The Bacteriophage λ Rex-Centric Mutualism Phenotype, Conditional Rex, and Other Novel Rex Phenotypes

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To Mary

my loving wife

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ABSTRACT

Rex-Centric Mutualism

The Rex Exclusion Phenotype, encoded by the *rexA-rexB* genes of bacteriophage lambda is defined as the inability of the mutant bacteriophage T4*rII* to generate plaques on a lysogenized *E. coli*(λ) host. Although the phenotype was first observed more than four decades ago, few advancements have been made in the elucidation of the Rex mechanism. The current model for Rex exclusion proposed in 1992 by Parma et al., states that the Rex system functions as an altruistic bacterial apoptotic module triggered by T4*rII* infection of the lysogenized host. We asked whether Rex exclusion confers a protective or a cell-killing phenotype to the host and found that following T4*rII* infection, the Rex system can channel lysogenic cells into a temporary arrested growth phase that gives an overall protective effect to the host even at enormous multiplicities of infection compared to nonlysogens, despite some associated killing. We termed this phenomenon Rex-centric mutualism. In 1989, Snyder and McWilliams demonstrated that the Rex-mediated arrest of cell growth can be triggered in the absence of infection by over-expressing *rexA* relative to *rexB.* We noted that plasmid expression of *rexA* in Rex^+ cells in the absence of infection resulted in similar cellular viabilities as that observed following T4*rII* infection. We visualized lambda Rex^+ lysogens, infected by T4*rII* and found that they were much delayed in colony formation, contracted in length, formed aggregates with adjacent cells, and released flagella. These phenotypes were accentuated in nonlysogenic cells carrying a specific multicopy *rexA-rexB* plasmid: cells were about two-fold contracted in length, expressed membrane-bound and secreted flagellar structures,

were odorous, were insensitive to infection by a variety of phages, and they extensively clumped/adhered when grown up in culture. Lysogenic cells mutated for *rpoS* stationary phase sigma factor were abrogated for Rex-centric mutualism, exhibiting more than 400-fold lower viability compared to the wild type, following infection by T4*rII*. These phenotypes show that the Rex system can impart a stationary phase like response that protects the host from T4*rII* killing.

RexB Inhibition of T4*rII* Lysis Inhibition Phenotype

We add to the activities and phenotypes of RexB. Expression of *rexB* from either a multicopy plasmid, or a *rexA*$^-$-*rexB*$^+$ phage is capable of suppressing the Lysis Inhibition Phenotype (LIN) seen upon infection of *E. coli* K strains by T4*rII* at high MOI. We also show that host mutations in either the periplasmic "tail-specific protease" *tsp*, or the 10Sa RNA *ssrA* completely abrogates the establishment of LIN in T4*rII* and T4 alike. We found that over-expression of *rexB* in cells suppresses both T4*t*$^-$ and λ*S*$^-$ holin mutations, increasing the plating efficiency of the mutant phages by up to 10^5 fold. Prophage level expression was noted to suppress the λ*S*$^-$ mutation to a lesser degree, but only from a *rexB*$^+$-*rexA*$^-$ prophage and co-expression of *rexA* with *rexB* on the prophage, or on a multicopy plasmid inhibited this activity of RexB. We also determined that nonlysogenic cells carrying the multicopy *rexB* plasmid are leaky for cytoplasmic proteins, whereas lysogenic (λ*rexB*$^+$-*rexA*$^-$) cells are not leaky but did reveal an unusually high concentration of cytoplasmic β-galactosidase in the periplasm. Electron microscopy was used to visualize cells transformed with a *rexB*, or *rexA-rexB* multicopy plasmid. The *rexB* plasmid conferred gross distortions to the outer surface of the cell, while the *rexA*$^+$-

rexB$^+$ plasmid imparted a shrunken, but otherwise normal appearance to cells. Our findings are consistent with RexB function as a pore forming unit, but RexB activity is inhibited by RexA. We propose a model for RexB suppression of T4*rII* lysis inhibition and the involvement of Tsp and 10Sa RNA in lysis timing and the establishment of LIN.

The Conditional Rex Exclusion Phenotype

The *cI-rex* operon of bacteriophage λ is expressed from the P_M maintenance promoter of the prophage as P_M-*cI-rexA-rexB-*t_{imm} message and confers a T4*rII* mutant phage *ex*clusion phenotype to the lysogen (Rex exclusion). Derepression of the prophage results in very strong P_E-*cI-rexA-rexB-*t_{imm} transcription, terminating at t_{imm}. Replication and excision defective, cryptic λ*cI*[Ts]857*cro*27 lysogens exhibit a conditional Rex[Ts] exclusion phenotype. At temperatures where the CI857 repressor is functional, *rexA-rexB* expression from P_M confers full Rex exclusion. However, upon thermal inactivation of the repressor little or no Rex exclusion is observed, despite a much higher level of transcription from P_E, stimulated by CII. The same conditional Rex exclusion phenotype was observed in cells harbouring a low copy plasmid encoding a P_{Tet}-P_M-*cI*857-*rexA-rexB-*t_{imm} fragment, but not with a *cI*$^+$ derivative plasmid, that imparted a Rex$^+$ phenotype. Thermally derepressed λ*cI*[Ts]857*cro*27 lysogens exhibited very high P_E transcription levels that abated dramatically toward the C-terminal of *rexA*, showing a powerful polar effect on downstream *rexB*. Renaturation of CI857 following prophage induction did not reestablish repressor activity, although there was a 20-30 fold increase in transcription compared to that seen from the repressed prophage. Introduction of a

rho mutation into our conditional Rex[Ts] strains partially suppressed Rex thermosensitivity, increasing Rex exclusion at 43°C by up to 10^4 fold, while mutation of *hflA* to stabilize CII and heighten CII-dependent P_E transcription conferred only a slight increase. Partial suppression of the conditional Rex[Ts] phenotype was also imparted by *ssrA*$^-$ and *clpP*$^-$ null mutations, which suggests that Rex may be subject to 10Sa RNA tagging and ClpP(X) degradation. We propose two possible models to account for *cI-rex* polarity and correlation between CI activity and Rex exclusion.

RexA:RexB Stoichiometry and the Rex Exclusion Phenotype

We examined the influence of disrupting Rex stoichiometric balance on Rex activity to account for how polarity in the P_{Tet}-P_M-*cI857*-*rexA*-*rexB*-t_{imm} operon can abrogate the Rex exclusion phenotype. Cultured λ *rex*$^+$ lysogenic cells were transformed with low-copy, and multicopy plasmids constitutive, or inducible for *rexA*$^+$, *rexB*$^+$, or *rexA*$^+$-*rexB*$^+$ expression. Lambda rex$^+$ lysogenic cells transformed with a low-copy plasmid constitutively expressing *rexA*$^+$, conferred only a minor attenuation of Rex exclusion, while transformation of *rex*$^+$ cells with a constitutive, or induced multicopy plasmid expressing *rexA*$^+$, or *rexB*$^+$ completely suppressed the Rex exclusion phenotype. In contrast, multicopy and low-copy *rexA*$^+$-*rexB*$^+$ and *rex*$^-$ plasmid derivatives did not abrogate Rex exclusion in transformed *rex*$^+$ cells. Furthermore, phage T4*rII* exhibited large rapid lysis plaques on the thermally induced Cro$^+$ conditional Rex exclusion phenotypic lysogen, resembling plaques formed on λ lysogens carrying the multicopy constitutive *rexB* plasmid. Plaques formed on the isogenic *cro*$^-$ derivative were tiny and nonsymmetrical; identical to

T4*rII* plaque morphology on λ lysogens carrying the multicopy constitutive *rexA*$^+$ plasmid. Our results suggest that an induced Cro$^+$ λ prophage escapes Rex exclusion by over-expressing *rexB* relative to *rexA*, while a derepressed λ*cro*$^-$ prophage suppresses Rex exclusion phenotype by over-expressing *rexA* relative to *rexB*.

CI Repressor Modulation of the Rex Exclusion Phenotype

Bacteriophage λ mutants defective for *ren* and *red* (*exo* or *bet*) are sensitive to restriction by λ *rex* genes, but exclusion is modulated by the *cI* repressor allele of the prophage. λspi156*nin*5 forms plaques with 10^5 fold higher efficiency on a *cI*$^+$-*rex*$^+$ lysogen than on *cI*[Ts]857, or *cI*[Ts]2 derivatives. Exclusion in *cI*[Ts] lysogens is suppressed by complementation with *cI*$^+$ plasmid.

CHAPTER ONE

Introduction

1.1 The Rex Phenotype of Bacteriophage λ

The T4*rII* exclusion (Rex) phenotype was discovered in the mid 1950's by Seymour Benzer who found that one particular rapid lysis mutant of bacteriophage T4 (*rII*) that generated large sharp-edged plaques on *E.coli* B was incapable of forming plaques on *E. coli* lysogenized with bacteriophage λ (Benzer, 1955). Benzer employed this classical selection technique to measure recombination frequencies between thousands of *rII* mutants of T4 that restored the rII^+ genotype, allowing him to map mutations within the *rII* genes. The *rII* genes remain the most thoroughly mapped genes in genetic history, and ultimately, Benzer's work played an invaluable role in our understanding of modern molecular genetics: gene divisibility, homologous recombination and the unpunctuated triplet nature of the genetic code. However, these triumphs in genetics are not without their ironies, since our understanding of how T4*rII* mutants are excluded by λ lysogenic cells, or why the *rII* genes of T4 suppress this exclusion phenotype is arguably no better than it was almost fifty years ago.

Several of the *rII* point mutants isolated by S. Benzer (1955) exhibited tiny plaques on λ lysogenic cells, suggesting that some *rII* function was retained, but the exclusion system has been shown to be particularly powerful versus *rII* null and deletion mutants with reversions on lysogenic cells occurring at frequencies of 10^{-8}, or lower. Benzer's observations led him to conclude that although various *rII* mutants are excluded to varying degrees on lysogenic cells, Rex exclusion ultimately results

in the death of the host cell. The Rex exclusion phenotype is encoded by λ genes *rexA* and *rexB* from a repressed lambda prophage (Landsman et al., 1982; Matz et al., 1982).

A lambda prophage (λ) is maintained in the lysogenic state through the expression of λ gene *cI*, which encodes a repressor that blocks further λ transcription and lytic development. The CI repressor functions by binding to operator sites O_L and O_R , whose sequences overlap promoters P_L and P_R, shown in Figure 1, reviewed by Meyer et al. (1980). The P_M promoter for *cI* also overlaps O_R. The normal binding of CI to O_R serves to block transcription from P_R and stimulate transcription from P_M (Heinemann and Spiegelman, 1970; Echols and Green, 1971; Reichardt, 1975; Meyer et al., 1980; Hawley and McClure, 1982; Li and Susskind, 1994).

The contiguous genes *cI-rexA-rexB* spanning λ bases 37,940-35,828 are transcribed in a repressed lysogenic cell from P_M (starting from base 37,940) through to terminator t_{Imm} at 35, 804 bp λ (Hayes and Szybalski, 1973; Daniels et al., 1983; Hayes et al., 1997), without continuing through the intervening 220 bp region between *rexB* and P_L. In the repressed prophage, the *cI-rexA-rexB* operon was formerly believed to represent the only expressed λ phage genes (Astrachan and Miller, 1972). The expression of *rexB-rexA* in a lysogenic prophage depends upon transcription initiated from P_M, and would therefore be controlled by the autoregulatory influence of CI at P_M. Hayes and Szybalski (1973) showed that the distal end nucleotides of *rex* were transcribed from a promoter termed P_{Lit} (late immunity transcription) that could bind RNA polymerase (Pirrotta et al., 1980). Later

it was revealed that there were in fact two P_{Lit} promoters (P_{Lit1} and P_{Lit2}), one constitutive and one regulated (Hayes et al., 1997). The *rexB* gene was eventually revealed by sequence analysis (Landsmann et al., 1982). Promoter positions and λ gene expression occurring in a derepressed prophage are shown in Figure 2. Some constitutive independent expression of *rexB* from the late immunity transcription promoter(s) occurs from the prophage, since a P_M^- prophage in double lysogens {[(λ *c*I857 P_M116)(λ *gal*8 *rex*$^-$ *c*I857 *cII*68)] or [(λ *c*I857 P_ME37)(λ *gal*8 *rex*$^-$*c*I857 *cII*68)]} can complement for *rexB* mutations, but not for *rexA* mutations (Matz et al., 1982). Upon induction of a lambda lysogen, *cII* is expressed from the P_R rightward promoter. CII in turn stimulates transcription of the establishment message from P_E through the *cI-rexA-rexB* operon (Echols and Green, 1971; Figure 2). Late immunity transcription also occurs from the late immunity promoter P_{Lit1} within the C-terminal of *rexA*, independently expressing *rexB* in the absence of any measurable *cI,* or *rexA* message (Hayes et al., 1997).

Mutation of either *rexA*, or *rexB* results in the abrogation of the exclusion phenotype and restores full plating of T4*rII* (Matz et al., 1982). The involvement of *cI* in the regulation of Rex is not fully understood and is further described herein (Chapter 5), but is not required in Rex function, since plasmid constructs expressing *rexA-rexB* in the absence of *cI* exhibit Rex exclusion (Shinedling et al., 1987; Snyder and McWilliams, 1989). However it appears that the CI repressor may modulate the activity or expression of Rex since λ*cI*[Ts]857 lysogens consistently exhibit higher Rex activity against sensitive lambdoid derivatives (Toothman and Herskowitz, 1980b). Our understanding of the function of the Rex proteins however, remains

elusive. Belfort (1978) isolated a 28.5 KDa RexA protein, later identified as RexA. The *rexA* gene is presumed to encode a cytoplasmic protein due to the hydrophilicity of its primary amino acid sequence (Parma et al., 1992). The function of RexA is largely unknown and its amino acid sequence shares no significant homology with other proteins registered in the gene bank, although a weak similarity (24% identity) with a membrane bound VirB4 type IV ATPase from *Sinorhizobium meliloti* (E = 0.47, where E is a measure of probability of coincidental sequence similarity, decreasing in value as homology increases). The *rexB* gene encodes a 16 KDa protein comprised primarily of hydrophobic residues (Landsmann et al., 1982). This protein has been localized to the cytoplasmic membrane of the host cell by *phoA* fusions (Parma et al., 1992), traversing the membrane at least four times with positively charged hydrophilic residues residing in the cytoplasm in accordance with the "plus inside" rule (von Heijne, 1986). The function of RexB as an ion channel may be supported by reports that Rex exclusion is dependent upon the ionic composition of the external medium. T4*rII* exclusion by λ lysogenic cells requires the presence of monovalent cations such as H^+, Na^+, K^+, NH_4^+, or Cs^+ in culture (Garen, 1961; Sekiguchi, 1966), but is attenuated by the presence of divalent cations such as Mg^{2+} or Ca^{2+}, polyamines, sucrose (Garen, 1961; Ames and Ames, 1965; Brock, 1965), arginine, lysine spermidine and a number of diamines (Buller and Astrachan, 1968). These findings are consistent with the role of RexB as an ion channel, but also suggest that RexB-mediated ion exchange is non-specific, discriminating neither by ion specificity nor by size. Furthermore, since ion exchange appears to be regulated solely by concentration, it is conceivable that RexB functions to form a lesion in the host

membrane, disrupting the ionic differential across the cytoplasmic membrane.

Infection of a *rex*$^{+}$ lysogen by T4*rII* results in loss of membrane potential, proton motive force (Parma et al., 1992), and a rapid drop in cellular ATP levels (Colowick and Colowick, 1983) and within ten minutes of infection. The cellular manifestations of Rex exclusion are roughly coordinated with the initiation of T4 replication. The addition of Mg^{2+}, or polyamines to the external medium before the tenth minute of infection can reverse Rex exclusion of T4*rII*. Exclusion is reversed with limited success after the sixth minute. This is presumably due to the decreased uptake of magnesium, or polyamines at this time, which may be accounted for by the decrease in cellular energy (Buller and Astrachan, 1968). Rex exclusion of T4*rII* by λ lysogens mutated for ATPase *unc* genes maintain cellular energy (Colowick and Colowick, 1983) following infection, suggesting that the loss of ATP is mediated by ATPase activity. Presumably intracellular ATP levels are depleted due to ATP-dependent pumping of protons to the exterior of the cell by ATPase to restore the proton motive force. However, although the loss of cellular energy may be a consequence of the Rex phenotype, it appears not to be essential for exclusion since *unc* mutants lysogenized for λ still interfere with T4*rII* growth. Snyder and McWilliams (1989) demonstrated that Rex-mediated cessation of cellular macromolecular synthesis can be induced in the absence of infection by multicopy plasmid over-expression of *rexA*, relative to *rexB*. This effect was not seen by isolated plasmid expression of either *rexA*, or *rexB*, or by co-expression of *rexA-rexB*. These findings may suggest that the Rex phenotype is triggered by an alteration of Rex protein stoichiometry, with RexA in excess, resulting in the metabolic "shut-

down" of the cell. In contrast, the over-expression of RexB, relative to RexA exhibited the opposite effect, suppressing Rex exclusion and restoring T4*rII* plating on a λ lysogen (Parma et al., 1992). These observations led Parma et al. (1992) to propose the only existing mechanistic model of Rex exclusion, offering that the Rex system functions as an altruistic cell death module that is activated upon infection of the lysogenic host by the Rex-sensitive phage. Under this model, RexB functions as the "regulatory" pore-forming unit within the inner membrane of the host cell, but is inactive when expressed at equal concentrations with the "sensor" unit, RexA; i.e. from the uninfected prophage. They suggest that infection of the lysogenic host with T4*rII* results in an increase in the ratio of RexA to RexB and leads to the activation of the RexB pore through direct interaction with at least two RexA proteins. If RexB is over-expressed, then the probability of interaction with multiple RexA subunits is reduced, thus accounting for the alleviation of exclusion. They noted that upon induction of a λ prophage, *rexB* would be expressed from the P_{Lit1} promoter, presumably increasing the ratio of RexB to RexA, subsequently protecting λ from being restricted by its own exclusion system. Similarly, initiation of λ replication is coordinated with expression of the *oop* gene, which encodes a functional RNA that was reported to attenuate the Rex exclusion phenotype (Hayes et al., 1997). The *oop* promoter (P_O) possesses two LexA binding operators (Krinke et al., 1991), and the P_{Lit1} site and OOP RNA both share with P_o a common 9 bp sequence with strong homology to the SOS box, suggesting that OOP may derepress the P_{Lit} promoter upon induction of the prophage to result in excess transcription of *rexB* in the absence of *rexA* (Hayes et al., 1997).

