, which is defective for λ replication initiation, confers two phenotypes to the host cells grown at 42°C, including AFI's >1 *and* the appearance of auxotrophs within phenotypically RK^- colonies arising on TB at 42°C. We have no evidence that these two phenotypes are (or are not) interlinked. We nevertheless, describe a model that can link the two phenotypes in Section 4.5.

Table 23 Auxotrophic mutants arising within Y836[pCI], *594*::*(cIII-ren)*$^{\lambda}$ [pCI], and Y836 *dnaB*grpD55[pCI] cfu selected at 42^{o}C

Strain	Colonies picked from 42^{o}C TB plates	Stabbed Colonies able to Grow Up on		Auxotrophs per colonies picked from TB plates
		MM at 30^{o}C	MM at 42^{o}C	
Y836[pCI]	88	88	88	0/88
594::(*cIII-ren)*$^{\lambda}$ [pCI]	110	110	110	0/110
Y836 *dnaB*grpD55 [pCI]	100	100	100	0/100

Table 24 Auxotrophic mutants arising within Y836 *dnaB*grpD55 cfu selected at 42^{o}C

Strain	Colonies picked from 42^{o}C TB plates	Stabbed Colonies able to Grow Up on		Auxotrophs per colonies picked from TB plates
		MM at 30^{o}C	MM at 42^{o}C	
Y836 *dnaB*grpD55	96	92	92	4/96

3.12. *Further Characterization of RK⁻ Y836 Auxotrophic Mutants Isolated on Rich TB Agar.*

We characterized some of the RK⁻ mutants isolated on rich TB agar at 42°C. Individual RK⁻ colonies were stabbed to auxotroph typing plates (see Methods section 2.3.8.) to screen for unique auxotrophic mutations. The majority of the RK⁻ mutants isolated were temperature sensitive. They grew on minimal medium at 30°C but would not grow on minimal medium at 42°C. The RK⁻ colony forming units, for two separate RK assays, that would not grow on minimal medium at either 30°C and/or 42°C were stabbed into minimal medium plates supplemented with one amino acid. Fifteen phenylalanine auxotrophs were identified that would not grow on minimal medium at either 30°C or 42°C without the addition of phenylalanine to the minimal medium (Table 25). These 15 RK⁻ phe⁻ mutants were isolated together in one RK Assay. 13/15 were FI⁺ (refer to Materials and Methods Section 2.3.15) and 2/15 were FI⁻. These were further characterized by reversion analysis, which revealed one class that did not revert, and another class that could revert back (reversion frequencies ranging from 9.5×10^{-8} to 1.3×10^{-7}) to Phe⁺. Biotin auxotrophs were also isolated that would not grow on minimal medium at either 30°C or 42°C without the addition of biotin to the medium. We expected some RK⁻ biotin auxotrophs, since the *bio* operon is included in the *bio*275 substitution (in the cryptic prophage, Fig. 2b) and could be linked to the RK⁻ phenotype if the RK⁻ mutations were large deletions removing both λ DNA and contiguous *E. coli* DNA. A variety of other [Ts] auxotrophs requiring different amino acid supplements were also identified, as shown in Table 25.

Table 25 RK$^-$ Auxotrophs Characterized

Parental Strain	Auxotroph	Acquired [Ts] Auxotrophy	Isolate Strain Collection #
Y836*his* RK$^-$	phenylalanine	-	1b, NY1068
Y836*his* RK$^-$	phenylalanine	-	2a, NY1067
Y836*his* RK$^-$	phenylalanine	-	2c, NY1066
Y836*his* RK$^-$	phenylalanine	-	13c, NY1069
Y836*his* RK$^-$	phenylalanine	-	14b, NY1072
Y836*his* RK$^-$	phenylalanine	-	35c, NY1074
Y836*his* RK$^-$	phenylalanine	-	2b, NY1071
Y836*his* RK$^-$	phenylalanine	-	36a
Y836*his* RK$^-$	phenylalanine	-	31a
Y836*his* RK$^-$	phenylalanine	-	10a, NY1070
Y836*his* RK$^-$	phenylalanine	-	27c, NY1094
Y836*his* RK$^-$	phenylalanine	-	31a
Y836*his* RK$^-$	phenylalanine	-	7b, NY1093
Y836*his* RK$^-$	phenylalanine	-	9a, NY1075
Y836*his* RK$^-$	phenylalanine	-	8b, NY1073
Y836*his* RK$^-$	aspartic acid	+	510c, NY1079
Y836*his* RK$^-$	isoleucine	+	512e, NY1076
Y836*his* RK$^-$	aspartic acid	+	513f, NY1078
Y836*his* RK$^-$	alanine	+	518a, NY1077
Y836*his* RK$^-$	aspartic acid, asparagine	+	504c, NY1080
Y836*his* RK$^-$	aspartic acid, lysine	+	509d, NY1081
Y836*his* RK$^-$	aspartic acid, methionine	+	510f, NY1082
Y836*his* RK$^-$	aspartic acid, arginine	+	512b, NY1083
Y836*his* RK$^-$	aspartic acid, lysine	+	518h, NY1084
Y836*his* RK$^-$	methionine, threonine, isoleucine	+	503e, NY1085
Y836*his* RK$^-$	aspartic acid, methionine, proline	+	518d. NY1086
Y836*his* RK$^-$	aspartic acid, proline, phenylalanine	+	519d, NY1087
Y836*his* RK$^-$	aspartic acid, arginine, phenylalanine	+	522b, NY1092
Y836*his* RK$^-$	aspartic acid, methionine, proline, phenylalanine	+	504b, NY1091
Y836*his* RK$^-$	aspartic acid, serine, threonine, arginine	+	510b, NY1090
Y836*his* RK$^-$	aspartic acid, methionine, proline, phenylalanine, threonine, isoleucine	+	511g, NY1088
Y836*his* RK$^-$	aspartic acid, methionine, proline, phenylalanine, threonine, isloeucine	+	521a, NY1089
Y836*his* RK$^-$	biotin	-	42d
Y836*his* RK$^-$	biotin	-	42e
Y836*his* RK$^-$	biotin	-	43c
Y836*his* RK$^-$	biotin	-	49b