Upon activation of RexB, the cytoplasmic membrane is depolarized and loss of cellular energy, and macromolecular synthesis ensues. Given the severity of Rex-mediated effects on the host, Parma et al. (1992) suggested that the Rex system was altruistic, sacrificing the host and prophage in order to abort replication of the invading phage and serve the greater good of the species. It is important to note that the viability of T4*rII*-infected Rex^+ lysogens was not measured. Sauerbier et al. (1969) reported that the shut-off of host mRNA synthesis and preferential transcription of T4*rII* DNA in T4*rII*-infected rex^+ λ lysogens ceases 13 minutes after infection, after which time host and prophage transcription resumes. These findings may suggest that cell death may not be the purposeful outcome of the Rex phenotype.

RexB, although a membrane protein, stabilizes the λ O initiation of replication protein by inhibiting its degradation by the Hsp100 ClpPX protease (Schoulaker-Schwarz et al., 1991). RexB also inhibits the degradation of the Phd unstable antidote component of the P1 Phd-Doc addiction module (Engelberg-Kulka et al., 1998). Upon infection P1 produces the stable Doc (death upon curing) toxin, which is neutralized by the unstable Phd (protection from host death) antidote. This system ensures plasmid propagation. Whether RexB inactivates the protease, or is a substrate of ClpPX degradation itself remains to be tested. Similarly, RexB was reported to protect *E. coli* cells from their own bacterial apoptotic *relA-mazEF* addiction module through the inhibition of ClpPA protease function (Engelberg-Kulka et al., 1998). During amino acid starvation, ribosome dependent (p)ppGpp synthetase I (or stringent factor) encoded by *relA* rapidly increases the levels of

(p)ppGpp, inhibiting further expression of chromosomally encoded *mazE* and *mazF* (Aizenmann et al., 1996). MazF encodes a lethal toxin, but is neutralized by the unstable antidote MazE. Under conditions of amino acid starvation, MazE is limiting since no de novo synthesis occurs and is rapidly degraded by ClpPA protease, relieving antagonism of the MazF, which endures and kills the cell. By inhibiting the protease activity of ClpPA, RexB may protect cells from programmed cell death induced by amino acid starvation. RexB has subsequently been called an "anti-death gene" (Engelberg-Kulka et al., 1998). Is it possible that this "anti-death" property conferred by RexB is responsible for the observation that *E. coli*(λ) lysogens exhibit increased reproductive fitness during aerobic growth in glucose-limited chemostats compared to isogenic nonlysogens (Lin et al., 1977)? Whether RexB-mediated protease inhibition is directly involved in Rex exclusion or is an alternate activity of RexB requires further investigation. At present Rex exclusion and Hsp100 proteases are connected only by the fact that Rex "activation" results in the loss of cellular ATP, which is required for ClpPA and ClpPX activity.

Several other phenotypes have been discovered with respect to the *rex* genes that seem to obscure the mechanism of Rex exclusion rather than aid in its elucidation. By measuring *lacZ* expression in the presence of amplified photolyase, Li et al. (1992) found that *rex*$^+$ λ lysogenic cells confer accentuated gene inactivation following UV exposure, compared to *rex*$^-$ lysogens, or nonlysogens. They reported that the phenotype was abrogated by mutation of either *rexA*, or *rexB* and suggested that photolyase interaction with UV-induced pyrimidine dimers acts as a substrate for Rex activation, shutting down cellular macromolecular synthesis. The *rex* genes

have also been implicated in modification of UV and alkylating chemical responses to DNA damage, increasing cell sensitivity to these agents (Fix, 1993). In particular MNU (*N*-methyl-*N*-nitrosourea) treated cells were sensitized by *rexA-rexB* expression from a prophage, or low-copy plasmid, but were not stimulated for mutagenesis. This finding suggested to Fix (1993) that repair of UV and particularly alkylating agent-induced DNA damage was inhibited due to DNA:protein interactions arising during repair that serve as a signal for Rex exclusion. As a result, SOS responses could confer "self exclusion" in lysogenic cells. It is important to note that Fix (1993) did not examine the effects conferred by RexA, or RexB alone. Thus, the Rex-mediated sensitivity to DNA damage observed by Fix (1993) may be an extension of the Rex exclusion phenotype.

1.2 The *rII* Genes of Bacteriophage T4

Although the *rII* genes of T4 are probably the most thoroughly investigated genes in genetic history, and led to our modern understanding of genetic structure and punctuation (Benzer, 1955) and recombination, it is rather embarrassing that the function of RIIA and RIIB proteins remains to be determined. The *rII* genes fall into a category of T4 mutants named for the rapid lysis phenotype they exhibited on *E. coli* B (L. Gorini) cells. Upon infection of a host, T4 generally lyses the cell within 25-30 minutes. In the event that external T-even phage are "sensed" within three minutes of initial infection at 37°C , then lysis is delayed for up to several hours, the duration of which is dependent upon the multiplicity of infection (Doermann, 1948; Abedon, 1994). The lysis inhibition phenotype is characterized by continued growth of the

phage within the cell for several hours after which approximately a thousand progeny are released per cell. This number is 10 fold higher than under conditions of normal lysis. The establishment of lysis inhibition provides a genetic selective advantage to T4 phage under conditions of low host availability, maximizing the probability for phage propagation. T4 mutations defective in the establishment of LIN phenotype were originally mapped to the loci *rI, rII* (*rIIA* and *rIIB*), and *rIII* (Hershey, 1946; Benzer, 1955), with new complementation groups found later by temperature-sensitive mutations (Krylov and Zapadnaya, 1965). These mutants form sharp-edged, "rapid lysis" plaques on an *Eco* B cell lawn as compared to a wild type rough-edged plaque conferred by lysis inhibited hosts. While the *rII* genes (*rIIA rIIB*) are required for T4 lysis inhibition in *E.coli* B, mutation of either of the *rII* genes does not affect plaque morphology, or the establishment of lysis inhibition in non-lysogenic *E.coli* K-12 cells, but does render the phage sensitive to Rex exclusion (Doermann, 1948; Benzer, 1955). The *rV* mutations are allelic with T4 *t* gene (Dressman and Drake, 1999), and *rIV* may be allelic with the T4 "spackle" gene, which may function to repair membrane damage upon T4 DNA adsorption and DNA injection (Abedon, 1994; Kai et al. 1999). No function has been associated with *rIII*, although it has been proposed that RIII may stabilize the interaction between RI and gp*t* during lysis inhibition (Ramanculov and Young, 2001). Host lysis is mediated by the holin and endolysin of T4, gp*t* (T, gene product of gene *t*) and gp*e* (E, gene product of gene *e*) respectively. The T holin protein permeabilizes the cytoplasmic membrane permitting the egress of the endolysin E protein to the periplasm, where the muralytic enzyme attacks the peptidoglycan and degrades the cell wall (Josslin, 1970; Josslin, 1971;

Young 1992). Paddison et al. (1998) predicted that RI may also be secreted into the periplasm and may interact directly with T, delaying lysis in response to a signal of secondary phage adsorption. RI interaction with T was later confirmed by Ramanculov and Young (2001). They also predicted that this interaction may be further stabilized by RIII. Although there is no sequence similarity, the function and possibly regulation of T activity has been paralleled with that of the λ holin S. T4 *t* can functionally substitute for *S* when cloned into a plasmid (Lu and Henning, 1992), and induced λ(*t*) lysogens are also capable of establishing LIN in the presence of secondary T4 adsorption (Ramanculov and Young, 2001). Energy poisons cause the inhibitory S107 and active S105 form of the λ holin to become active, disrupting the precise timing clock and causing premature lysis (Young, 1992). Although no analogous inhibitory form of T has been found, loss of T4 lysis timing by energy poisons is also holin-dependent (Josslin, 1971; Ramanculov and Young, 2001).

While T4 lysis inhibition is unequivocally dependent upon the allelic state of *rI* and *t*, the requirement for *rII, rIII* and *rIV* genes seems to be host-dependent. The requirement for RII in the establishment of lysis inhibition is conferred by the presence of a cryptic P2 related prophage harboured by the *Eco* B strain (Benzer, 1957; Rutberg and Rutberg, 1964). Paddison et al (1998) suggested that T4*rII*$^{-}$ rapid lysis on P2 lysogens may be connected to Rex exclusion seen in lambda lysogens. They proposed that P2 may possess a rudimentary, less efficient form of the Rex system that induces premature lysis of T4*rII*-infected lysogens and that Rex exclusion may function similarly except more efficiently, lysing the infected cell prior to phage maturation. Although a reasonable assumption, no evidence of Rex-induced cell

lysis has yet been reported. Furthermore, the absence of leakage of intracellular compounds during exclusion (Sekiguchi, 1966) tends not to support this hypothesis.

Both the *rIIA* and *rIIB* genes are expressed early in T4 infection and are essential for T4 viability on λ lysogenic cells. The RIIA and RIIB proteins were reported to associate with the inner membrane (Weintraub and Frankel, 1972; Ennis and Kievitt, 1973; Huang, 1975; Takacs and Rosenbusch, 1975). Mosig et al. (1984) proposed that one of the functions of the RIIA, RIIB proteins is to anchor the T4 replication complex to the inner membrane. The localization of both the RII proteins of T4 and the RexB protein of λ leads one to ask: is RII subversion of Rex exclusion due to interaction, or interference with RexB in the cytoplasmic membrane?

How the *rII* genes are involved in replication is unknown, but a variety of interactions with T4 replication proteins and RII-mediated replication phenotypes have been reported in nonlysogenic *E. coli*:

i) mutation of either *rIIA*, or *rIIB* has been reported to suppress f T4 ligase mutants (*30*) (Berger and Kozinski, 1969; Karam, 1969), or T4 single-stranded protein (*32*). These proteins are involved in recombination and replication (Mosig et al., 1984; Mosig, 1987). Mosig and Breschkin (1975) proposed that the RII proteins may sequester or inhibit host ligase, preventing host ligase complementation for the T4 ligase defective mutant.

ii) T4 *30*, or T4*32* replication/recombination mutants reverse the *r*-type (rapid lysis) plaque morphology of T4*rII* mutants on *Eco* B back to wild type fuzzy edged plaques.

iii) RII proteins interact with DNA and T4 gp*32* single-stranded binding protein (Manoil et al., 1977).

iv) *rII* mutations were reported to alter the phenotype of endonuclease VII, encoded by T4 *49*, severely compromising phage DNA packaging (Mosig et al., 1984).

How could the involvement of T4 RII proteins in DNA processing/packaging, be connected to their essential role to subvert Rex exclusion in a λ lysogenic host? A suitable hypothesis is that gp32, or gp49 bound to DNA in the absence of RII proteins may be "sensed" as a trigger for Rex exclusion.

The connection between recombination genes and Rex exclusion also extends to other phage species. Phage λ*red* (*exo bet*) recombination mutants and λ*ren* (Rex escape) mutants are susceptible to growth restriction by their own Rex exclusion system (Toothman and Herskowitz, 1980). In this case the *exo* gene encodes an exonuclease involved in recombination. T5*l* mutant phage are restricted for growth on λ lysogens, which has been ascribed to Rex exclusion (Jacquemin-Sablon and Lanni, 1973). Gene *I* is required for the full injection of T5 DNA into the host and is also responsible for the breakdown of host DNA.

T7 *20* phage mutants are similarly restricted for growth on λ*rex*$^+$ lysogens (Pao and Speyer, 1975). Like the *rII* genes, *20* is essential only for growth on λ *rex*$^+$ lysogenic cells and can also suppress defects in T7 ligase. Rex-mediated interference in the growth of T7*20* mutant phage occurs late in the lytic cycle, probably during DNA packaging (reviewed in Molineux, 1991). Cumulatively, these data support an hypothesis that RexA-RexB can sense phage recombination intermediates as DNA:protein interactions, or nucleolytic activity involved in DNA recombination/repair. This hypothesis is further advanced by the reports that the RexA and RexB proteins reduce host DNA solubilization by colicin E2 (Saxe, 1974),

and can increase the half-life of cellular mRNA (Sauerbier et al., 1969).

The RII proteins are not essential for T4 growth on a λ*rex*$^+$ lysogenic cell until 10 minutes post infection, before which the *rII* mutant functions indistinguishably from *r*$^+$ phage with respect to oxygen consumption, DNA synthesis and protein synthesis. Host metabolism becomes strongly reduced in T4*rII*-infected cells after ten minutes (Sauerbier et al., 1969; Parma et al., 1992).

The suppression of Rex exclusion by the *rII* genes of T4 occurs in a gene dosage-dependent manner. Over-expression of *rexA-rexB* on a multicopy plasmid excludes not only T4*rII*, but also T4 and a variety of wild-type phages including T5 and T7, but not λ (Shinedling et al., 1987). Colowick and Colowick (1983) noted that lysogenic cells infected with T4 exhibit a drop in cellular ATP immediately following infection, but ATP levels are quickly restored. These results imply that the consequence of T4 adsorption to the cell wall and DNA injection into the host is the immediate loss of cellular energy. Infection of λ Rex$^+$ lysogens by T4*rII* does not result in the recovery of cellular ATP.

The T4 *motA* gene is termed the *rim* (Rex immunity gene) because *motA*$^-$ defects alleviate Rex restriction of T4*rII* (Mattson et al., 1974) and delay the loss of membrane potential following infection of a λ*rex*$^+$ lysogen by T4*rII* (Parma et al., 1992). MotA is required for early replication of T4 and replication is dramatically delayed in *motA* mutants (Johnson and Hall, 1974; Mattson et al., 1974). The *motA* gene (modifier of transcription) may stimulate the expression of genes *rIIB*, *43* (T4 DNA polymerase), *45* (processivity enhancing protein), and *32* (single-stranded protein) as noted by low level early synthesis of these proteins in T4*motA* infections

(Mattson et al., 1974). However, while *rIIB* expression may be dependent upon MotA, *rIIA* levels were seen to increase as infection of T4*motA* progressed. Do these data imply the involvement of a T4 DNA structure or recombination intermediate, or a DNA:protein interaction in the "activation" of the Rex phenotype?

The mechanism behind Rex exclusion has proven to be complex and difficult in its elucidation, despite numerous reports with respect to the phenotype. Several models regarding the trigger and mechanism of Rex exclusion have been formulated, none of which have yet been tested. What is the "trigger" that results in the harsh cellular manifestations of the Rex phenotype? Does Rex exclusion rely solely on the disruption of Rex stoichiometry and RexA over-expression? Is RexA, or RexB modified from an inactive to an active form by phosphorylation, methylation, or proteolytic modification? Do the RexA, RexB proteins interact and function as a complex and is this complex modified from an inactive to an active form? In the following compilation of reports we begin by investigating the model of Parma et al. (1992) and testing the fate of T4*rII*-infected λ*rex*$^+$ lysogens. We also uncover several new phenotypes associated with the Rex proteins of lambda with respect to cellular manifestations of the Rex phenotype, the influence of RexA, RexB stoichiometry on Rex exclusion activity activity, novel aspects of λ *rex* gene regulation and the modulation of Rex exclusion by the λ repressor, in an attempt to unravel the Rex mystery.

1.3 Directions, Strategies and Experimental Objectives

Parma et al. (1992) proposed that the *rexA-rexB* genes of λ encoded an

altruistic death module, whereby *E. coli* cells infected by T4*rII* commit suicide in order to abort phage propagation. In order to test this hypothesis lambda Rex^+ lysogens will be infected with T4*rII* and the viability of these cells will be determined compared to Rex^- lysogenic and nonlysogenic cells.

The T4 *rIIA rIIB* genes allow T4wt to overcome Rex exclusion. RIIA and RIIB proteins are also required for the establishment of lysis inhibition (LIN) on *Eco B* cells. Using the establishment of LIN phenotype as an assay system, I will examine the relationship between the λ*rexA, rexB* and T4 *rII* genes and determine whether the expression of *rexA* and *rexB* in *E. coli* K-12 cells can reverse the establishment of lysis inhibition.

The stoichiometry of RexA:RexB has been proposed as an important aspect of functionality for explaining the Rex exclusion phenotype. Cryptic λrex^+cI[Ts]cro^- lysogens exhibit a loss of Rex exclusion activity upon thermal induction of the prophage, termed the conditional Rex exclusion (Cdl-Rex) phenotype. I will examine whether the Cdl-Rex phenotype is due to a stoichiometric imbalance in expression of *rexA* relative to *rexB*. Further effects of stoichiometric RexA:RexB imbalance will be examined by multicopy plasmid over-expression of *rexA*, *rexB*, or *rexA-rexB*, in lysogenic Rex^+ cells.

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1.5 Figure Legends

Figure 1. Bacteriophage Lambda Gene Expression from Repressed Prophage.

Figure 2. Bacteriophage Lambda Gene Expression from Induced Prophage.