Y836*his* RK$^-$	biotin	-	54e

3.13. Assessing if a λ Gene Product(s) is Responsible for the Appearance of Auxotrophic Mutations.

RK$^-$ mutants were isolated from strain Y836 and the mutations responsible for the *ori*λ replication defect were genetically mapped (Hayes and Hayes 1986; Hayes and Hayes unpublished). The (*cIII-cI[Ts]857-ren*)$^{\lambda}$ fragments from these mutants were transduced into strain 594 by the previous technique in order to eliminate possible co-selected mutations arising within the Y836 RK$^-$ mutants. Each of the 594 transductants plated with essentially equal efficiency on TB agar at 30^{o}C and 42^{o}C (Table 26) showing that the (*cIII-cI[Ts]857-ren)*$^{\lambda}$ fragments were defective for *ori*λ replication initiation. Among the *O*$^-$*P*$^+$, *O*$^+$*P*$^-$ and *O*$^-$*P*$^-$ isolates, only those expressing *P*$^+$ at 42^{o}C exhibited a reduction in plating on MM. These results indicate that the AFI phenotype linked to the induced (*cIII-cI[Ts]*857-*ren*)$^{\lambda}$ fragment depends upon the expression of lambda gene *P*. When λ *P* expression at 42^{o}C from 594(*cIII-cI[Ts]*857-*O*$^-$*P*$^+$-*ren*)$^{\lambda}$ was prevented by transforming the cells with plasmid pCI$^+$ the AFI phenotype of *P* expression was suppressed (Table 26). The expression of λP at 42^{o}C during the formation of 594(*cIII-cI[Ts]*857-*O*$^-$*P*$^+$-*ren*)$^{\lambda}$ colonies was seen to increase the appearance of auxotrophic mutations (Table 26). Colony forming units isolated on TB agar plates at 42^{o}C were picked and stabbed into MM that had been warmed to the designated temperature and growth was checked after 48 hours of incubation. Table 27 shows that only cells harbouring a P$^+$ prophage resulted in colony forming units that had acquired auxotrophic mutations in nutritional genes inhibiting growth on MM. Indicating that both the mutator phenotype and the AFI phenotype are dependent on expression of λ gene *P* and are not strictly dependent on *ori*λ replication. This suggests that the two phenotypes may share a

Table 26 Plating of 594(*cIII-cI*857-*ren*)$^{\lambda}$ Defective in λO or λ*P* on TB and MM.

Strain	Cell Titer at 30°C		RK$^-$ 42°C Titer		Freq. Cfu 42°C/ 30°C		Freq. on TB/ Freq. on M.M.
	TB	MM	TB	MM	TB	MM	
Cryptic fragment expressing λ*P*							
594::*(cIII-ren)*$^{\lambda}$ [O^-P^+]	7.5x10^8	9.5x10^8	1.5x10^8	2.1x10^6	0.2	2.2x10^{-3}	90
594::*(cIII-ren)*$^{\lambda}$ [O^-P^+]	2.5x10^9	3.2x10^9	2.2x10^9	4.7x10^7	0.9	1.5x10^{-2}	60
Cryptic fragment expressing λ*O*							
594::*(cIII-ren)*$^{\lambda}$ [O^+P^-] sc5	3.9x10^8	3.7x10^8	6.5x10^8	6.1x10^8	1.7	1.6	1.0
594::*(cIII-ren)*$^{\lambda}$ [O^+P^-] sc5	1.2x10^9	1.8x10^9	1.5x10^9	1.3x10^9	1.2	0.7	1.6
594::*(cIII-ren)*$^{\lambda}$ [O^+P^-] sc6	1.1x10^9	1.2x10^9	6.7x10^8	1.0x10^9	0.6	0.9	0.7
594::*(cIII-ren)*$^{\lambda}$ [O^+P^-] sc6	1.6x10^9	1.6x10^9	1.4x10^9	1.0x10^9	0.9	0.7	1.3
Cryptic fragment defective for λ*O* and λ*P* expression							
594::*(cIII-ren)*$^{\lambda}$ [O^-P^-]	2.1x10^8	2.0x10^8	1.7x10^8	1.8x10^8	0.8	0.9	0.9
Preventing the expression of λ*P* from cryptic fragment							
594::(*cIII-ren*)λ [O^-P^+] [pCI]	1.8x10^9	1.8x10^9	1.5x10^9	1.4x10^9	0.8	0.7	1.1

Table 27 Stab Assay for Unselected Auxotrophic Mutations in 42°C Survivor Clones in Strains 594::*(cIII-ren)*$^{\lambda}$ [O^-P^+], 594::*(cIII-ren)*$^{\lambda}$ [O^+P^-], and 594::*(cIII-ren)*$^{\lambda}$ [O^-P^-].

Strain	Colonies picked from 42°C TB plates	Stabbed Colonies able to Grow Up on		Auxotrophs per colonies picked from TB plates
		MM at 30°C	MM at 42°C	
594::*(cIII-ren)*$^{\lambda}$ [O^-P^+]	100	100	99	1/100
594::*(cIII-ren)*$^{\lambda}$ [O^-P^+]	238	238	236	2/238
594::*(cIII-ren)*$^{\lambda}$ [O^+P^-] sc5	140	140	140	0/140
594::*(cIII-ren)*$^{\lambda}$ [O^-P^-]	100	100	100	0/100
594::*(cIII-ren)*$^{\lambda}$ [O^-P^+] [pCI]	150	150	150	0/150

common mechanism for their expression or alternatively may reflect separable outcomes from λ*P* expression.

3.14. Independent Assay For Mutator Effect.

We sought an independent measure of the mutator effect of an induced cryptic λ prophage by examining the frequency for culture cells becoming resistant to rifampicin. We found that spontaneous rifampicin-resistant (Rif^R) colonies arose in Y836 culture cells at frequencies between 38-130x10^{-9} (average = 6x10^{-8}), and between 5-112x10^{-9} (average = 9.5x10^{-8}) for 594 culture cells grown at 30^{o}C (Table 28). The appearance of Rif^R mutants among RK^- mutants from Y836 arose at a frequency of about 4x10^{-3} or one per each 250 RK^- mutants (Table 29). If the spontaneous RK^- and Rif^R mutations arose independently in each cell, we would expect that double RK^- Rif^R mutants would arise at a frequency of about (9.85x10^{-6} x 6x10^{-8}) 5.91x10^{-13}. We conclude that the mutator phenotype of an induced cryptic λ prophage increased the frequency of Rif^R mutants among the RK^- clones.

Table 28 Rifampicin Resistant Frequencies for Y836, Y836[pCI], Y836 *dnaB*grpD55, 594, 594::(*cIII-ren*)$^{\lambda}$, and 594::(*cIII-ren*)$^{\lambda}$[pCI]

Date	Strain	TB+rif 30^{o}C freq. x10^{-9}
May18,04	Y836*his*$^{-}$	38*
May28, 04	Y836*his*$^{-}$	130*
May28,04	Y836*his*$^{-}$	43*
May28,04	Y836*his*$^{-}$	57*
May25,04	Y836*his*$^{-}$	42*
May25,04	Y836*his*$^{-}$	72*
Jun 07,04	Y836*his*-	23*
May18,04	Y836*his*$^{+}$	186
May20,04	Y836 *dnaB*grpD55	83
July02,04	Y836 *dnaB*grpD55 [pCI]	1
Jun11,04	Y836 [pCI]	19
July02,04	Y836[pCI]	29
May18,04	594	83
May26,04	594::*(cIII-ren)*$^{\lambda}$	89
May19,04	594::*(cIII-ren)*$^{\lambda}$	112
July01,04	594::*(cIII-ren)*$^{\lambda}$[pCI]	5

* values used for averages in section 3.11.