Figure 1

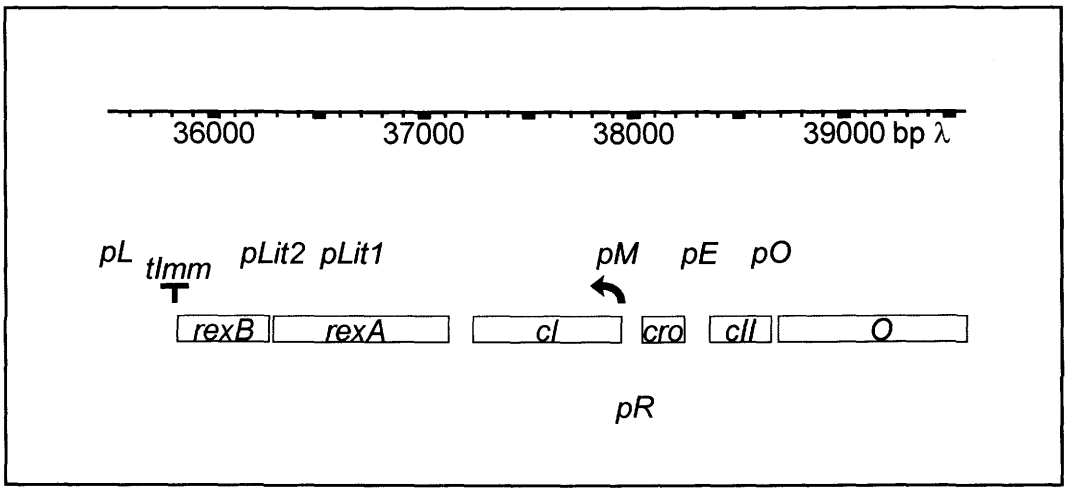
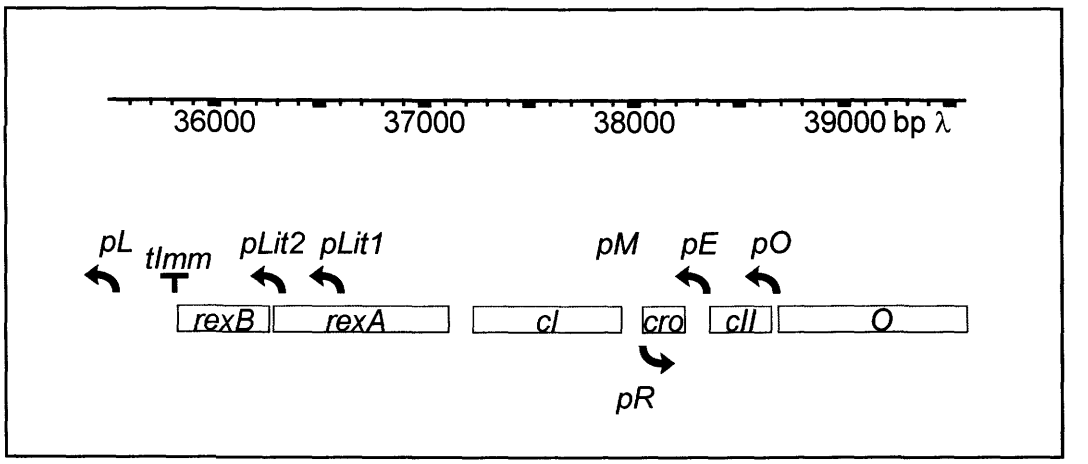


Figure 2



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CHAPTER TWO

Rex-Centric Mutualism.

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Running title: Rex-Centric mutualism

2.1 Abstract

We asked whether Rex exclusion encoded by a lambda prophage confers a protective or a cell-killing phenotype. We found that the Rex system can channel lysogenic cells into an arrested growth phase that gives an overall protective effect to the host despite some associated killing.

2.2 Introduction

The term Rex phenotype connotes generalized phage exclusion by λ lysogens, restricting plaque formation by *rII* mutants of T4 (Benzer, 1955), certain T7 and T5 mutants, and particular variants of lambdoid phages (Toothman and Herskowitz, 1980; Molineux, 1991). The *rex* locus of coliphage λ encoded by genes *rexA* and *rexB* (Matz et al., 1982) is co-transcribed as part of the p_M-*cI*-*rexA*-*rexB*-t_{imm} operon expressed by a repressed λ prophage (Hayes et al., 1997). The model of Parma et al. (1992) predicts that RexB protein forms an inner membrane pore that is opened upon direct interaction with at least two RexA proteins, resulting in a cellular apoptotic response termed "altruistic cell death." The degree of cell death was unreported. We asked if the Rex phenotype confers a protective or a cell killing response to phage attack.

2.3 Materials and Methods

2.3.1 Cells and Phage

We utilized *E. coli* K-12 strains R594 F^- *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD*-*rrnE*)1 λ^- (Bachmann, 1987); W3350A F^- *lac*-3350 *galK*2 *galT*22 IN(*rrnD*-*rrnE*)1 λ^- (Bachmann, 1987), and SA500 F^- *his*-87 *relA*1 *strA*181 *tsx*-83 λ^- to prepare lysogens. λ wild type was from our stock (#271), λ*rexB*5A and λ*rexA*30A were from G. Gussin (Matz et al., 1982) via W. Szybalski. The phages T4*rIIA* (point mutation in *rIIA* gene of T4), T4*rII*Δ1589 (deletion spanning the *rIIA* and *rIIB* genes that generates a *rIIA*-*rIIB* gene fusion and exhibits a $RIIB^+$ phenotype), and T4D were obtained from G. Mosig.

2.3.2 Measuring Viability of T4rII-infected Cells

Cultures of *Eco* K cells were prepared in TB (10 g Bacto Tryptone, 5 g NaCl per litre) by incubating at 30°C for 16 -18 hours. Subcultures were then prepared by 0.1 dilution of the original culture(s) into fresh TB, with incubation in shaking water baths at 30°C, 34°C, 37°C, 40°C, or 43°C to A_{575nm}= 0.8. These cultures were diluted 100-fold in TN buffer (0.01M NaCl, 0.01M Tris, pH 7.6) and aliquots (0.2 ml) were mixed (MOI 10) with a sterile ($CHCl_3$-treated) T4*rIIA* lysate prepared on *EcoK* cells. The infected cells were incubated at the culture growth temperature(s) for 10 min, washed twice, and suspended in 1 ml TN buffer. The washed cells were diluted and aliquots (0.1 ml) were mixed with 3 ml TB soft agar (7.5 g/L Bacto agar) and poured onto TB agar plates (11g/L Bacto agar) that were preheated at the incubation temperature, and then incubated inverted for 48 hours.

2.4 Results

Cellular Viability of T4rII-infected Cells

Cellular viability was determined following T4*rII* infection (MOI = 10) of the Rex^+ lysogen R594(λ) and Rex^- lysogens R594(λ*rexA*30A), R594(λ*rexB*5A) and nonlysogenic R594 cells (Fig. 1A). Optimal infectivity was between 37 – 43°C, with a reduction of >10^3-fold infectivity at 30°C, in agreement with earlier studies (Anderson, 1948; Conley and Wood, 1975). The examination of spread plates from mock infections without T4*rII* showed that virtually 100% of the colonies arose by 24 hours plate incubation at temperatures between 30° and 43°C. No survivors

were seen for the T4*rII*-infected cells during the same interval. We continued incubation for an additional 24 hours, during which the colonies from mock infections continued to grow larger; and tiny surviving colonies appeared between 36-48 hours for the T4*rII* infected Rex^+ lysogens revealing a prolonged growth arrest. The colonies arising from CFU that survived T4*rII* infection were examined for retention of the Rex^+ phenotype and sensitivity to T4. All tested colonies remained Rex^+ and T4-sensitive. In contrast, we found that the viability of Rex^- R594, R594(λ*rexA*) and R594(λ*rexB*) culture cells infected between 37°C and 43°C was <0.001%. Similar results were found for R594(λ) cells infected with T4D (RII^+) (Fig. 1B). The Rex^+ R594(λ) lysogens survived T4*rII* infections with ≥ 40% viability between 37°C and 43°C. Identical infections of Rex^+ lysogens SA500(λ) and W3350A(λ) yielded the same level of survivors as for R594(λ), whereas the viability of their Rex^- derivatives was <0.01% . This experiment revealed that the Rex^+ phenotype can confer an enormous (>10^4-fold) protective advantage to infected λ lysogenic cells. We also followed the viability of Rex^+ and Rex-defective lysogenic and nonlysogenic cells infected in solution with T4Δ*rII* at an MOI of 5. Both R594 and R594(λ*rex*) culture cells were reduced in titer by more than 10^3-fold (assay minimum) within the first hour and surviving CFU were not subsequently detected. Whereas infected R594(λ) cells showed a 10-fold drop in cell titer within the first hour of infection, a lag in cell growth, followed by an increase in CFU. None of the R594(λ) surviving colonies tested were found resistant to T4. In all of the infection experiments, we observed that the surviving cells from samples appeared as colonies after a prolonged lag in cell growth, and were considerably smaller than

the colonies arising from parallel mock infections.

2.5 Discussion

Our findings suggest that the *rex* genes of λ confer symbiotic protection to the lysogenic host against secondary infection. Previous studies have shown that high cellular levels of Rex expression restricts plaque formation by phages T2, T4, T5, T6 and T7 (Shinedling et al., 1989), and thus its advantage in the wild may be more widespread than is appreciated. However, mechanistically, it is far from clear that the Rex phenotype evolved, is maintained, or functions in the wild for the purpose of host protection against secondary lytic infection. It is our view that the cellular manifestations of Rex exclusion that are triggered upon infection may be severe enough to result in cell death, but in balance, may also provide the necessary intolerable environment to eliminate invading phage DNA.

The question of how a Rex^+ cell avoids lethal gene expression from infecting T4*rII* remains unanswered. The stationary phase of *E. coli* host cells has been shown to prevent the growth of T4 phage (Delbruck, 1940; Heden, 1951), and these cells maintain a lower proton motive force (Kashket, 1981). Also during starvation, the stringent response prevents macromolecular synthesis (Sands and Roberts, 1952; Borek et al., 1956) and may lead to bacterial apoptosis (Aizenman, et al., 1996). The Rex system acquired by lambda channels lysogenic cells into an arrested growth phase, resembling stationary phase or stringent response, both of which have levels of cell killing associated with them but on the whole, the responses of this system exhibit mutualism, conferring a protective ability to the

host. The hypothesis that the λ Rex phenotype triggers an altruistic response in excluding the plating of T4*rII* requires that the *rexB-rexA* genes function as a suicide module. Our study does not support this model but rather suggests that Rex exclusion of invading phage is a protective mechanism which results in the increased survival of the infected cells and in turn defends the cell population as a whole from subsequent phage exposure.

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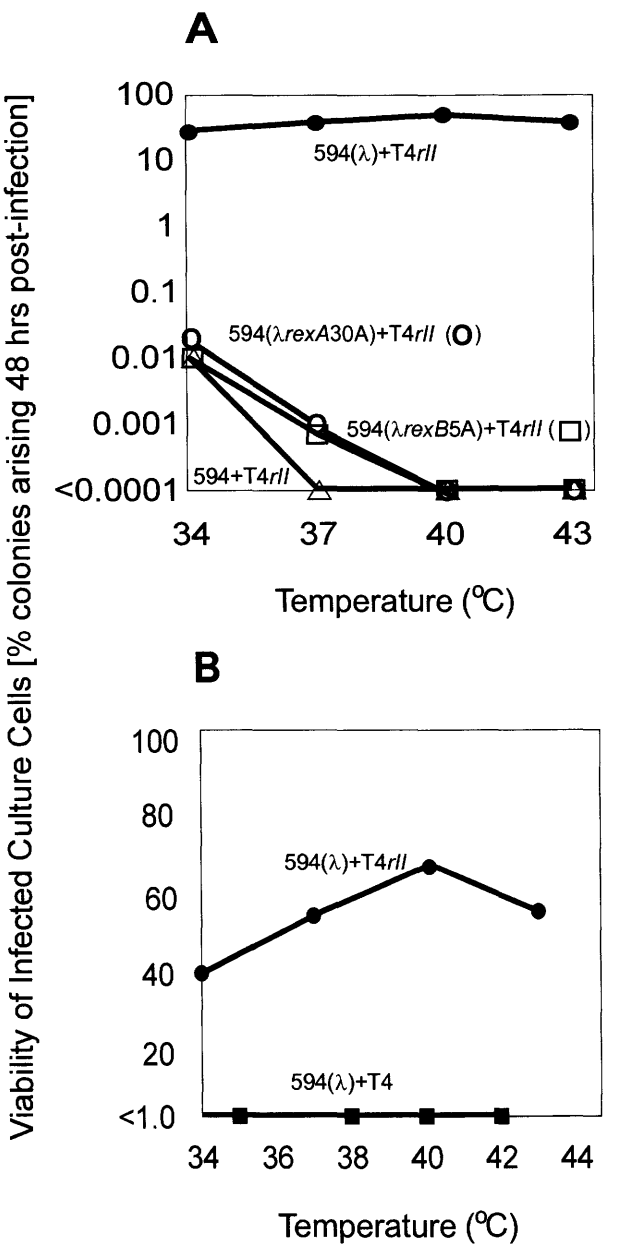
2.7 Figure Legend

Figure 1. Measurement of the cellular viability of *Eco* K strain R594 and lysogens R594(λ), R594(λ*rexA*30A) and R594(λ*rexB*5A) infected: **A.** with phage T4*rIIA*, or **B.** with T4D (*rII*$^{+}$) or T4*rIIA*, as described in text. Viability was determined by counting CFU arising from infected cells after 48 hours indicated temperature.

Figure 1


A
100
10
1
0.1
0.01
0.001
<0.0001
594(λ)+T4rII
594(λrexA30A)+T4rII (O)
594(λrexB5A)+T4rII (□)
594+T4rII
34
37
40
43
Temperature (°C)
B
100
80
60
40
20
<1.0
594(λ)+T4rII
594(λ)+T4
34
36
38
40
42
44
Temperature (°C)
Viability of Infected Culture Cells [% colonies arising 48 hrs post-infection]

CHAPTER THREE

Stationary phase-like properties of the bacteriophage λ Rex-centric mutualism phenotype.

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3.1 Abstract

The *rex* genes of bacteriophage λ protect the lysogenized host against T4*rII* killing, termed Rex-centric mutualism at phage:cell ratios as high as 10^4. This protective effect was abrogated upon mutation of the host stationary phase sigma factor *rpoS*. The T4*rII*-infected Rex^+ lysogens were contracted, formed aggregates and released flagella, resembling cells entering stationary phase. These phenotypes were accentuated in nonlysogenic cells carrying a specific multicopy *rexA-rexB* plasmid: cells were about two-fold contracted in length, expressed membrane-bound and secreted flagella, emitted an odor, were insensitive to infection by a variety of phages and they extensively clumped/adhered when grown up in culture. Our observations support an hypothesis that the Rex system can impart a stationary phase like response that protects the host against T4*rII* infection.

3.2 Introduction

The term Rex phenotype connotes generalized phage exclusion by λ lysogens, restricting plaque formation by *rII* mutants of T4 (Benzer 1955), certain T7 and T5 mutants, and particular variants of lambdoid phages (Toothman and Herskowitz 1980b; Molineux 1991). The *rex* locus of coliphage λ is comprised of two genes, *rexA* and *rexB*, which are co-transcribed as part of the P_M-*cI-rexA-rexB-*t_{imm} operon, expressed by a repressed λ prophage (Matz et al. 1982; Hayes et al. 1997). Although the Rex exclusion phenotype was identified more than forty years ago (Benzer 1955), the mechanism behind this powerful exclusion system remains to be deciphered. How the *rII* genes (*rIIA rIIB*) of T4 allow the phage to escape exclusion is also unknown, but the effect is gene dosage dependent since plasmid over expression of *rex* genes also excludes T4 (Shinedling et al. 1987). Infection of a Rex^+ lysogen with T4*rII* results in the loss of cellular membrane potential, proton motive force and cellular energy (Colowick and Colowick 1983; Parma et al. 1992). Snyder and McWilliams (1989) reported that the *rex* genes can inhibit cell function in the absence of phage infection by over-expression of *rexA* relative to *rexB* in nonlysogens. The RexB protein may function in the inner membrane of the host and shares sequence similarity with ion channel proteins, while the amino acid composition of RexA suggests that it may reside within the cytoplasm (Parma et al. 1992). The Rex phenotype is supported by Na^+ ions in the external medium, and can be abrogated by loss of external sodium, or by the addition of divalent cations, or polyamines (Garen 1961; Ames and Ames 1965; Brock 1965). These observations are consistent with an ion exchange function of RexB.

The infection of a *rex*$^+$ λ lysogen with T4*rII* was previously predicted by Parma et al. (1992) to result in host altruistic cell death by RexA activation of RexB pore(s) in the inner membrane. We have shown that the λ *rex* genes confer protection to lysogenic cells against T4*rII* infection when infected at MOI of 10, termed Rex-centric mutualism (Slavcev and Hayes 2002).

In this study we examine: i) the threshold for Rex-centric mutualism and the influence of stationary phase regulatory genes; ii) the lethality of *rexA* over-expression on Rex$^+$ cells; and iii) the morphological changes to λ lysogenic cells infected with T4*rII*, and nonlysogenic cells expressing plasmid encoded *rexA*$^+$-*rexB*$^+$ genes.

3.3 Materials and methods

3.3.1 Cells and phage

We utilized derivatives of *Eco* K strains of *E.coli*: R594 F$^-$ *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1 λ$^-$ (Bachmann 1987); W3350A F$^-$ *lac*-3350 *galK*2 *galT*22 IN(*rrnD-rrnE*)1 λ$^-$ (Bachmann 1987), SA500 F$^-$ *his*-87 *relA*1 *strA*181 *tsx*-83 λ$^-$; MC4100 F$^-$ *araD*139 Δ(*argF-lac*)205 *flbB*5301 *ptsF*25 *relA*1 *rpsL*150 *deoC*1 λ$^-$ (B. Bachmann, personal communication) to prepare lysogens and JM101 F' *traD*36 *lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$ / *supE thi* Δ(*lac-proAB*) λ$^-$ (Bachmann 1987) for blue/white screening of plasmid inserts on X-gal plates. MC4100*lrp*201::Tn*10* (RO64), MC4100Δ*relA*251::*kan spoT*207::*cat* (MJ155), MC4100Δ*himA*82-Tn*10* (MJ150), MC4100Δ*cya*851 *ilv*::Tn*10* (RH74) and W3110 *rpoS*::Tn*10* (ZK1171) were obtained from R. Salomon (Chiuchiolo et al. 2001). The marker *rpoS*::Tn*10* was

introduced into MC4100 by P1 transduction, using strain ZK1171.