Table 29 Frequency of Co-formation of RK^- and Rif^R Mutants

Strain	Cfu at 42°C on TB/Cfu at 30°C on TB	Cfu at 42°C TB+rif/ 42°C Cfu at 42°C on TB
Y836*his*$^-$	$5.7x10^{-6}$*	$6.2x10^{-3}$
Y836*his*$^-$	$1.4x10^{-5}$*	$2.3x10^{-3}$
Y836 *dnaB*grpD55	0.93	$1.7x10^{-7}$
Y836 *dnaB*grpD55[pCI]	0.68	$1x10^{-9}$
Y836[pCI]	0.89	$3.5x10^{-8}$
Y836[pCI]	0.57	$2.5x10^{-8}$
594::*(cIII-ren)*$^{\lambda}$	$1.6x10^{-5}$	$5.2x10^{-3}$
594::(*cIII-ren)*$^{\lambda}$[pCI]	0.76	$4x10^{-9}$
594::(*cIII-ren)*$^{\lambda}$[O^+P^-]sc11	$8.3x10^{-1}$	$3.4x10^{-8}$
594::(*cIII-ren)*$^{\lambda}$[O^-P^+]sc5	0.2	$2.1x10^{-6}$
594::*(cIII-ren)*$^{\lambda}$ [O^-P^-]sc10	$4.4x10^{-1}$	$3.0x10^{-8}$

* Average RK^- frequencies used in section 3.11.

4. Discussion.

4.1. Identification of a Mutator Effect of Induced Cryptic λ Prophage.

Host cell survival in the presence of high concentrations of λ P protein produced from a plasmid encoding λ *P-nin-Q* was examined by Klinkert and Klein in 1979. They hypothesized that high levels of P protein might interfere with cellular DNA replication and thus with cell division if λ P protein had an analogous role to host DnaC in replication initiation. Therefore, the authors devised a system where the concentration of P protein was regulated under the control of a *lac* promoter-operator where *P* expression was inducible by the addition of IPTG to cells. They found that within minutes following the induction of *P* expression the average cell size increased, filament formation occurred at a higher than normal rate and stationary growth phase was reached at a comparatively low cell density. The authors compared the effects of naladixic acid (an inhibitor of DNA chain elongation) and chloramphenicol (an inhibitor of protein synthesis) on bacterial DNA synthesis. The results showed that the effects of chloramphenicol and the induction of P protein synthesis were indistinguishable. The results suggested that each inhibited the initiation but not elongation stage of cellular DNA synthesis. Upon the addition of IPTG (to induce *P* expression) or chloramphenicol, DNA synthesis came to a "slow-stop" as evident in the growth curve where ^{3}H-thymine incorporation slowly ceased over approximately 30 minutes. This study resulted in the concept of "P-lethality". In 1991, Maiti *et al* showed that low levels of P protein (as condition after infection of cell by λ) are not lethal to the host cell, but when the expression of *P* is elevated, it is lethal to the host. Three *dnaB*groP mutants were examined by Maiti *et al.*

These mutants in the DnaB helicase have no effect on *E. coli* replication but do not allow λ DNA replication. Presumably, there is a reduction or loss in the ability of altered groP version of DnaB to interact productively with λP, nevertheless, the *dnaB*groP cells remained susceptible to killing by λP protein. Maiti *et al* (1991), concluded that 'P-lethality" did not involve an interaction of P with the host DnaB helicase protein, an essential protein for λ DNA replication. The cause of P-lethality is still in debate (Bull, 1995). It has been hypothesized that high levels of P protein bind the available host DnaB inhibiting its interaction with DnaC, and therefore prevents further cellular replication initiation from *oriC,* thus killing the cell.

The start of my project began when we asked whether host mutations arise upon the induction of the cryptic λ prophage within the *E. coli* chromosome. In an attempt to answer this question, we considered the strategies and results from other mutator assays. An advantage to measuring forward mutation frequencies (i.e. the change from wild type gene to an altered gene) often is its simplicity (Drake, 1983). A single forward mutation measurement detects most or all types of mutations simultaneously. Thus, the simple detection of a mutant frequency increase reveals a mutagenic effect, but does not identify the DNA changes involved. Therefore, forward mutation is the measurement of choice in the absence of information about the type(s) of mutation to be induced (Drake, 1983). When considering mutation studies in bacteria, viruses or animal cells, the normal mutation frequencies encountered are on the order of 10^{-7} to 10^{-9} per base pair per round of replication (Goodman, 1983). Based on this frequency, we can calculate the expectation that an RK^- mutant arising on rich TB agar would have acquired an additional mutation making the cell an auxotroph as per following assumptions:

a) The *E. coli* genome has 4, 639, 221 base pairs (bp) of DNA and 4288 protein coding genes (Blattner *et al*, 1997).

b) It is estimated that out of the 4288 protein coding genes in *E. coli*, 620 are essential (i.e. approximately 15%) (Eisenstein, 2004).