Phage λ wild type was from our stock (#271), λ*rexB*5A, λ*cI*857[Ts]*rexA*amQ were from G. Gussin (Matz et al. 1982) via W. Szybalski. The T4 phages were obtained from G. Mosig and include T4*rIIA* (point mutation in *rIIA* gene of T4), T4*rII*Δ1589 (deletion spanning the *rIIA* and *rIIB* genes that produces an in frame fusion of *rIIA* and *rIIB* and renders the phage $RIIA^-$ $RIIB^+$), and T4[D] wt.

3.3.2 Plasmids

Plasmids used in this study are shown in Fig. 1. pRS2 was constructed as follows. λ DNA 38,103 bp through 35,711 bp was obtained via *Bgl*II digest of pCH1 (Hayes et al. 1997) and was ligated into pACYC184 tet^R, digested with *Bam*HI at 1,869 bp. pRS2 carries λ P_M-*cI*857-*rexA*-*rexB*-t_{imm} downstream from promoter for tet^R. pRS10 was constructed as follows. Primers *im*3 (5' AAGTCGACAGTGAGTTGTATCTATTT 3') and *im*5 (5' TTGGATCCTTAACGTATGAGCACAAA 3') were used to amplify λ sequences 35,731 bp – 37,948 bp from pRS2 encoding λ gene interval *cI*857-*rexA*-*rexB*-t_{imm}. The PCR amplified fragment was digested at its ends with *Bam*HI and *Sal*I and ligated into pACYC184 double digested with *Bam*HI and *Sal*I at 1,869 bp and 2,145 bp respectively. pRS10 carries the λ *cI*857-*rexA*-*rexB*-t_{imm} downstream of promoter for tet^R. pUC18 and pUC19 were obtained from New England Biolabs (NEB). pRS7 was constructed by digesting pRS2 with *Mfe*I and ligating the resulting λ DNA 35,764bp – 37,186 bp fragment into the MCS of pUC19, digested at 396 bp with *Eco*RI. Inserts were screened by blue/white colony formation on IPTG + X-gal plates in JM101. pRS7 carries λ *rexA*-*rexB*-t_{imm} downstream of the promoter for

lacZ'. pRS13 was constructed as follows. Primers *im*3 (5' AAGTCGACAGTGAGTTGTATCTATTT 3') and *im*5 (5' TTGGATCCTTAACGTATGAGCACAAA 3') were used to amplify λ sequences 35,731, bp – 37,948 bp from pRS2. The amplified fragment was double digested with *Mfe*I (λ 37,186 bp) and *Ase*I (λ 36,276 bp) yielding λ fragment 37,186 bp – 36,276 bp, and this was ligated into pUC18 double digested with *Nde*I (at 183 bp, within *lacZ'*) and *Eco*RI (at 396 bp, within the MCS). In pRS13 *rexA* is downstream of promoter for *lacZ'*. pRS14 was made by *Hin*dIII digestion of pRS7 at 36,895 bp and within MCS, removing λ DNA 36,895 bp – 37,186 bp, and religation. pRS14 carries λ DNA 35,764 bp – 36,895 bp corresponding to genes *rexA*[partial Δ]-*rexB*-t_{imm}, deleting the translation initiation site of *rexA*. The *rexB* insert for pRS14, which includes the low-level constitutive P_{Lit2} promoter (Hayes et al. 1997), is downstream of the promoter for *lacZ'*. Plasmid pRS4 was constructed by digesting pCH1 (Hayes et al. 1997) with *Bgl*II and ligating λ DNA 38,103 bp through 35,711 bp into pUC18, digested with *Bam*HI in MCS 486 bp. Plasmid pRS4 carries λ P_M-*cI*857-*rexA*-*rexB*-t_{imm} downstream from the promoter for *lacZ'*. pRS11 was constructed by digesting pRS4 with *Mfe*I to remove λ DNA 37,186 bp – 35,764 bp. pRS11 carries λ P_M-*cI*[Ts]857 downstream of the promoter for *lacZ'*. pRλ*cI*[Ts]857was constructed as follows. pRS11 was double digested with *Sal*I and *Eco*RI yielding chimeric fragment *Eco*RI-MCS-λ*cro*-P_R-P_M-*cI*[Ts]857-*Sal*I (22 bp pUC18 MCS—970 bp λ DNA – 12 bp MCS); the fragment was ligated into pBR322 double digested with *Ava*I and *Eco*RI at 1,429 bp and 4,359 bp respectively. pRλ*lacZ'* was constructed by double digesting pUC19 with *Sma*I and *Aat*II at 412 bp and

2,617 bp respectively and ligating *lacZ'-*$t_{lacZ'}$ fragment into pRλ*cI*[Ts]857 double digested with *Sma*I and *Aat*II in MCS generating the chimeric gene interval $t_{lacZ'}$-*lacZ'* MCS*cro-*P_R*-*O_R*-*P_M*-cI*[Ts]857. Inserts were screened by blue colony formation on IPTG + X-gal plates in JM101 conferred by the in-frame λ*cro-lacZ'* fusion. pRS5 was constructed by digesting λDNA (NEB) with *Mfe*I and ligating λ DNA 35,764 bp – 37,186 bp into pUC19 digested with *Eco*RI at 396 bp in the MCS. pRS7 carries λ t_{imm}-*rexA-rexB* downstream of the promoter for *lacZ'*. pRS6 was constructed identically to pRS7 with the exception that λ*cI*857*rexA*amQ DNA was digested with *Mfe*I and pRS6 carries λ t_{imm}-*rexB-rexA*amQ downstream of the promoter for *lacZ'*. pRS5 was constructed identically to pRS6 and pRS7 with the exception that λ*cI*857*rexB*5A was digested with *Mfe*I and pRS5 carries λ t_{imm}-*rexB*5A-*rexA* downstream of the promoter for *lacZ'*. pRS15 was constructed by double digesting pRS7 with *Aat*II and *Sma*I and ligating the chimeric $t_{lacZ'}$-*lacZ'- rexA-rexB* fragment into pRλ*lacZ'* double digested with *Aat*II and *Sma*I. Inserts were screened by blue/white colony formation on IPTG + X-gal plates in JM101. pRS15 carries λ *rexA-rexB* genes downstream of CI[Ts]857-regulated λ promoter P_R and is repressed at 30°C and induced at 35-40°C (see Fig. 1 legend). pRS16 was constructed in the same manner as pRS15 except that pRS6 was digested with *Aat*II and *Sma*I. pRS16 carries λ *rexA*amQ-*rexB* genes downstream of the λ P_R promoter. pRS17 was constructed in the same manner as pRS15 and pRS16 except that pRS5 was digested with *Aat*II and *Sma*I. pRS17 carries λ *rexB*5A-*rexA* genes downstream of the λ P_R promoter. pHBRex was prepared by H. Bull from pHB25 (Hayes et al. 1997) and includes λ DNA between the *Bgl*II cut at 35,711 bp

(left of t_{imm}-*rexB*) and a rightward *Hin*dIII cut at 37,459 bp (within *cI*), directionally cloned into the MCS of pTZ19R (Pharmacia). Low level constitutive *rexB* transcription occurs from promoter P_{Lit2} present on pHBRex and is located within the distal end of *rexA* (Hayes et al. 1997). Some expression of the cloned λ fragment in pTZ19R, i.e., genes *rexA-rexB* in pHBRex must also arise from the *lacZ'* promoter. Since the Rex effects observed were found to be IPTG-independent, the transcription from $P_{LacZ'}$ was explained by the high plasmid copy number titrating out the cellular LacI repressor. The orientation of the λ DNA inserts within plasmids was confirmed by restriction pattern analysis following *Hin*dIII digestion.

3.3.3 Viability of infected log phase cells

Cultures of *EcoK* cells were prepared in TB (10 g Bacto Tryptone, 5 g NaCl per litre) by incubating at 30°C for 16 -18 hours. Subcultures were then prepared by diluting 0.1 ml of the original culture(s) into fresh TB, with incubation in shaking water baths at 39°C to A_{575nm}= 0.1, or 0.4-0.8. These cultures were diluted 100-fold (4-8 X 10^6 CFU/ml) , or 10^5 fold (4-8 X 10^3 CFU/ml) in TN buffer (0.1M NaCl, 0.01M Tris, pH 7.6) and aliquots (0.2 ml) were mixed (MOI 0 (mock infection), 10, 10^4, 10^5, or 10^6) with a sterile ($CHCl_3$-treated) T4*rIIA* lysate prepared on *EcoK* cells in tryptone broth. The cells were incubated at 39°C for 10 min with the phage to allow adsorption, washed twice by centrifugation, and suspended in 0.2 ml TN buffer. The suspended cells were mixed with 3 ml TB-soft agar (TB + 7.5 g/L Bacto agar) and poured onto TB-agar plates (TB + 1 mg thiamine HCl /L + 11g/L Bacto agar) that were preheated at 39°C. Plates were incubated inverted for 48 hours at the

corresponding original cell incubation temperature. Viability of stationary phase mutants was conducted identically to the above procedure (as employed in Slavcev and Hayes 2002), except that culture cells were grown to A_{575} = 0.1 prior to dilution (rather than to A_{575} = 0.4-0.8).

3.3.4 Plasmid transformation

Cell cultures were grown overnight at 30°C in fresh TB, pelleted, and resuspended in 0.5 volume of cold 0.01M NaCl, pelleted again and suspended in an equal volume of cold 0.03M $CaCl_2$, and incubated on ice for 30 minutes. The incubated cells were pelleted and resuspended in 0.2 volume of cold 0.03M $CaCl_2$. Transformation involved mixing one microgram of plasmid DNA with 0.2 ml of cell suspension, incubating on ice for 1 hour, and then suspension at 42°C in a circulating water bath for 2 minutes. One ml of TB (preheated to 37°C) was added to each transformation reaction tube and incubated with gentle shaking in a 37°C water bath for 90 minutes. Transformant CFU were selected on TB-agar plates with Ampicillin (100 μg/ml) plus IPTG ($1.14X10^{-5}$ M) and total CFU were enumerated on TB-agar plates. Plates were incubated for 36 to 48 hours at 37°C. Transformation frequency was scored as CFU titer on TB-agar plates [+Amp] divided by the CFU titer on TB-agar plates [no Amp]. Single colony isolates of R594(λ)[pRS13] and R594(λ)[pRS14] were grown in TB and the resulting culture cells were diluted and replica plated onto TB-agar and TB-agar [+ Amp] plates to screen for Amp^S colonies, i.e. lysogens that had lost the plasmid. These cured lysogens were assayed for Rex exclusion of T4*rII* to determine if plasmid transformation selected for Rex^- prophage.

3.3.5 Viability of Rex$^+$ cells carrying temperature inducible rex plasmids

Cultures of nonlysogenic Rex$^-$ R594 and Rex$^+$ R594[pRS10] were transformed with temperature inducible multicopy plasmids pRλ*lacZ'*, pRS15, pRS16, or pRS17 and grown overnight at 25°C in TB plus antibiotic(s). The cultured cells were diluted and plated at 30, 34, 37, and 40°C on preheated TB + antibiotic(s) plates and incubated overnight. The number of CFU arising at 34, 37, and 40°C was divided by that at 30°C (repressed) for each strain to quantify cellular viability following thermal induction of cloned genes in the multicopy plasmids.

3.3.6 Visualization of cells

Light microscopy and electron microscopy were used to visualize cells. For electron microscopy aliquots (0.1ml) were taken from cell cultures grown at 37°C to A_{575}=0.1. Cells were negatively stained with phosphotungstic acid (Leduc and Frehel 1990), and visualized with a Philips 410LS transmission electron microscope. Infections were carried out by mixing cells (A_{575}=0.1) and phage (MOI 5) grown up on a R594 *filC*::Tn*10*, and incubating for one hour with shaking at 37°C prior to removing aliquot(s) (see Fig's. 2,3).

3.3.7 Biological assays for Rex exclusion

The Rex exclusion phenotype encoded by λ prophage genes *rexA-rexB* was measured in lysogenic cells grown at 37°C. The relative e.o.p. for each phage was determined by dividing the relative e.o.p. of T4*rII* on the assayed host cells by the titer obtained in parallel on the permissive host cells R594. For each assay, the cultures were grown overnight at 30°C, diluted (0.1X) into fresh TB broth (10g Bacto Tryptone, 5g NaCl / liter). Assays were performed at 37°C by first

transferring culture aliquots (about 3 X 10^8 CFU) to a heated water bath and adding 0.1 ml of dilutions of phage lysates of T4*rII*Δ1589. Three ml of TB top agar (TB plus 6.5g Bacto agar/liter) was added and the mixtures were poured onto TB bottom agar plates (TB plus 11g Bacto agar and 1 mg thiamine·HCl / liter) pre-warmed to the assay temperature. The plates were incubated inverted at the assay temperature for 16 hr. Phage T4D [wt] infections were used as a control for Rex exclusion to show that host cells remained sensitive to T4.

3.4 Results

3.4.1 Survival of T4rII-infected lysogens

Cellular viability was determined following T4*rIIA* infection at phage to cell ratios of 10, 10^4, 10^5 and 10^6 of *EcoK* cells of a Rex^+ lysogen R594(λ), Rex^- lysogen R594(λ*rexB*5A), and nonlysogenic R594 cells (Table 1). The examination of plates from mock infections (no phage added) showed that all plated cells formed CFU by 24 hours at 39°C, which continued to increase in size upon incubation for an additional 24 hours. No survivor CFU appeared by 24 hours for any of the noted culture cells infected in parallel with T4*rII*. However, tiny survivor CFU appeared between 30-48 hours for T4*rIIA*-infected Rex^+ lysogens (revealing a prolonged growth arrest), but no CFU arose from the infected Rex^- lysogen or nonlysogen. The CFU from the Rex^+ lysogen surviving T4*rIIA* infection were examined for retention of their Rex^+ phenotype and sensitivity to T4D [wt]. All or the infected R594(λ), W3350(λ) and SA500(λ) lysogens remained Rex^+ and T4-sensitive. The Rex^+ (λ) lysogens survived T4*rIIA* infection at 20-34% viability at a phage to cell

ratio of 10^4, and 4-9% at a phage:cell ratio of 10^5 (Table 1). No survivor of R594(λ), or W3350(λ) CFU were detected following T4*rIIA* infections at a phage:cell ratio of 10^6. These experiments revealed that the *rexA-rexB* genes conferred to cells lysogenized by λ an enormous survival advantage against T4*rII* killing.

3.4.2 Plasmid-mediated alteration of rexA:rexB stoichiometry

The altruistic cell death model proposed by Parma et al. (1992) predicts that T4*rII*-infection of a λ lysogen results in RexA activation of RexB pores at a 2:1 stoichiometric ratio, killing the cell. We tested this model by measuring the transformation frequency of a λ lysogen with a multi-copy plasmid expressing *rexA*. The transformation frequencies of R594 nonlysogens, and R594(λ) lysogens, were determined for pUC18 (*rex*$^-$), pRS7 (*rexA*$^+$-*rexB*$^+$), pRS13 (*rexA*$^+$) and pRS14 (*rexB*$^+$) plasmids at 37°C (Table 2). While each plasmid transformed nonlysogenic R594 cells with relatively equal frequency (~10^{-4}), distinct differences in the frequency for transformation of R594(λ) were observed. The transformation frequency of a R594(λ) by pUC18[*rex*$^-$] was five-fold lower than R594, suggesting that the integrated λ prophage interfered with plasmid establishment. The frequency for transformation of a λ lysogen of R594 with a *rexA*$^+$ plasmid was lower (five fold) than with *rexB*$^+$ and *rexA*$^+$-*rexB*$^+$ plasmids. The CFU transformed by a *rexA*$^+$ plasmid did not appear until ~30 hours incubation. (Parallel experiments with cell transformation and incubation at 30°C yielded similar results-data not shown.) We attribute the delay in CFU formation of the p*rexA*$^+$ transformants of R594(λ) to the disruption of the RexA:RexB stoichiometric balance, since no delay in CFU formation was seen for nonlysogenic R594 cells.

We wanted to learn whether the R594(λ)[p*rexA*$^+$] transformants retained the Rex exclusion phenotype upon loss of the p*rexA*$^+$ plasmid in order to ascertain if the transformants had acquired a mutation that nullified Rex expression. R594(λ)[p*rexA*$^+$] transformants were grown up in culture without antibiotic selection in order to enable cells that had lost the plasmid to grow. Individual CFU were screened for loss of the plasmid. We found that all the R594(λ) CFU that had spontaneously lost p*rexA*$^+$ exhibited full Rex activity. Thus, the p*rexA*$^+$ transformed lysogens arose without co-selection for a mutation in host or phage that conferred a Rex$^-$ phenotype. The delayed emergence of R594(λ)[p*rexA*$^+$] CFU (which arose at a frequency of 19% of pUC18 transformants of R594(λ) is comparable with the viability seen for Rex$^+$ lysogens infected with T4*rIIA* at a 10^4:1 phage:cell ratio (20-30% survival, Table 1).