c) If we assume that 10% of the total 4288 genes within the *E. coli* genome are nutritional genes, there would be 429 nutritional genes.

d) An *E. coli* gene, on average, codes for 317 amino acids (Blattner *et al*. 1997) i.e. 951 base pairs.

e) Therefore, if you multiply the total number of nutritional genes, 429, by the average gene size in base pairs, 1000, this would equal ~ 4.1×10^5 base pairs, or fractionally 0.08838 of the total base pairs in an *E. coli* genome.

f) Thus, 4.1×10^5 base pairs would be the target size if all of the nutritional genes were included as a target for mutagenesis.

g) If we take the mutation frequencies noted above, 10^{-7} and 10^{-9} per base pair, and multiply them by the target size, 4.1×10^5 (=0.08838 X 4.639221 $\times 10^6$ bp):

$4.1 \times 10^5 \times 10^{-7} = 0.041 = 1$ target site mutation per 24 cells

$4.1 \times 10^5 \times 10^{-9} = 0.00041 = 1$ target site mutation per 2439 cells

h) Therefore, this tells us that 1/24 cells or 1/2439 cells will have a mutation in a nutritional gene based on the mutation frequencies noted above.

i) RK^- mutants arise from strain Y836 at a frequency of about 3×10^{-6} on rich TB agar at 42^oC. (Hayes, 1990)

j) Therefore we can assume that within the population of RK^- mutants arising at a frequency of 3×10^{-6}, about 1/24 to 1/2439 of these will have acquired at least one

mutational change in a nutritional gene, i.e. a frequency of $1.2x10^{-7}$ to $1.2x10^{-9}$. The actual frequency for auxotrophs arising within the RK^- mutant population would be much lower since only a few of the actual base changes would inactivate a given nutritional gene.

The first question we addressed was whether mutations that conferred an auxotrophic phenotype to the cell by inactivating a nutritional gene arose during the selection for RK^- survivor mutants at 42^oC. The plating frequency of the RK^- survivors isolated on rich TB agar was compared to the plating frequency of the RK^- survivors isolated on minimal medium. Results from four experiments showed that RK^- mutants arise at a higher frequency on rich compared to minimal medium, supporting a model that auxotrophic deficiencies (incapacitating lesions and mutations within nutritional genes) arise in parallel during the selection for RK^- mutant cells. When a casamino acids supplement (not vitamin-free) was added to the minimal medium agar, the RK^- mutation survivor frequency became equivalent to the plating frequency on rich TB agar, again supporting a model that auxotrophic deficiencies arise within the mutant RK^- population. We hypothesize that the reduction in RK^- survivor frequency on MM is due to an inability of cells with auxotrophic deficiencies appearing within the RK^- population to grow without providing the missing nutrient.

An auxotroph formation index (AFI) was used to reflect the difference in plating frequencies seen on MM and TB agar media and represents the RK^- mutant survivor frequency on rich TB at 42^oC divided by the RK^- mutant survivor frequency on minimal medium at 42^oC. Note: Each of these separate RK^- mutant survivor frequencies on MM or TB was determined as the plating titer of RK^- survivor mutants forming colonies at

42°C divided by the titer of the RK^+ starting cells on plates incubated at 30°C per medium.

We used a stab assay to screen for the presence of auxotrophic mutations among all of the well isolated Y836 RK^- cells appearing on rich TB agar plates. The results for two independent assays showed that some of the RK^- mutant survivor colonies had acquired auxotrophic mutations that prohibited their growth on minimal medium at 30°C and/or 42°C.

We tested whether the RK^+ assay strain Y836 had an intrinsic mutator phenotype by transducing the cryptic λ prophage from Y836 into two other prototrophic *E. coli* K12 strains, 594 and W3101. Both transductants exhibited AFI's of >1. This result strongly demonstrated that the mutator phenotype moved along with the λ fragment from Y836 into 594 and W3101 and was not an intrinsic property of the Y836 RK^+ assay strain cells.

4.2. Host Gene Requirements for Mutator Phenotype.

One theory behind replicative killing is that upon the induction of the cryptic λ prophage, one of the bidirectional replication forks initiated from *oriλ* collides with a replication fork initiated from the *E. coli* origin of replication or *oriC*. The colliding forks will stall *E. coli* and λ replication progression. In bacteria, stalled replication forks trigger the host SOS response (Snyder and Champness, 2000) which involves RecA being converted to RecA* upon binding single stranded DNA fragments, and the LexA repressor cleaving itself in the presence of the RecA* co-protease. (Friedberg et al, 1995) The induction of an SOS response is prevented by a *recA*[Def] mutation, i.e. by the deletion of the *recA* gene, and by a *lexA*[Ind] mutation i.e. by the introduction of a noncleavable *lexA* allele. Plating frequencies on rich TB and minimal medium at 42°C

were compared for both *recA*[Def] and *lexA*[Ind] variants of Y836. We also assayed for the participation of SOS induced gene products in the appearance of the auxotrophic mutations within the RK^- population. The *E. coli* polymerases, PolIV encoded by *dinB* and PolV encoded by *umuC* and *umuD*, rescue stalled replication forks at the expense of an increased mutation frequency and conveying a mutator phenotype to cells where SOS regulon expression is induced. The effects of host mutations in *umuC/D, dinB, lexA*[Ind], and *recA*[Def] on RK^- mutant survivor frequencies obtained on rich TB agar or on minimal medium were compared. The appearance of clones with auxotrophic deficiencies within the population of RK^- mutant survivor colonies arising on rich medium did not require induction of the SOS response, or the polymerases PolIV or PolV, or the *recA* gene product. This is not to say that these genes do not have some indirect effect on both the RK frequency or the formation of auxotrophic mutations. We note for the first time (Table 16) that blocking SOS induction (with a *lexA*[Ind] allele) lowered the frequency for appearance of RK^- colonies by about 10-fold, but an AFI >1 was observed. Presumably, some SOS response gene product is involved in the process whereby RK^- mutations arise. In contrast, deletion of *recA* did not perturb the RK^- frequency but again AFIs >1 were observed.

4.3. Lambda Gene Requirements for Mutator Activity.

Up to this point our results have suggested that a mutator effect is associated with the thermal induction of a cryptic λ fragment, resulting in expression of the included λ genes and in the initiation of replication from *oriλ*. In order to confirm that induction of the λ fragment was important for the effect we introduced into the RK^+ assay strains a mid-copy CI^+ plasmid that expresses wild-type CI repressor at both 30°C and 42°C. In

cells with the plasmid, gene expression from the cryptic λ prophage and replication initiation from *oriλ* are inhibited at both 30°C and 42°C. The CI^+ plasmid completely prevented replicative killing of Y836, and of 594 and W3101 with a cryptic λ prophage fragment when the cells were shifted to 42°C, as shown by their near-identical efficiency of plating at both 30°C and 42°C. In cells with pCI^+ the AFI was approximately 1.0, showing that prophage induction or replication was required for the AFI effect. Prophage induction or λ replication initiation was also found to be a requirement for the mutator phenotype. In a stab assay of the colonies forming at 42°C on rich TB agar, all the cfu were able to grow on minimal medium at both 30°C and 42°C. Therefore, we conclude that blocking gene expression and replication initiation from the cryptic λ prophage eliminates the AFI effect and the mutator phenotype. In order to distinguish between *oriλ* replication and λ gene expression, a mutation called grpD55 within the *dnaB* gene of *E. coli* was transduced into Y836 in order to help decide if replication initiation from *oriλ* was required for the mutator effect. Cells with a *dnaB*grpD55 allele do not support the replication of λ at 42°C. Thus we are able to inhibit all replication initiation from *oriλ* but nevertheless permit the induction of λ gene expression from the single cryptic prophage copy in cells shifted to 42°C. We found that Y836 *dnaB*grpD55 formed colonies at approximately the same efficiency at 30°C and 42°C on rich TB agar, indicating that the grpD allele essentially suppressed the RK phenotype seen when Y836 RK^+ cells are shifted to 42°C. However, the absence of *oriλ* replication initiation did not suppress the appearance of auxotrophic mutations within Y836 *dnaB*grpD55 colonies arising on TB agar at 42°C as shown in the stab assay. When λ gene expression *and* λ replication initiation was prevented in Y836 *dnaB*grpD55 cells by the addition of pCI^+: a) the cells

formed colonies on both TB and MM at equal efficiency at 30°C and 42°C and b) no auxotrophic mutations were detected within the colonies arising at 42°C. We conclude that the putative mutator effect accounting for the reduction in colony formation on minimal medium plates at 42°C and the appearance of auxotrophic mutations is linked to the induction of gene expression from the cryptic λ fragment but does not require actual replication initiation from *ori*λ.

These results strongly suggested that the expression of a λ gene product was responsible for the appearance of auxotrophic mutations within the survivor colonies isolated at 42°C on rich TB agar. Therefore, we initiated a search for the responsible λ gene(s) product. We selected from our laboratory collection RK^- mutants derived from strain Y836 that were shown by complementation analysis to be either O^+P^-, O^-P^+ or O^-P^-. The cryptic λ fragments were transduced into 594 cells in order to eliminate unselected host mutations, which might have arisen during the selection for the RK^- mutants. Each of the λ fragment-carrying transductants plated with equal efficiency on TB agar at 30°C and 42°C indicating that the λ fragment mutation was sufficient to confer an RK^- phenotype to the transduced 594 cells. We then compared the ability of the 594 transductants to form colonies at 30°C and 42°C on rich TB and on MM agar. Only the transductant expressing *P* at 42°C plated with reduced efficiency on MM plates. These results suggest that the AFI effect associated with the reduced plating efficiency on MM and the appearance of auxotrophic mutations is linked to the expression of lambda gene *P* from the cryptic λ fragment.