A second approach was utilized to examine the potential lethality of *rexA* over-expression in *rexA*$^+$-*rexB*$^+$ cells exhibiting the Rex exclusion phenotype. For this experiment we used pRλ*lacZ'* to construct inducible *rexA*$^+$-*rexB*$^+$ (pRS15), *rexA*amQ-*rexB*$^+$ (pRS16), and *rexA*$^+$-*rexB*5A (pRS17) plasmids under the control of the λ P_R promoter and CI857[Ts] repressor (Fig. 1). These plasmids were transformed into Rex$^-$ R594 and Rex$^+$ R594[pRS10=*rexA*$^+$-*rexB*$^+$] (see Fig. 1) cells at 30°C. In plasmids pRS15, pRS16 and pRS17 the transcription of *rexA-rexB* positioned downstream from the P_R promoter was repressed by CI[Ts]857 repressor between 30-37°C and fully induced at 40°C (see legend to Fig. 1). The results in Table 3 suggest that the induction of *rexA*$^+$-*rexB*5A from pRS17 at 40°C in R594[pRS10/pRS17] cells disrupts RexA:RexB stoichiometry, with RexA

becoming in excess. The increased *rexA* expression results in a transient growth arrest phenotype and a 10-fold reduction in cell viability.

3.4.3 Cell Morphology: influence of rex plasmids or T4rII infection

Both light and electron microscopy were used to observe R594(λ) cells infected with T4*rII*. Gram stains of cell aliquots taken pre- and one hour post-infection (data not shown) revealed a cellular transition from the usual rod shape to a smaller, spherical shape. Uninfected cells with an axial ratio (length/width) of 3.7-4.0 (Fig. 2a,c) changed within one hour after infection with T4*rII* to a contracted morphology with an axial ratio of about 1.7 (Fig. 2b,d; insert, Fig. 3a). Secreted flagella-like structures were observed for T4*rII* infected R594(λ) cells (note, the 6-micron-long filament beneath the cell in Fig. 2b), but were not observed in micrographs of parallel, noninfected R594(λ) culture cells (Fig. 2a). We introduced a null mutation into *fliC*, the gene encoding the structural component for flagellin, i.e. making R594 *fliC*::Tn*10* (λ). The R594 *fliC*::Tn*10* (λ) lysogenic cells (Fig. 2c) were observed to undergo a similar contraction upon T4*rII* infection (Fig. 2d) as seen with T4*rII* infected FliC$^+$ Rex$^+$ lysogens (Fig. 2b). The appearance of flagellar structures was not seen in the noninfected, or T4*rII*-infected Rex$^+$ lysogenic cells defective in *fliC* (Fig. 2c,d). We conclude that the flagella-like structure(s) appearing after T4*rII* infection and associated with the contracted spherical cells (Fig. 2b) were *fliC*-dependent products.

These results add additional Rex phenotypes that are encoded by the *rexA-rexB* genes of λ expressed in lysogenic cells infected with T4*rII*: a) contraction and rounding of cells, b) secretion of *fliC*-dependent flagella-like structures, and c)

appreciable cell survival after a period of prolonged growth arrest. We questioned if these three new phenotypes required phage infection, or if they could arise in the absence of viral infection and were dependent on *rexA-rexB* expression. We found that both Rex exclusion and the new phenotypes a, b, and c arose in noninfected R594 cells that had been transformed with plasmid pHBRex (Fig. 3). The R594[pHBRex] transformants: a) were much smaller (Fig. 3a) than R594[pTZ19R] control cells (Fig. 3b); b) expressed membrane-anchored as well as secreted flagellar structures (Fig. 3a); c) grew slowly in culture; d) were insensitive to infection by a variety of phages, e.g., T4, T4*rII*, λvir, ϕ80, and P1vir (data not shown for c and d); and e) were extensively aggregated (Fig. 3a) and appeared to secrete a mucoid-like polymer, which may explain their clumped appearance. The clustered, flagellated, R594[pHBRex] cells (Fig. 3a) were about 0.4 to 0.7 of the length of the contracted, spherical, T4*rII*–infected cells (insert in Fig. 3a). The R594[pHBRex] cells were up to 5-fold shorter than the R594[pTZ19R] cells (Fig. 3b). The mechanism for these additional Rex phenotypes remains to be explained.

We also observed that no cellular fimbriae appeared on any of the R594 cells transformed with pHBRex (e.g., Fig. 3a). In contrast, about half of the observed culture cells of R594 (not shown), R594(λ) (Fig. 2a), R594 *fliC* (λ) (Fig. 2c), and R594[pTZ19R] (top cell in Fig. 3b), were fimbriated. Fimbriation variation in *E. coli* is regulated by an on-off switch at the rate of approximately 1 change per 1000 bacteria per generation (Brinton, 1959). This observation suggests that the appearance of fimbriae is Rex-independent in λ lysogens, but that increasing the copy number of the *rexA-rexB* genes inhibits cellular fimbriation. Both the

fimbriated and nonfimbriated R594(λ) lysogens were seen to form contracted spherical cells after infection with T4*rII* (Fig. 2b,d). We also note that the extensive clumping of R594[pHBRex] cells grown up in culture (Fig. 3a) was observed by light microscopy for T4*rII*-infected R594(λ) cells as well (data not shown). Cell clumping can interfere with accurate CFU measurements of cellular viability.

3.4.4 Rex phenotypes and cellular stationary phase

E. coli MC4100 strains defective in stationary phase gene regulation were lysogenized with phage lambda to make the cells *rexA*$^+$-*rexB*$^+$. We examined the influence of stationary phase defects on the Rex exclusion phenotype and the viability of T4*rII*-infected cells (Table 4). Again, CFU arising from T4*rII*-infected lysogenic cultures were not visible until after 30 hours incubation at 37°C, while those arising from mock infections were readily visible after 16 hours incubation. The MC4100(λ) lysogens mutated for the *rpoS* stationary phase sigma factor, σ^S, exhibited at least a 400 fold lower viability (T4*rII* cell killing) following T4*rIIA* infection compared to the wild type (29% viability). MC4100Δ*relA* Δ*spoT*(λ) lysogenic cells, mutated for ribosome-dependent (p)ppGpp synthetase I and ribosome-independent (p)ppGpp 3'-pyrophosphohydrolase-(p)ppGpp synthetase II, are incapable of synthesizing (p)ppGpp and were seven fold more susceptible to T4*rII* killing compared to the wild type. In contrast, T4*rII*-infected cells with mutation in regulatory genes *himA*, *cya* (data not shown), and *lrp*, which are each involved in stationary phase gene expression, survived at the same level as MC4100(λ) wild type. These data suggest that σ^S, and to a lesser degree (p)ppGpp, are required for the survival of T4*rII*-infected Rex$^+$ lambda lysogens following a period of

prolonged growth arrest.

3.5 Discussion

We previously found that the Rex system can channel lysogenic cells into an arrested growth phase that gives an overall protective effect to the host despite some associated killing (Slavcev and Hayes 2002). The arrested growth of infected Rex^+ λ lysogens remains to be explained. In the present study we examined the fate of λ lysogenic host cells following T4*rII* infection at higher MOI's, and multi-copy *rexA* plasmid disruption of RexA:RexB stoichiometric balance. We observed that a significant proportion of λ lysogens survived T4*rII* infection at an MOI of 10^4, and survived *rexA* multi-copy plasmid transformation. These observations support the model that the *rex* genes encode a protective rather than an altruistic death module. We cannot rule out that MOI's greater than 10^4 diminish cellular viability due to membrane trauma induced by excessive T4 adsorption to the outer membrane of the cell and its associated lysozyme-5 activity.

We show herein that the derepression of *rexA* from a multicopy plasmid in cells already expressing the Rex^+ phenotype resulted in 10% survival. This observation is in agreement with a model whereby the disruption of the normal RexA:RexB stoichiometric balance can influence cell viability and growth. Our proposal for Rex-centric mutualism (Slavcev and Hayes 2002) suggested that triggered Rex^+-dependent growth arrest, likely representing a cessation of macromolecular synthesis, sufficed to suppress the lethality of T4*rII* infection.

The cellular phenotypes we show herein resulting either from T4*rII* infection

of a λ lysogen, or from excess *rexA-rexB* expression from a multicopy plasmid support a model where Rex exclusion is activated/triggered. The hypothesis that Rex exclusion is triggered leading to altered cellular morphology (i.e., a change from rod-shaped to contracted-spherical shape as seen upon T4*rII* infection of R594(λ) agrees with an observation of Toothman et al. (1980c), who noted that a Rex^+ λ*cI*[Ts] ren^- lysogenic cell changed from rod-shaped to spherical morphology upon thermal induction of the λ prophage.

We suggest that the cellular manifestations of triggered Rex exclusion resemble those of stationary phase. At the onset of stationary phase, *E.coli* cells express flagella, develop a spherical morphology (Ingraham et al. 1983; Lange and Hengge-Aronis 1991; Amsler et al. 1993) and maintain a lower proton motive force (Kashket 1981). Previous studies have also shown that cells in a post-exponential growth phase do not support the growth of T4 (Delbruck 1940; Heden 1951). We found that Rex^+ lysogenic cells infected with T4*rII* were shunted into a state of temporary growth arrest, during which culture cells were temporarily insensitive to T4D killing (data not shown). We have shown that lysogenic Rex^+ cells mutated for *rpoS* stationary phase sigma factor exhibited a drastic loss of viability following T4*rII* infection compared to wild type lysogens. We note that ClpPX is responsible for the degradation of stationary phase sigma factor (σ^S) during normal log phase growth (Schweder et al. 1996), and that RexB inhibits ClpPX activity (Schoulaker-Schwarz et al. 1991; Engelberg-Kulka et al. 1998) and thus may indirectly stabilize σ^S. Furthermore, σ^S stability can be osmotically stimulated (Lange and Hengge-Aronis 1994). This suggests that the perturbation of ions across the inner

membrane following T4*rII* infection of a lambda Rex$^+$ lysogen (Parma et al. 1992) could stimulate the induction of σ^S-dependent genes.

(p)ppGpp is involved in the expression of several stationary phase genes, the stringent response (Sands and Roberts 1952; Borek et al. 1956), and the regulation of starvation-induced apoptosis (Aizenman et al. 1996). The intracellular level of (p)ppGpp increases following the entry of cells into stationary phase. In addition, amino acid starvation increases intracellular (p)ppGpp levels due to the function of the ribosome-dependent (p)ppGpp synthetase I (RelA) and can result in apoptosis by the *relA-mazEF* "addiction module" (Aizenman et al. 1996). Apoptosis is stimulated by ClpPA protease degradation of the MazE "antidote" and favours the accumulation of the stable MazF toxin. RexB has been shown to inhibit ClpPA activity, thereby stabilizing MazE and preventing apoptosis (Engelberg-Kulka et al. 1998). We show herein that Δ*relA* Δ*spoT* mutations (making cells incapable of (p)ppGpp synthesis) reduced the viability of T4*rII*-infected Rex$^+$ lysogenic cells. We propose that the Rex system may function to shunt cells into a phase simulating the stringent response, while inhibiting the induction of MazEF-mediated apoptosis. We also suggest that infection of a λ lysogen by T4*rII* triggers the *rexA-rexB* gene products to channel the infected cells into a cellular phase resembling a state of stationary phase, resulting in a temporary arrest of celular growth and inhibition of phage replication.

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3.7 Figure Legends

Figure 1. Genetic Map of Plasmids Used in This Study.

a Constitutive Expression Plasmids. **b** Temperature Inducible Plasmids.

Temperature-sensitive CI857 repressor activity of pRλ*lacZ'*, pRS15, pRS16 and pRS17 was determined by e.o.p. of λ*cI*72 at 30°C, <2.4X10^{-6}; 34°C, <2.4X10^{-6}; 37°C, <2.4X10^{-6}; and 40°C, 0.27-0.34. Temperature controlled *rexA-rexB* gene expression from the λ P_R promoter of pRS15 was determined by e.o.p. of T4*rII*Δ1589 at: 25°C, 0.57; 30°C, 0.25; 34°C, 8.5X10^{-3}; 37°C, <1.1X10^{-6}; and 40°C, <1.1X10^{6}. All plasmids confer Ampicillin resistance ecept pRS10, which confers chloramphenicol resistance.

Figure 2. Cellular Changes Following Infection of Rex^{+} Lambda Lysogens with T4*rII*.

Electron micrographs of *E. coli* cells grown in TB. Aliquots of *E. coli* log phase (A_{575} =0.1) cells were taken one hour following infection with T4*rII*Δ1589 (MOI 5) and adsorbed to EM grids for two minutes. Grids were then washed with water for two minutes prior to negative staining with phosphotungstic acid (PTA) for two minutes: **a** noninfected R594(λ); **b** unlysed R594(λ) after culture cells were infected for 1 hour at 37°C with T4*rII*Δ1589 (MOI 5); **c** noninfected R594 *fliC*::Tn*10* (λ); and **d** unlysed R594 *fliC*::Tn*10* (λ) after culture cells were infected for 1 hour at 37°C with T4*rII*Δ1589 (MOI 5).

Figure 3. Cellular Changes Mediated by Multicopy Rex Plasmid.

Electron micrographs of *E. coli* cells grown in TB and negatively stained with phosphotungstic acid: **a** R594[pHBRex] taken at a culture absorbance (575nm) of 0.4. The insert is at an identical magnification as that in the main panel of Fig. 3a [with four attached, empty T4*rII* phage heads, and two filled T4*rII* phage heads]; and **b** R594[pTZ19R] at identical culture absorbance of 0.4 [one cell fimbriated and

one not].

Table 1. Cell Viability Following Infection of *E. coli* K Cells with T4*rIIA*

Strain	Cell Viability Following T4*rIIA* Infection (39°C)			
	phage:cell 10^7:10^6	10^7:10^3	10^8:10^3	10^9:10^3
R594	$<7.7 \times 10^{-7}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
R594(λ*rexB*5A)	8.6×10^{-6}	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
R594(λ)	0.60 [a]	0.20 [a]	0.04 [a]	$<10^{-3}$
W3350(λ)	n.t.	0.26 [a]	0.08 [a]	$<10^{-3}$
SA500(λ)	n.t.	0.34 [a]	0.09 [a]	0.003 [a]

nt not tested at 39°C.

[a] No colonies visible after 20 hours. Colonies became visible after >30 hours.

Table 2. Influence of Rex Stoichiometry on Frequency of Transformation

Strain	Plasmid	Plasmid Genotype	Transformation Frequency [a]
R594	pUC18	*rex*$^-$	1.8×10^{-4}
R594	pRS7	*rexA*$^+$-*rexB*$^+$	1.6×10^{-4}
R594	pRS13	*rexA*$^+$	1.6×10^{-4}
R594	pRS14	*rexB*$^+$	2.0×10^{-4}
R594(λ)	pUC18	*rex*$^-$	3.3×10^{-5}
R594(λ)	pRS7	*rexA*$^+$-*rexB*$^+$	2.8×10^{-5}
R594(λ)	pRS13	*rexA*$^+$	6.1×10^{-6} [b]
R594(λ)	pRS14	*rexB*$^+$	3.8×10^{-5}

[a] Frequency of transformation with 1 μg of plasmid DNA after 20 hour incubation at 37°C. Frequency of transformation was scored as number of Amp^R CFU / total number of $CaCl_2$ treated CFU.

[b] No colonies visible after 20 hours. Colonies became visible after >30 hours.

Table 3. Influence of *rexA* Gene Over-expression on Cell Viability

Strain [a]	Cell viability	
	30°C	40°C
Controls:		
R594	1.0	1.0
[pRS10]	1.0	0.8
[pRλ*lacZ*']	1.0	1.0
[pRS15]	1.0	0.6
[pRS16]	1.0	1.0
[pRS17]	1.0	1.0
[pRS10] + [pRλ*lacZ*']	1.0	1.0
[pRS10] + [pRS15]	1.0	1.0
[pRS10] + [pRS16]	1.0	1.0
Experiment:		
[pRS10] + [pRS17]	1.0	0.1 [b]

[a] Culture cells of R594 and R594[pRS10] transformed with the inducible *rex* plasmids were grown to log phase (A_{575}~0.1) at 30°C, then diluted and plated at 30°C, [34°C, 37°C—data not shown] and 40°C on preheated TB + antibiotic(s) plates.

[b] no colonies were present after 20 hours incubation. Colonies arose after 30+ hours incubation at 40°C. The same delay in colony emergence was also seen upon incubation at 37°C, but viability was scored at 0.94 at 37°C (data not shown).

Table 4. Does Mutation of Stationary Phase Regulation Genes Modulate Rex Phenotypes?

Host Cells	Rex Exclusion Phenotype [a]	Cell Viability Following Infection with T4*rII* (MOI 10) [b]
MC4100 (λ)	+	0.3
MC4100 *rpoS*::Tn*10* (λ)	+[c]	$<6.0 \times 10^{-4}$
MC4100 Δ*relA* Δ*spoT* (λ)	+	0.04
MC4100 Δ*himA* (λ)	+	0.3
MC4100 *lrp*::Tn*10* (λ)	+	0.4

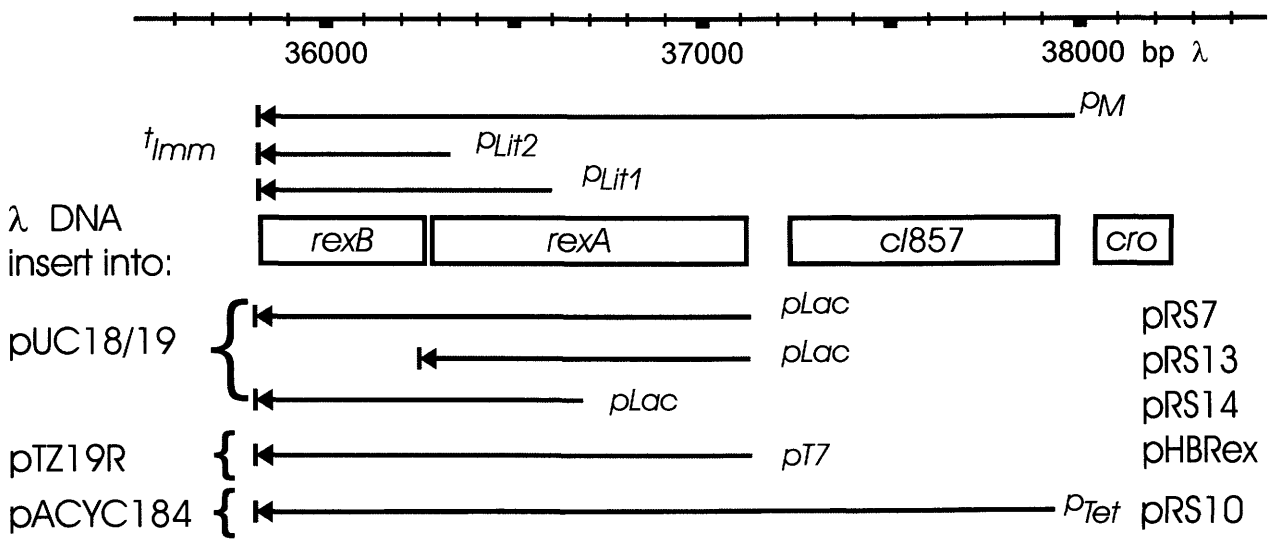
[a] Rex exclusion (+) was denoted as $<10^{-6}$ e.o.p. of T4*rII*Δ1589 at 37°C. The T4*rII*Δ1589 e.o.p. was 0.88-1.0 for all nonlysogenic derivatives at 37°C. The T4D [wt] e.o.p. was 0.2-1.0 for all lysogenic derivatives at 37°C.