4.4. ***Auxotroph-Independent Assay for Mutator Activity.***

An auxotroph independent screen for the λ mutator activity was sought. Y836 spontaneous rifampicin resistant colonies arose at a frequency between 38-130 $x10^{-9}$ at $30^{o}C$. 594 spontaneous rifampicin resistant colonies arose at a frequency between 5-112 $x10^{-9}$ at $30^{o}C$. Rifampicin resistant Y836 RK^- colonies arose at a frequency of $3x10^{-3}$ at $42^{o}C$ (i.e., 1 per 333 RK^- mutants) rather than at a frequency of $5x10^{-8}$ per RK^- mutant (i.e., 1 per $2x10^7$ RK^- mutants) as would be expected if the RK^- and Rif^R mutations arose independently of the other. Preventing λ gene expression by the addition of pCI^+ to Y836 or Y836 *dnaB*grpD55 suppressed the increase in Rif^R mutants seen without the plasmid. Thus, the λ mutator effect increased the frequency of Rif^R mutants among the RK^- clones.

4.5. Hypothesis for λ Mutator Activity.

The initiation of λ DNA replication is known to involve the activities of λ initiator proteins O and P and several *E. coli* proteins as DnaB, DnaJ, DnaK, GrpE, DNA Polymerase III, and DnaG (Hoffman *et al*, 1992; Dodson *et al*, 1989; Liberek *et al*, 1990). Many studies have attempted to elucidate the nature of the protein interactions occurring at *oriλ.* The λ P initiator protein is known to act as a tether for bringing the DnaB protein to the O-some formed at *oriλ* (Dodson *et al,* 1989). The binding of λP to DnaB inactivates the helicase activity of DnaB (LeBowitz *et al,* 1986). The *E. coli* heat shock proteins DnaJ, DnaK and GrpE are required to release λP from the pre-initiation complex and restore the helicase activity of DnaB, which can then proceed to unwind the duplex DNA in both directions from *oriλ* (Dodson *et al*, 1989; Liberek *et al*, 1990).

The following model is proposed to explain the appearance of untargeted auxotrophic mutations within the RK^- mutant population isolated at 42°C. Upon replication initiation, DnaB helicase unwinds DNA at replication forks which move away from *ori*λ into the straddling *E. coli* chromosomal DNA sequence. We hypothesize that unsequestered λP protein within the cell recognizes and binds to a DnaB helicase localized at a replication fork. This perturbs the progressing replication fork and causes base mispairing events e.g. polymerase slippage or stalling, which could eventually produce untargeted transitions, transversions, deletions, or insertions in the replicated *E. coli* chromosome. It has been shown *in vitro* that λP activity is sequestered by binding to DnaK, but in the cell, an overexpression of λP must at some point overwhelm the cellular concentration of DnaK, especially at 42°C when the activity of DnaK becomes essential for refolding denatured host proteins. Therefore, an unmanageable increase in λP concentration would sequester DnaB i.e. would not be removed, and cause cell death.

The model proposed for Y836 *dnaB*grpD55:

The *E. coli* GrpD55 mutant isolated by Saito and Uchida (1977) was found to prevent the growth of λ at 42°C and was suggested to inhibit λ replication. Genetic analysis by Saito and Uchia (1977) suggested that the GrpD55 strain contained a mutation mapping at 71.5 minutes on the *E. coli* linkage map. However, an inability to functionally complement the grpD55 mutation with DNA from the 71.5 minute region suggested (Bull,1995; Bull and Hayes,1996) that the wild type of the grpD55 mutation was incorrectly mapped. They remapped the mutation to 91.5 minutes on the *E. coli* linkage map, a position suggesting that grpD55 was likely an allele of *dnaB*. This was confirmed by DNA sequence analysis (Horbay and Hayes, unpublished).

Replication initiation from *oriλ* at 42°C was shown to be completely inhibited from induced Y836 cells with a *dnaB*grpD55 allele (southern blot analysis, Hayes and Hayes, unpublished). In the absence of *oriλ* replication initiation, the level of λP protein expressed from Y836 *dnaB*grpD55 cells at 42°C was tolerated by the *E. coli* cells as shown herein by the near identical plating frequencies of Y836 *dnaB*grpD55 at 30°C and 42°C on rich TB agar. Several hypotheses may explain the inhibition of *oriλ* replication in a *dnaB*grpD55 host cell:

1) At the restrictive temperature the conditional DnaBgrpD55 protein will bind to λP protein less tightly than to wild type DnaB. The conformation of DnaBgrpD55 protein will change at 42°C and this will reduce its affinity for λP but not for DnaC. As a consequence, host DnaC should displace λP from the DnaBgrpD55-λP complex enabling *oriC* replication initiation at 42°C.

2) DnaBgrpD55 protein interacts with λP but the complex cannot interact with λO at the O-some formed at *oriλ*.

3) λP is not removed from the pre-primosomal *oriλ*-O-P-DnaBgrpD55 complex by the heat shock proteins at 42°C. Therefore, the helicase activity of DnaB is never restored and replication initiation is inhibited at *oriλ* at the nonpermissive temperature.

We would expect that a reduction in the affinity of the λP-DnaB interaction in DnaBgrpD55 cells would reduce the mutator effect of P-expression if it depends upon the arrest or perturbation of replication fork progression. We would predict that as the concentration of λP increases in a cell, there would be a parallel increase in replication fork perturbation and copy errors. We presume λP can be removed from a DnaB-polymerase complex at a replication fork enabling continued fork progression until

another λP binds. We found less mutagenesis in cells with *dnaB*grpD55 than with the wild type *dnaB* allele, i.e. out of 100 RK^- Y836 *dnaB*grpD55 survivors, only 4 auxotrophic mutants were isolated whereas in separate assays with the Y836 RK^- mutants, either 43/98 or 22/99 auxotrophic mutants were obtained.

It has been reported that high levels of λP expression in a cell is lethal, e.g., when λP is overexpressed from a plasmid (Maiti *et al*, 1991). We suggest that a reduced level of λP expression, such as during the induction of a cryptic prophage, confers a mutator effect manifested via the appearance of auxotrophic mutations arising within selected RK^- mutants. In order to determine the number of auxotrophic mutations within the population of RK^- survivor clones appearing at 42°C a stab assay was used. The ratio of RK^- auxotrophs / RK^- clones appearing at 42°C on rich TB agar was presented. We concluded from the stab assays that mutations resulting from chromosomal perturbations (DNA lesions) occur within nutritional genes, but such cells are not handicapped for growth on TB because the missing nutrient is provided by the medium. We propose that during the growth of these cells on rich medium, the inhibitory lesions are either correctly repaired or are misrepaired. Only cells with misrepaired lesions in a nutritional gene would appear as a double mutant i.e. a cell with both a RK^- phenotype and an auxotrophic mutation. We used an auxotroph formation index (AFI = RK^- frequency on rich TB agar divided by the RK^- frequency on MM) to reveal the frequency of RK^- mutants that were handicapped for growth on MM. Upon comparing the actual RK^- auxotrophs per RK^- mutant ratio with the AFI we are led to suggest that most of the chromosomal perturbations (DNA lesions) arising within nutritional genes during the RK^- selection are correctly repaired as the RK^- mutants form colonies on rich media at 42°C. For example,

in Tables 8 and 11, the AFI was 90 and the number of auxotrophs isolated within the RK^- mutant population was 43/99. From the AFI results we would expect that virtually all the RK^- mutants would also have acquired an auxotrophic mutation. Since this was not found, we conclude that the λ-dependent copy errors are mostly corrected during cell growth on rich medium. The hypothesis we have made for repair/misrepair of chromosomal perturbations (DNA lesions) to account for the difference between the AFI and the actual proportion of auxotrophs observed during the selection requires future examination and proof.

Up to this point, we have observed a mutator phenotype of an induced cryptic lambda prophage which is linked to the expression of included lambda gene *P*. We have not yet investigated whether λP has a mutator effect during the normal infectious λ life cycle toward either its own chromosome or its host. Klinkert and Kline (1979) and Maiti *et al.* (1991) showed that an overexpression of λP from a plasmid is inhibitory to the host *E. coli* cell metabolism. The effects of λP during a normal phage burst is still unknown. We do not know if λP accumulates following cell infection in sufficient amounts to have a mutator effect, as is seen upon the induction of a cryptic λ fragment. The participation of λP in both theta and sigma replication is still unclear. In 1997, Learn *et al*. showed that λP protein is required to transfer the DnaB helicase onto the DNA during initiation of theta replication. Whether this activity is required during sigma replication is unknown. The turnover rate of λP during the rather short infectious λ life cycle remains to be determined. Therefore, it is unknown whether λP accumulates in the cell sufficiently during an infectious cycle to evoke damaging effects to its own or to the host genome

though one would assume that natural selection has directed evolution of expression of P to a level most advantageous to the phage.

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