[b] cells were washed, diluted and ~10^6 CFU were mixed with 10^7 PFU of T4*rII*, incubated for 10 min. at 37°C, washed of excess phage, and CFU were plated as described in Slavcev and Hayes (2002).

[c] Although extensive cell killing was evident at 37°C, no defined T4*rII* plaques were observed.

Figure 1

a.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37000
38000 bp λ
pM
tImm
pLit2
pLit1
λ DNA
insert into:
rexB
rexA
cI857
cro
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pLac
pLac
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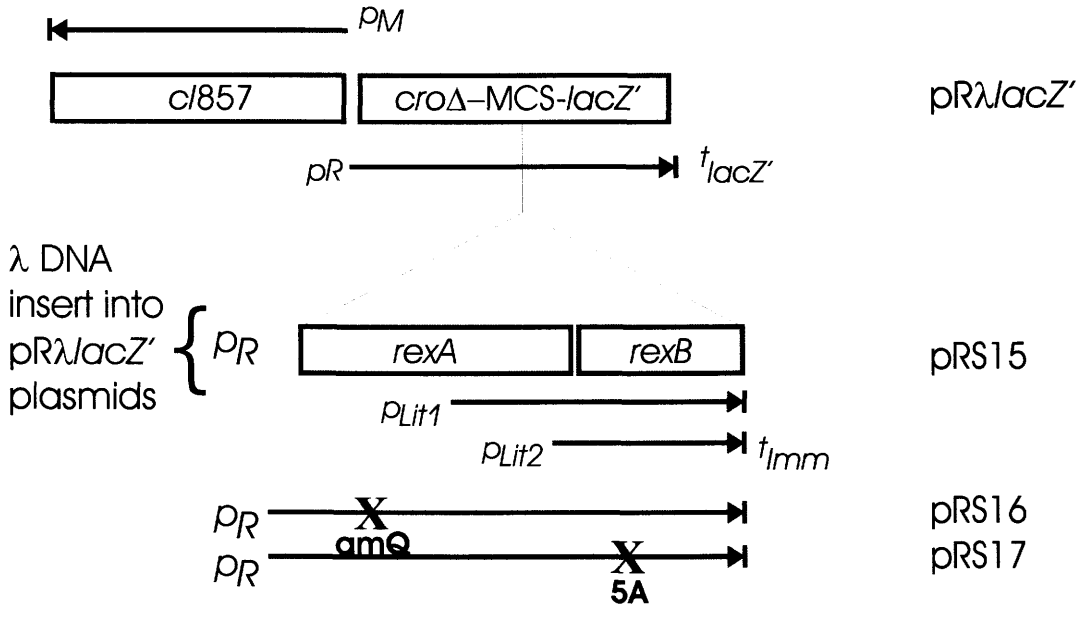
pHBRex
pACYC184
pTet
pRS10

b.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croΔ–MCS-lacZ′
pRλlacZ′
pR
tlacZ′
λ DNA
insert into
pRλlacZ′
plasmids
pR
rexA
rexB
pRS15
pLit1
pLit2
tImm
pR
amQ
pRS16
pR
5A
pRS17

Figure 2

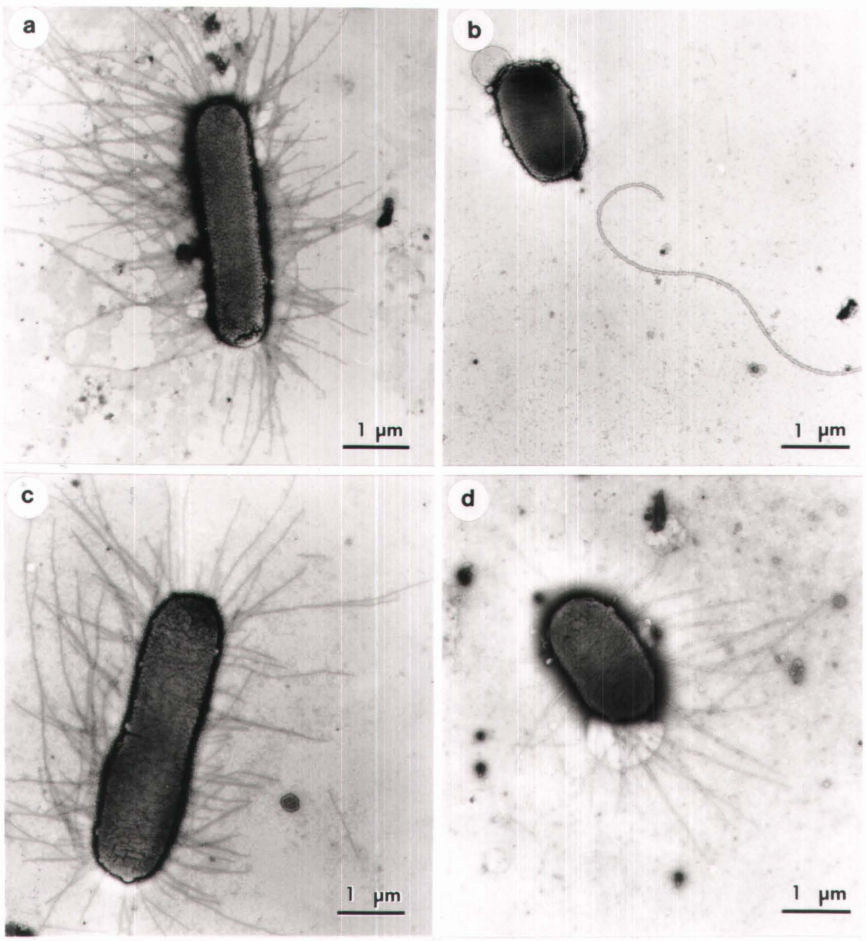
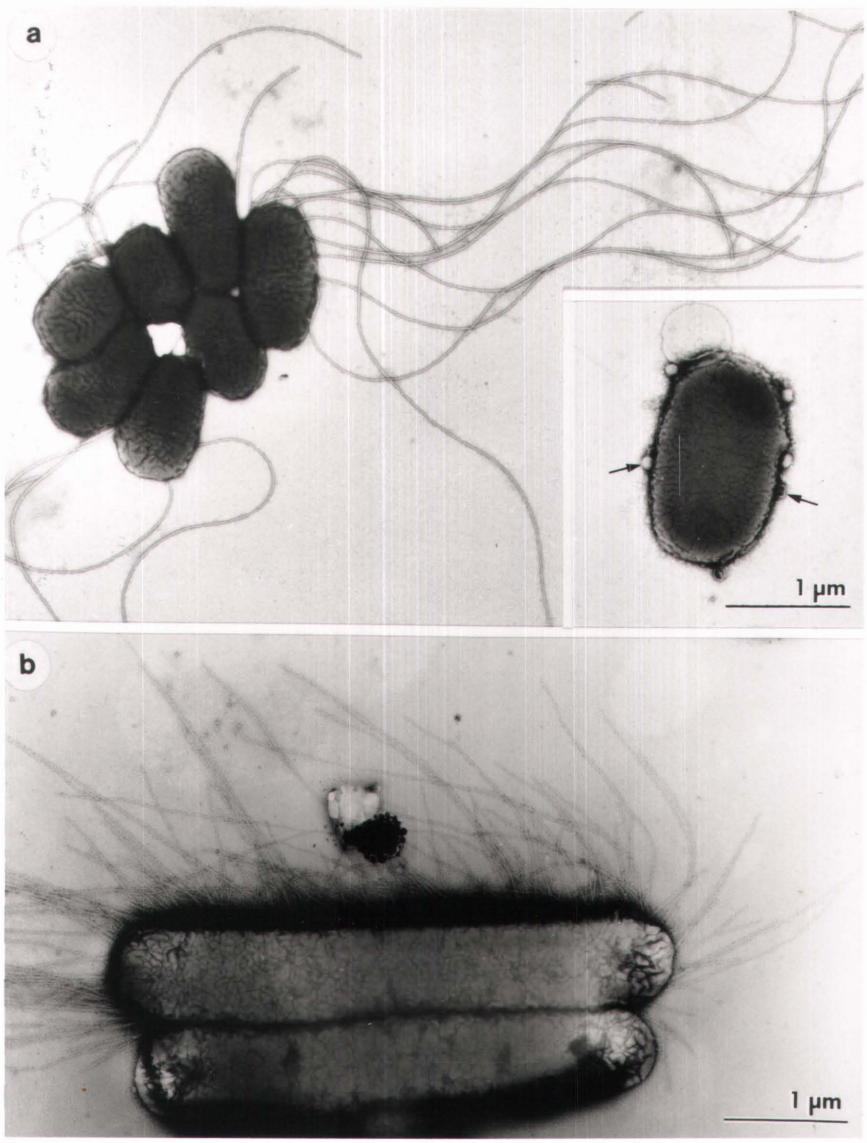


Figure 3



CHAPTER FOUR

Bacteriophage λ RexB Suppression of T4 Lysis Inhibition Phenotype

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Abbreviations: ATCC, American Type Culture Collection; bp, base pairs(s); βGal, β-galactosidase; CGSC, *E. coli* Genetic Stock Center; Cm, chloramphenicol; gp preceding gene, gene product of that gene; IPTG, isopropyl β-D-thiogalactopyranoside; LIN, T4 Lysis Inhibition Phenotype; MOI, multiplicity of infection; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PTA, phosphotungstic acid; TB, Bacto tryptone broth; XGal, 5-bromo-4-chloro-3-indole β-D-galactopyranoside; Δ, deletion; ( ), denotes prophage(lysogenic) state; [ ], denotes plasmid-carrier state; ::, novel junction (fusion or insertion).

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4.1 Abstract

The delay in the lysis of T4*rII* infected cells termed lysis inhibition (LIN) was found to be abrogated by expression of lambda *rexB* from prophage or plasmid. In addition, we found that host mutations in either the periplasmic "tail-specific protease" *tsp*, or the 10Sa RNA *ssrA*, completely abrogated the establishment of LIN following both T4*rII* and T4 infections. Nonlysogenic cells carrying a multicopy *rexB* plasmid were found leaky for a cytoplasmic protein whereas, lysogenic ($\lambda rexB^{+}$-$rexA^{-}$) cells were not. The over-expression of *rexB* suppressed both $T4t^{-}$ and λS^{-} holin mutations, increasing their plating efficiency on otherwise nonpermissive hosts by up to 10^5 fold. Electron microscopy was used to visualize cells transformed with a *rexB*, or *rexA-rexB* multicopy plasmid. The *rexB* plasmid altered surface morphology, while the rex^{+} plasmid imparted a shrunken, but otherwise normal appearance to cells. Our findings are consistent with the possibility that RexB functions as a pore forming unit, inhibited by RexA. We propose models for RexB suppression of T4*rII* lysis inhibition and the involvement of Tsp and 10Sa RNA in lysis timing and the establishment of LIN.

4.2. Introduction

When *E.coli* cells are singly infected with bacteriophage T4, they lyse within 25-30 minutes at 37°C in rich media. However, at a high multiplicity of infection in high-density cell cultures, lysis is delayed for up to several hours during which time the infecting phage continues to replicate and develop vegetatively within the host (Doermann, 1948; Abedon, 1994), before releasing about ten times the normal burst of progeny particles (Josslin, 1970). This T-even phage phenomenon, termed the lysis inhibition (LIN) phenotype, is the result of secondary T-even phage adsorption, three minutes or more following primary infection. LIN provides a selective advantage to the phage by significantly increasing burst size to maximize the probability of progeny finding new hosts under conditions of low host availability. T4 mutations defective in the establishment of LIN phenotype were mapped to the loci *rI, rII* (*rIIA* and *rIIB*), *rIII*, *rIV* and *rV*, forming sharp-edged, "rapid lysis" plaques on an *Eco* B cell lawn as compared to a wild type rough-edged plaque conferred by lysis inhibited hosts (Hershey, 1946; Benzer, 1955). While the *rII* locus (*rIIA rIIB*) is required for T4 lysis inhibition in the canonical (L.Gorini) *E.coli* B strain, mutation of either the *rIIA* or *rIIB* genes does not affect plaque morphology on a nonlysogenic *E.coli* K cell lawn; however, these mutations render T4 sensitive to Rex exclusion encoded by a lambda prophage (Benzer, 1955). The *rIIA rIIB* gene products have been reported to localize to the inner membrane of the host (Weintraub and Frankel, 1972; Takacs and Rosenbusch, 1975). RIIA and RIIB have also been reported to be involved in T4 late replication (Ennis and Kievitt; Huang, 1975), and were suggested to associate the replication complex with the host membrane

(Mosig et al., 1984; Mosig, 1994). The *rV* mutations are allelic with T4 *t* gene (Dressman and Drake, 1999), and *rIV* may be allelic with the T4 "spackle" gene, which functions to repair lysozyme-5 damage to the inner membrane upon T4 DNA injection (Abedon, 1994; Kai et al., 1999). While T4 lysis inhibition is unequivocally dependent upon the allelic state of *rI* and *t*, the requirement for *rII, rIII* and *rIV* genes seems to be host-dependent.

Host lysis is mediated by the holin and endolysin of T4, gp*t* (T, gene product of gene *t*) and gp*e* (E, gene product of gene *e*) respectively. The T holin protein permeabilizes the cytoplasmic membrane permitting the egress of the endolysin E protein to the periplasm, where the muralytic enzyme attacks the peptidoglycan and degrades the cell wall (Josslin, 1970; Josslin, 1971; Young 1992). Paddison et al. (1998) predicted that RI may also be secreted into the periplasm and may interact directly with T, delaying lysis in response to a signal of secondary phage adsorption. RI interaction with T was later confirmed by Ramanculov and Young (2001). They also predicted that this interaction may be further stabilized by RIII. Although there is no sequence similarity, the function and possibly regulation of T activity has been paralleled with that of the λ holin S. T4 *t* can functionally substitute for *S* when cloned into a plasmid (Lu and Henning, 1992), and induced λ(*t*) lysogens are also capable of establishing LIN in the presence of secondary T4 adsorption (Ramanculov and Young, 2001). Energy poisons cause the inhibitory S107 and active S105 form of the λ holin to become active, disrupting the precise timing clock and causing premature lysis (Young, 1992). Although no analogous inhibitory form of T has been found, loss of T4 lysis timing by energy poisons is also

holin-dependent (Josslin, 1971; Ramanculov and Young, 2001).

Although the *rII* genes have undergone decades of extensive genetic analysis and have contributed to the elucidation of the triplet nature of the genetic code, the mechanism by which they subvert exclusion in a Rex^+ λ lysogen is still unclear. The T4*rII* exclusion phenotype of λ is encoded by the *rexA-rexB* genes, which are co-transcribed with the *cI* repressor from the λ prophage P_M promoter. The RexB protein is extremely hydrophobic and has been located to the inner membrane of the host (Parma et al., 1992), traversing the membrane five times and adhering to the "plus inside" amino acid rule for transmembrane proteins (von Heijne, 1986). RexB has also been reported to inhibit ClpPA and ClpPX proteases activity (Engelberg-Kulka et al., 1998). However, it is unclear whether RexB is substrate of ClpPA and ClpPX, directly inhibits protease activity through interaction, or inhibits SsrA tagging of peptides for degration. Host *ssrA* encodes a 10Sa RNA (tmRNA) that functions as both an mRNA and as a t-RNA when ribosomes are paused at the 3' end of an mRNA lacking a stop codon. 10Sa RNA tags such peptides with an 11 a.a. C-terminal sequence that targets them for degradation by ClpPX, ClpPA (cytoplasmic), or Tsp (periplasmic) [Keiler et al., 1996; Gottesman et al., 1998]. Tsp is a periplasmic endoprotease (tail-specific protease) that recognizes 10Sa RNA tagged, or hydrophobic C-terminal residues of peptides and endoproteolytically degrades periplasmic peptides (Silber et al., 1992; Gottesman et al., 1998). Parma et al. (1992) proposed that RexB forms an inactive pore in the inner membrane of the host since it shares sequence similarity with various pores and ion channels, and requires RexA for activation. The model of RexB as a potential

ion channel is supported by observations of Rex-induced depolarization of the inner membrane (Parma et al., 1992), a requirement for monovalent ions in the culture medium for Rex exclusion (Garen, 1961), and the attenuation of the effect by substitution of Na^+ with Mg^{2+}, Ca^{2+}, sucrose, or polyamines (Garen, 1961; Brock, 1965; Ames and Ames, 1965). The relative hydrophilicity of the RexA protein suggested to Parma et al., (1992) that it was cytoplasmic. The *rIIA* and *rIIB* genes of bacteriophage T4 suppress the Rex exclusion phenotype. The enhanced exclusion of wild type T4 by cells expressing *rexA-rexB* from a multicopy plasmid suggests that RII suppression of Rex exclusion is gene dosage-dependent (Shinedling et al., 1987).

In this study we report that: 1) *rexB* expressed either from a multicopy plasmid, or a *rexA*$^-$-*rexB*$^+$ prophage, is capable of suppressing the lysis inhibition phenotype (LIN), seen upon infection of *E.coli* K strains by T4*rII* at high MOI; 2) mutation of periplasmic "tail-specific endoprotease" *tsp* (Silber et al., 1992), or *ssrA* completely abrogates the establishment of LIN by T4; 3) *rexB* expressed from a prophage, or a multicopy plasmid can suppress mutation of λ*S*$^-$ and T4*t*$^-$ holin genes and restore plating efficiency of these phages by up to 10^5 fold; 4) *rexB* expression results in β–galactosidase entering the periplasm, while *rexB* expression from a multicopy plasmid allows β–galactosidase to escape from the cell; and 5) *rexB* expression from a multicopy plasmid alters cell surface morphology. Our findings are consistent with the idea that RexB function as a pore-forming unit. Furthermore, we note that RexB activity is inhibited by RexA. We propose a model for RexB suppression of lysis inhibition establishment by T4*rII*

as well as for Tsp and 10Sa RNA involvement in T4 lysis timing and establishment of lysis inhibition.

4.3 Materials and methods

4.3.1 Cells and phage

We utilized derivatives of *Eco* K strains of *E.coli* (Table 1), namely, R594: F^- *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1 λ^- (Bachmann, B., 1987); MC4100: F^- *araD*139 Δ(*argF-lac*)169 *flhD*5301 *fruA*25 *relA*1 *rpsL*150 *rbsR*22 *deoC*1 λ^- (*E.coli* Genetic Stock Center CGSC# 6152); W3101: *galT*22 IN(*rrnD-rrnE*)1 (Bachmann, B., 1987); Y-Mel: F^+ *mel*-1 *supF*58 (*tyrT*58, su^+); and JM101: F' *traD*36 $lacI^q$ Δ(*lacZ*)M15 $proA^+B^+$ / *supE thi* Δ(*lac-proAB*) λ^-. The *Eco* B strains of *E.coli* used were: EMG31 (from CGSC), ATCC #11303 and the *Eco* B strain L.Gorini (CGSC #5713; Table 1). *Eco* B restriction/modification systems were verified via relative efficiency of plating (e.o.p.) assays with bacteriophage λpapa and φ80. We utilized λ, λ*rexB*go293, λ*rexA*30A, and λ*imm*434T lysogens of R594 and W3101. Phages employed in this study are listed in Table 1. Wild-type bacteriophage λ was from laboratory lysate #271; λ*rex* phages were from G. Gussin (Matz et al., 1982) via W. Szybalski, λ*imm*434T, λ*cI*857Sam7 and λ*imm*434*cII*2002Sam7 lysates #539, # 759 and #873 respectively were from our collection. The T4 phages were obtained from G. Mosig and include T4*rIIA* (point mutation in *rIIA* gene of T4), T4*rII*Δ1589 (deletion spanning the *rIIA* and *rIIB* genes that fuses *rIIA* and *rIIB* rendering the phage $RIIA^-RIIB^+$), T4*t*A3 (point mutation in holin *t*) and T4D. Recombinant φ80*imm*λ*rex* phages were constructed using the functional immunity

(FI) assay described in Hayes and Hayes, (1986).

4.3.2 Plasmids

Plasmids employed in this study are listed in Table 1. Plasmid pHBRexB was prepared by H. Bull from pHB25 (Hayes et al., 1997). pHBRexB includes λ DNA between the *Bgl*II cut at 35,711 bp (left of t_{imm}-*rexB*) and a rightward *Hin*dIII cut at 36, 895 bp (in *rexA*) (Hayes et al., 1997), directionally cloned into the MCS of pTZ19R (Pharmacia). Low level constitutive *rexB* transcription occurs from promoter P_{Lit2} located within the distal end of *rexA* (Hayes and Szybalski, 1973; Hayes et al., 1997). Some expression of the cloned λ fragment in pTZ19R may also arise from the *lacZ'* T7 promoter. pUC19 was obtained from New England Biolabs. Since formation of JM101[pUC19] blue colonies on XGal plates was found to be IPTG independent, the transcription from $P_{LacZ'}$ was explained by the high plasmid copy number titrating out the cellular LacI repressor. pRS7 was constructed by digesting λ with *Mfe*I and ligating λ DNA 35,764bp – 37,186 bp into the MCS of pUC19, digested at 396 bp with *EcoR*I. Inserts were screened by blue/white colony formation on IPTG + XGal plates in JM101. Plasmid pRS7 includes λ *rexA-rexB-*t_{imm} under the control of the *lacZ'* promoter. pRS13 was constructed: primers *im*3 (5' AAGTCGACAGTGAGTTGTATCTATTT 3') and *im*5 (5' TTGGATCCTTAACGTATGAGCACAAA 3') were used to amplify λ sequences 35,731, bp – 37,948 bp from pRS2; the amplified fragment was double digested with *Mfe*I (λ37,186 bp) and *Ase*I (λ36,276 bp) yielding λ fragment 37,186 bp – 36,276 bp; and this was ligated into pUC18 double digested with *Nde*I (at 183 bp within *lacZ'*) and *Eco*RI (at 396 bp within the MCS). In pRS13 *rexA* is downstream

of promoter for *lacZ'*. pRS14 was constructed by *Hin*dIII digestion of pRS7 removing λ DNA 36,895 bp – 37,186 bp (second cut at *Hin*dIII site in MCS of pRS7) and religation. Plasmid pRS14 was constructed by *Hin*dIII digestion of pRS7 removing λ DNA 36,895 bp – 37,186 bp (second cut at *Hin*dIII site in MCS of pRS7) and religation. Plasmid pRS14 carries λ DNA 35,764 bp – 36,895 bp corresponding to genes [partial Δ*rexA*]-*rexB*-t_{imm} , with *rexB* transcription under the control of the pUC19 P_{Lac} promoter as well as under the present low-level constitutive P_{Lit2} promoter within the C-terminal of *rexA*. The presence and orientation of λ DNA inserts was confirmed by restriction digest analysis with *Hin*dIII.

4.3.3 Construction of protease mutants

Protease mutants in this study are listed in Table 1. Protease mutant strains SG12045 (*clpA::kan*), SG22100 (*clpB::kan*), SG22159 (*clpP::kan*--polar on *clpX)*, X9368 (*hflA::kan*), and SG22069 (*ssrA::cat*) were received from S. Gottesman (Gottesman et al., 1998). KS1000 [Δ(*tsp*)::*kan*] was from New England Biolabs (E4128S). Mutations were transduced into R594, and R594(λ) with P1vir, or P1clr100Cam^R, selecting for the antibiotic resistance marker. Recipient strains transduced with P1clr100Cam^R were further screened on TB (10 g Bacto Tryptone, 5 g NaCl per litre) + Cm (20 μg/ml) plates to ensure that they were not P1 lysogens.

4.3.4 T4rII "Lysis Curves"

Cells were grown (16-18h) overnight in TB. Six Klett flasks with 10 ml TB were inoculated with 0.1 ml of an overnight culture and the diluted cells were grown up at 37°C to A_{575} = 0.1. T4*rIIA* phage (2X10^{10} PFU/ml), or T4D phage (4X10^{10}

PFU/ml) lysate was added to the flasks to give MOI's of 0 (mock infection), 1, 3, 5, 7 (data not shown), and 10, based on an assumed cell titer of $1X10^8$ CFU/ml at A_{575} = 0.1. Culture absorbance was recorded with a Spectronic 20 spectrophotometer hourly for eight hours, following the addition of phage (see Fig. 1, 2, 3). Zero time (t=0 hours) implies time of mixing cells plus phage.

4.3.5 Plaque morphology on Eco K and Eco B strains

Cultures of *Eco* B and *Eco* K strains were grown overnight in 10 ml of TB at 30°C. 0.3 ml of culture cells was mixed with 3 ml of molten TB soft agar (7.5 g/L Bacto agar) and the contents were poured onto fresh TB agar (or bottom agar) plates (TB with 11g/L Bacto agar) and left to solidify for 3 minutes. This was done in duplicate for each strain. To examine plaque morphology, 50 μl of a 10^{-7} dilution (approximately 100 PFU) of sterile T4D or T4*rII* (T4*rIIA* or T4*rII*Δ1589) lysate was streaked onto the cell overlay. Plates were incubated for 16 hours at 37°C.

4.3.6 Plating of λcI857Sam7 and T4tamA3 mutant phages

Cultures were grown overnight in 10 ml of TB at 30°C. 0.1 ml of phage dilution was added to 0.1 ml of cell culture and mixed with 3 ml of molten TB soft agar and the contents were poured onto fresh TB plates and left to solidify for 1 minute. λ*Sam7* infection plates were incubated for 16-18 hours at 39°C and T4*t*amA3 plates were incubated for 10 hours at 37°C. The relative e.o.p. for each phage was determined by dividing phage titer on the assayed culture cells by the titer obtained in parallel on *supF* Y-Mel permissive host cells.

4.3.7 Measuring cytosolic protein leakage: A) inducing osmotic shock in cells; B) measuring units of β-galactosidase

Cells were osmotically shocked as described by Nossal and Heppel, (1966). Overnight cultures of cells were diluted 1/100th in 3 ml fresh TB + 0.2 ml 0.02M IPTG and grown at 37°C to A_{575} = 0.2. A 1 ml aliquot was removed from each culture, pelleted and resuspended in same volume of SET Buffer [20% sucrose, 0.03M Tris-HCl, (pH 8.0), 1mM EDTA] and incubated at 30°C for 10 min. Cells were pelleted and resuspended in 100μl SET buffer, rapidly added to 1 ml of ice-cold water and left on ice for 10 min with occasional gentle mixing. Cell suspension aliquots of 0.4 ml were added to 3.2 ml of sodium phosphate buffer (0.08 M, pH 7.7) plus 0.4 ml ONPG (2.5 mM). The mixture was vortexed and incubated at room temperature for 30 minutes. The reaction was stopped by adding 0.4 ml Na_2CO_3 (1M) and the absorbance (420nm) was recorded. Units of βGal were calculated from the equation: Units βGal = 1000 X A_{420} / reaction time (min) X culture volume (ml) X A_{575} culture absorbance (Miller, 1972).

4.3.8 Visualization of cells

Light microscopy and electron microscopy were used to visualize cells. Aliquots (0.1ml) were taken from cell cultures grown at 37°C to A_{575}=0.1 in TB and the cells were negatively stained with PTS (Leduc and Frehel, 1990).

4.4 Results

4.4.1 RexB and Eco B strain-dependent suppression of the T4rII lysis inhibition phenotype

The requirement for T4 *rIIA rIIB* gene products in the establishment of lysis inhibition has been reported to be exclusive to the *Eco* B strain; whereas, RIIA and

RIIB are not required for LIN in *Eco* K strains, or the Bc (prophage-cured) derivative of B (Benzer, 1957; Rutberg and Rutberg, 1964). We confirm our method for assaying LIN in Fig. 1, showing that *Eco* B strains EMG31 and ATCC11303 exhibit lysis inhibition upon infection with T4*rII*, but the *Eco* B L. Gorini strain was defective in LIN, and was rapidly lysed following infection with T4*rII* as shown by abrupt loss of culture turbidity within an hour. These results were corroborated by plaque morphology examination (Table 2). T4*rII* exhibited well-defined rapid lysis (r-type) plaques on the L. Gorini strain, and fuzzy wild-type plaques on EMG31, or ATCC11303, indicating that the P2-related cryptic prophage that confers an RII requirement for lysis inhibition resides within the *Eco* B L. Gorini, but not the EMG31, or ATCC11303 strains.

A minimum delay of three hours in cell lysis (LIN) for T4*rII*-infected cultures (MOI 10) of *Eco* K R594, R594[pTZ19R], R594(λ*rexB*go293) and R594(λ*imm*434T) following T4*rII* lysate addition. However, the strains R594(λ*rexA*30A) and R594[pHBRexB] showed rapid loss of culture turbidity following addition of T4*rII* (Fig. 2). We measured cellular viabilities in parallel to A_{575} readings for R594 and derivatives by removing aliquots from infected and noninfected (mock infection) cultures and determining the CFU titer. We found that that CFU viabilities from infected cultures were <.01% that from parallel mock infections after 15 minutes incubation with T4*rII*. Our results indicate that RexB suppresses the T4*rII* lysis inhibition phenotype in R594. Strain R594(λ*imm*434T) also exhibited lysis inhibition when infected with T4*rII*, indicating that imm^{λ} was required. We carried out infections of the R594 culture series in parallel with T4D and observed lysis

inhibition in R594(λ*rexA*30A) (Fig. 3). The *rII* genes of T4 reversed RexB inhibition of LIN by the λ*rexA*$^-$-*rexB*$^+$ prophage, but not from the multicopy pHBRexB plasmid shown in Fig. 3.

T4*rII* phages exhibited rapid lysis plaques on R594(λ*rexA*30A) compared to T4D infection of the same strain (Table 2). Both T4 and T4*rII* phages exhibited less defined r-type plaque morphologies on R594[pHBRexB] than equivalent plaques on L. Gorini *Eco* B cell lawns. Both T4 and T4*rII* formed well-defined r-type plaques on R594 carrying the pRS14 *rexB* multicopy (pUC) plasmid. We also noted that all plaques on this strain exhibited a "halo" of cell killing beyond the boundary of cell lysis.

4.4.2 Influence of host tail specific protease and 10Sa RNA mutations on the lysis inhibition phenotype

We examined the influence of host protease mutations on the lysis inhibition phenotype to determine whether RexB suppression of LIN was due to the reported RexB-dependent ClpPX, or ClpPA protease inhibition (Schoulaker-Schwarz et al., 1991; Engelberg-Kulka et al., 1998). The establishment of lysis inhibition was observed following T4 infection (MOI 10) of *clpA*$^-$, *clpB*$^-$, *clpP*$^-$ (polar on *clpX*) derivatives of R594; whereas, *ssrA*$^-$ and Δ*tsp* mutant cells were lysed within an hour of infection (Fig. 4). The abrogation of lysis inhibition by *ssrA* and *tsp* mutations was not influenced by the lysogenic state of the mutant. The results indicate that RexB suppression of T4*rII* establishment of LIN is not due to ClpPX, ClpPA inhibition and that *ssrA* and *tsp* host genes are required for the LIN establishment.

Next, we examined plaque morphology of T4D on cell lawns of the R594

protease defective strains (Table 2). T4 formed rough-edged LIN^+ plaques on the Hsp100 mutant derivative strains, but formed very sharp rapid lysis plaques on the Δ*tsp* mutant. The Δ*tsp* mutant reduced the relative T4 e.o.p dramatically (e.o.p.=0.03) and the r-type plaques formed by T4 were heterogeneous in size. The heterogeneity of plaque formation and attenuated plating efficiency of T4 on the Δ*tsp* mutant implies that Tsp protease is involved in some aspect of T4 vegetative growth. Due to the inability of the R594*ssrA*$^-$ mutant to generate adequate cell lawns, we concentrated the culture cells three fold prior to plating T4. We noted that the T4 plaques formed on the *ssrA* mutant were tiny and appeared to have well defined edges, although the plating efficiency of T4 was not altered by the *ssrA* mutation (e.o.p.=1.0). T4 plaque formation on the *ssrA* mutant was slightly heterogeneous in size with one percent of the plaques appearing much larger than the rest (tiny), and clearly exhibiting a rapid lysis phenotype. Our results indicate that that Tsp and 10Sa RNA are required by T4 in the establishment of lysis inhibition, possibly by tagging a peptide(s) for degradation by Tsp.

4.4.3 RexB suppression of λS and T4t mutations

We hypothesized that if *rexB* functions as a pore-forming unit it might suppress holin mutations in λ*S*$^-$, or T4*t*$^-$ that are incapable of lysis due to the inability of the endolysin to gain access to the peptidoglycan layer. We assayed plating efficiencies of λ*cI*[Ts]857*S*am7 and T4*tam*A3 mutant phages in a non-permissive MC4100 strain expressing *rexB* from a multicopy plasmid, or from a prophage (Table 3,4). The *rexB* multicopy plasmid (pRS14) increased λ*cI*[Ts]857*S*am7 plating efficiency by more than 10^5 fold and T4*tam*A3 plating

efficiency by greater than 10^4 fold in the MC4100 Pm^- strain. In contrast, the co-expression of *rexA-rexB* from a multicopy plasmid (pRS7) did not permit the plating of λ*cI*[Ts]857*S*am7 while the S^+ parent phage plated with a relative e.o.p. of 0.7 (Table 3). The relative e.o.p's of T4D and T4*t* on MC4100[pRS7] (p*rexA-rexB*) were $<10^{-6}$ in agreement with the results of Shinedling et al. (1987) who reported that high levels of *rexA-rexB* expression increased the range of Rex exclusion.

Since pRS14 is a pUC based high copy plasmid and excessive expression of membrane-bound RexB may compromise the integrity of the inner membrane, we also assayed the plating efficiency of λ*imm*434*S*am7 on hybrid φ80*imm*λ lysogens of MC4100 that express the *rex* genes at prophage levels from the λ P_M promoter. We found that *rexB* expression from the $RexA^-RexB^+$ prophage increased λ*imm*434*S*am7 plating by more than 250 fold compared to the nonlysogen (Table 4). In contrast, $RexA^+RexB^-$ and $RexA^+RexB^+$ prophages did not suppress the S^- mutation, indicating that co-expression of *rexA-rexB* inhibits the RexB suppression phenotype. Hybrid phage were constructed to exclude the possibility of marker rescue of the wildtype *S* allele from the prophage. We assayed *clpP*$^-$ mutants in order to test the idea that RexB suppression was due to its reported inhibition of ClpPX, or ClpPA activity (Schoulaker-Schwarz et al., 1991; Engelberg-Kulka et al., 1998). We found that introduction of a *clpP*$^-$ allele into the host did not suppress λ, or T4 holin mutations.

4.4.4 RexB$^+$ cells release cytoplasmic protein

Streaking M13mp19 phage on an overlay of JM101[pRS14] culture cells poured on TB plus XGal plates yielded blue plaques due to the phage expression

of the *lacZ'* and complementation with the *lac*ZΔM15 allele from JM101. A halo of XGal hydrolysis extended various lengths beyond the perimeter of the edge of each plaque. This observation suggested that cytoplasmic protein, i.e. β-galactosidase was escaping from these cells. This observation was followed up by quantitatively assaying cellular leakage of cytoplasmic βGal from cultured W3101 *lac*$^+$ cells expressing *rex* gene(s) from a multicopy plasmid, and comparing the result to a lysogen with a single prophage copy (Fig. 5). Cells carrying the pRS14 multicopy *rexB* plasmid exhibited high levels of βGal in the external medium compared to the pUC19 (*rex*$^-$) control (Fig. 5A), or co-expression of *rexA-rexB* from a multicopy plasmid, which showed six-fold lower extracellular βGal from untreated cells than cells carrying the *rexB* plasmid). An alternative experiment involved osmotic shock treatment to release periplasmic protein (Fig. 5B). There was more than a two fold increase in βGal levels released from *rexB*$^+$-*rexA*$^-$ lysogen versus a *rexB*$^+$-*rexA*$^+$ lysogen following osmotic shock treatment of cells to release periplasmic protein. Our results indicate that: a) high level expression of *rexB* results in the escape of cytoplasmic protein from the cell; and b) RexA inhibits this activity of RexB.

4.4.5 Rex plasmid-induced cell changes

Electron microscopy was used to visualize log-phase R594 cells transformed with multicopy plasmids expressing *rexA-rexB*, *rexA*, *rexB* and plasmid control (pUC19) [Fig. 6]. The *rexB* plasmid conferred an abnormal external morphology to cells with formations in the outer membrane that resemble pores. The cells grew normally in TB culture and on TB bottom agar plates, exhibited a translucent "halo" circumscribing the cell colony (data not shown) indicative of cell

leakage and the log-phase cells were slightly shorter than the control with an average axial ratio (length/width) of 2.4-3.0 for three cells measured. In contrast, cells carrying the *rexA-rexB* plasmid were contracted in length with an axial ratio of 1.7-2.0 (roughly a third the size of control cells) and they did not exhibit the circular "pore" formations seen cells making RexB (compare Fig's. 6B and 6D), and were noted to grow slowly in TB and on TB bottom agar plates. Cells over-expressing *rexA* were not visibly altered in appearance (axial ratio of 3.9-4.2) for three cells measured, although they exhibited extensive cellular aggregation (Gram stain data not shown).

4.5 Discussion

The lysis of T4-infected cells requires T4 gene *e* lysozyme mediated attack of the peptidoglycan layer. The egress of this lysozyme to the peptidoglycan layer is mediated by a holin, the product of T4 gene *t*. During lysis inhibition (LIN) holin-dependent cell lysis is delayed and was proposed by Paddison et al. (1998) to involve *rI* inhibition of gp*t* holin function. We show that RexB expressed either from a plasmid, or a *rexB*$^+$-*rexA*$^-$ prophage is capable of suppressing the lysis inhibition phenotype seen upon infection of *E.coli* K strains by T4*rII* at high MOI. The effect of individually expressing RexB in these *Eco* K cells closely resembles the T4*rII* rapid lysis phenotype seen on the L. Gorini *Eco* B strain. The rapid lysis phenotype following the infection by T4*rII* on *Eco* B strain L. Gorini, or on an *Eco* K strain expressing λ*rexB* is suppressed in T4 *rIIA*$^+$*rIIB*$^+$ infections. Further similarities between an *Eco* K strain expressing *rexB*$^+$ strains and the L. Gorini *Eco* B strain

were seen for T4*t* holin suppression: a) multi-copy plasmid expressing *rexB* suppressed an amber mutation of T4*t,* or λ*S*, increasing plating efficiency by more than 10^4 fold, and b) Josslin (1970) reported that T4*t* mutants are capable of growth on log-phase *Eco* B cells. We examined the hypothesis that the P2-related cryptic prophage harboured by the L. Gorini *Eco* B strain expresses a *rexB*-like gene, which confers a requirement for RII in T4 establishment of the lysis inhibition phenotype. We searched for a homologue of λ*rexB* in P2, but found no similar sequences, nor were we able to complement for Rex exclusion by lysogenizing L. Gorini *Eco* B with a ϕ80*imm*λ*rexA*$^+$*rexB*$^-$ prophage (data not shown).

RexB has been localized to the inner membrane and has been proposed to act as an inducible ion channel, resulting in loss of membrane potential upon "activation" of Rex exclusion (Parma et al., 1992). The loss of the potential difference across the membrane caused by various energy poisons has been shown to convert the inactive form of λS holin into the active form, resulting in rapid lysis of the host (Smith et al., 1998). Similarly, energy poisons induce premature lysis in T4 phage, although there is no evidence of an inactive or inhibitory form of the *t* product. Conceivably, RexB suppression of lysis inhibition may be due to a comparable membrane depolarization event that precludes the establishment of lysis inhibition upon T4*rII* infection. Paddison et al. (1998) proposed that P2 possesses a rudimentary *rex* system that inhibits T4*rII* establishment of LIN by triggering premature membrane depolarization and lysis. Even if the P2 prophage confers rapid lysis in this fashion, it is unlikely that RexB suppresses LIN by a similar mechanism since "activation" of the Rex exclusion phenotype requires the

expression of both *rexA* and *rexB*, which we find to be an inhibiting condition for the RexB activities investigated herein. Co-expression of *rexA-rexB* was seen to inhibit RexB function with respect to a) suppression of holin mutations, b) leakage of cytoplasmic protein, and c) suppression of lysis inhibition. Results suggest that RexA inhibits RexB pore formation. But, both RexA and RexB are required to trigger Rex-mediated membrane depolarization. Furthermore, the expression of *rexB* in the absence of *rexA* confers a Rex$^-$ phenotype (Matz et al., 1982) and over-expression of *rexB* relative to *rexA* has been shown to suppress Rex activity (Parma et al., 1992). Rolfe and Campbell (1977) also reported differing lytic patterns between effectively λ*rexA*$^+$*B*$^+$Sam7 and λ*rexA*$^-$*rexB*$^+$Sam7 phage under anaerobic conditions, although at the time it was unknown that the *rex* locus was comprised of two genes (their mutant mapped in *rexA*). We would suggest (but have not tested whether) RexA can suppress RexB inhibition of Clp proteases (Schoulaker-Schwarz et al., 1991; Engelberg-Kulka et al., 1998).

The model we propose (Fig. 7) involves RexB suppressing T4*rII* establishment of lysis inhibition. RexB, serving as a cytoplasm to periplasm pore-forming unit can provide an alternative route by which the endolysin gp*e* of T4 may egress to the periplasm. An alternative model is that T4 RI directly interacts with gp*t* monomers (Paddison et al., 1998), serving as a negative dominant inhibitor of gp*t* oligomers forming an active transmembrane lesion (Ramanculov and Young, 2001; Fig 7A). In the second step (Fig. 7B) RexB bypasses the establishment of T4 lysis inhibition apparatus by allowing an auxiliary channel for T4 endolysin to gain entry to the periplasm in a gp*t* -independent manner. In agreement with Paddison

et al. (1998) that T4 RIIA RIIB proteins have no immediate role in the establishment of lysis inhibition in an *E. coli* K host, we suggest (Fig. 7C) that they nevertheless function in the auxiliary (RexB pore) lytic pathway by inhibiting RexB activity and thus restoring T4 capacity to establish lysis inhibition. The RII membrane proteins could block RexB function through direct interaction (as RI is proposed by Paddison et al. 1998, to inhibit gp*t*); although, so far neither direct protein interaction nor inhibition of transcription or translation have been observed. Our suggestion that RII can block auxiliary egress of gp*e* through a RexB pore is supported by finding that *rexB* expression from a multicopy plasmid blocks the establishment of lysis inhibition by T4*rII* and T4 alike. We suggest this to indicate that RII inhibition of RexB activity is gene dosage-dependent. A dosage relationship between *rII* and *rexB* is also supported by the earlier studies showing that over-expression of *rexB-rexA* in a cell excludes not only T4*rII,* but also T4 (Shinedling et al., 1987). The RII-mediated inhibition of RexB function with respect to suppression of lysis inhibition may provide insight into the mechanism of T4 RII-dependent suppression of λ Rex exclusion. If the RIIA and RIIB proteins act to "cork", or otherwise inhibit RexB pore activity, then a $RexB^-$ phenotype would be conferred to the λ lysogen and Rex exclusion would be averted.

RexB is involved in three activities associated with the inhibition of the ATP-dependent ClpP family of proteases. 1.) It stabilizes the O replication protein of λ by inhibiting the proteolytic degradation of gp*O* by the ATP-dependent serine protease ClpPX (Schoulaker-Schwarz et al., 1991). 2.) RexB has also been shown to inhibit ClpP-directed proteolysis of two known antitoxic proteins, preventing

starvation-induced cell death by the *rel mazEF* addiction module (Aizenman et al., 1996; Engelberg-Kulka et al., 1998). The MazE antitoxic protein is readily degraded by ClpPA, and leads to the lethal accumulation of toxic MazF in the absence of RexB (Engelberg-Kulka et al., 1998). 3.) Cellular loss of P1 leads to cell death from its encoded stable Doc toxin. P1 encodes an unstable antidote, Phd, that neutralizes the Doc toxin. In the P1 plasmid-prophage addiction module, the antidote protein Phd is readily degraded by ClpPX. RexB inhibits the proteolytic degradation of PhD by ClpPX (Engelberg-Kulka et al., 1998).

Herein we showed that mutation of any of the *clp* protease genes had no effect on the establishment of T4 lysis inhibition, suggesting that the RexB suppression of LIN was independent of Hsp100 protease inhibition activity (Fig. 4). However, we noted a requirement for 10Sa RNA (*ssrA*) and tail-specific protease (*tsp*) in the establishment of lysis inhibition. *E.coli* 10Sa RNA tags peptides stalled in translation with an 11 amino acid sequence (AANDENYALAA) that is recognized and degraded by ClpPA, ClpPX (cytoplasmic), or Tsp (periplasmic) proteases (Gottesman et al., 1998). Tsp is a periplasmic protease that recognizes peptide hydrophobic C-terminal residues and cleaves endoproteolytically (Silber et al., 1992).

The Δ*tsp* mutation used here abrogated the establishment of lysis inhibition, reduced the relative e.o.p. of T4 by 10 fold and conferred heterogeneous T4 plaque sizes. These findings suggested to us that Tsp may be involved in lysis timing. The T4 *t* holin sequence encodes a potentially strong hairpin-like secondary structure within the mRNA (Fig. 8), which has previously been suggested to be

involved in gp*t*-dependent lysis timing. By isolating several *t* alleles, which altered lysis timing, Ramanculov and Young (2001) proposed that the N-terminal 96 amino acids of the gp*t* protein contained the transmembrane region and function to permeabilize the membrane, while the remaining 121 were residues are involved in lysis timing and the lysis inhibition phenotype, with the C-terminal domain beyond position 50 residing in the periplasm. Based on the observed requirement for host Tsp and 10Sa RNA in the establishment of lysis inhibition we propose that the hairpin structure within *t* serves to prevent early lysis by pausing translation, resulting in 10Sa RNA tagging of the truncated gp*t* peptide for degradation by periplasmic Tsp (Fig.8A). By late infection, the hairpin structure within *t* may be relieved such that translation progresses beyond the hairpin to termination, after which gp*t* is resistant to Tsp degradation and sensitive to RI-dependent establishment of lysis inhibition (Fig. 8B). Unlike the λ *S* gene, no alternative translational start site or regulatory form of *t* has been identified in lysis timing. We propose that expression of the C-terminal end of gp*t* is regulated by 10Sa RNA tagging and Tsp degradation.

The abilities of RexB to inhibit lysis inhibition, suppress T4 *t* and λ *S* holin mutations, and allow the translocation of cytoplasmic protein into the periplasm support the role of RexB as a constitutive pore-forming protein, the activity of which is inhibited in the presence of RexA, or RII. We have proposed that the inhibition of Rex activity by the RII proteins of T4 is due to their targeting and inactivation of RexB. We have shown that the host 10Sa RNA and Tsp protease are involved in the establishment of T4 lysis inhibition, they act independently of RexB, and their

effects are not reversible by RII. These studies provide new insight into the mechanism of the T4 lysis clock.

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4.6 References

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4.7 Figure Legends

Figure 1. Lysis inhibition phenotype of *Eco* B strains.

Culture absorbance at 575nm, following infections with T4*rIIA* (MOI 10) at 37°C. *Eco* B strains were verified by plating phage ϕ80 lysates prepared by growth on either *Eco* K, or *Eco* B strains. Phage lysates prepared on *Eco* K strain TC600 had e.o.p. of 10^{-4} to 10^{-5}. Phage lysates prepared on *Eco* B ATCC#11303 plated with e.o.p. of 1.0 on all three *Eco* B strains.

Figure 2. RexB inhibition of T4*rII* lysis inhibition phenotype.

Culture absorbance at 575nm, following infections with phages T4*rIIA*, or T4D (MOIs of 0 [mock infection], and 10) at 37°C in tryptone broth (see Materials and Methods). The results for infections at MOI's of 3, 5 and 7 (not shown) were similar to MOI 10.

Figure 3. RII^+ reversal of RexB suppression of LIN.

Culture absorbance at 575nm, following infection with phage T4D (MOI 10) at 37°C in tryptone broth (see Materials and Methods). Zero time represents time of phage/cell mixing.

Figure 4. Influence of host protease mutations on T4 lysis inhibition phenotype.

Culture absorbance at 575nm, following infection with T4D (MOI 10) at 37°C in

tryptone broth (see Materials and Methods). Zero time represents time of phage/cell mixing. Nonlysogenic R594 cells are indicated by "λ^-". Lambda lysogenic R594 derivatives are denoted by "λ^+".

Figure 5. RexB expression confers a leaky phenotype to cells.

Units of βGal released into external medium as determined by hydrolysis of ONPG and measurement of absorbance (420nm) following treatment of culture cells. Units of βGal were calculated as: 1000 X OD_{420} / reaction time X volume culture X OD_{575} culture absorbance (Miller, 1992). (**A**) Toluene (solid bars), or no treatment of nonlysogenic cells (grey bars) carrying multicopy plasmids at 37°C. pUC plasmids do not require induction with IPTG presumably due to the diluting out of cellular LacI. (**B**) Toluene (solid bars), osmotic shock (open bars), or no treatment of lysogenic cells (grey bars) at 37°C induced with IPTG (see Materials and Methods).

Figure 6. Manifestations of λ *rex* gene expression on cellular morphology.

Electron micrographs of noninfected *E. coli* culture cells grown in tryptone broth at 37°C to A_{575} = 0.1, then negatively stained with PTS: (**A**) R594[pUC19]; (**B**) R594[pRS7]; (**C**) R594[pRS13] and (**D**) R594[pRS14].

Figure 7. Model of RexB suppression of T4*rII* lysis inhibition and RII inhibition of RexB Activity.

(**A**) Simplification of the model proposed by Paddison et al. (1998) and Ramanculov and Young (2001) to explain the role of RI in establishment of T4 lysis inhibition. (**B**) Independently of the LIN apparatus, RexB, encoded by the prophage, is proposed to act as an auxiliary translocation pathway through which the T4

endolysin gp*e* may gain access to the periplasm, suppressing LIN. (**C**) The function of RII (RIIA RIIB) proteins of T4 is proposed to inhibit the pore-forming activity of RexB, blocking the auxiliary pathway and establishing LIN.

Figure 8. Model of T4 lysis clock regulation in *E. coli* K host.

(**A**) The gp*t* (holin) mRNA is proposed to form a hairpin secondary structure during early infection. Translational pause causes host 10Sa RNA trans-translation of the degradation signal AANDENYALAA to the C-terminus of the truncated holin targeting it for degradation by periplasmic Tsp protease and inhibiting lysis. (**B**) The hairpin in *t* mRNA is relieved late in infection allowing translation through the C-terminus to the translational stop. The completely translated holin product is not sensitive to Tsp degradation allowing egress of gp*e* and cell lysis; or holin activity is prevented by an interaction with RI resulting in establishment of LIN (Fig. 7).

Table 1. Strains and Plasmids.

Strains	Characteristics or Genotype	Source/Reference
***E. coli* K**		
R594	F⁻ *lac*-3350 *galK2 galT22 rpsL*179 IN(*rrnD-rrnE*)1	Bachmann, 1987
clpA::*kan*	*clpA*⁻	this study
clpB::*kan*	*clpB*⁻	this study
clpP::*kan*	*clpP*⁻	this study
ssrA::*kan*	*ssrA*⁻	this study
Δ*tsp*::*kan*	*tsp*⁻	this study
MC4100	F⁻ *araD*139 Δ(*argF-lac*)169 *flhD*5301 *fruA*25 *relA*1 *rpsL*150 *rbsR*22 *deoC*1	Bachmann, 1987
clpP::*kan*	*clpP*⁻ (insertion is polar on downstream *clpX*)	this study
ssrA::*kan*	*ssrA*⁻	this study
Δ*tsp*::*kan*	*tsp*⁻	this study
W3101	*galT22* IN(*rrnD-rrnE*)1	Bachmann, 1987
Y-mel	F⁺ *mel*-1 *supF*58 (*tyrT*58, su⁺)	Bachmann, 1987
JM101	F' *traD*36 *lacI*^{q} Δ(*lacZ*)M15 *proA*⁺*B*⁺/*supE thi* Δ(*lac-proAB*)	Bachmann, 1987
***E. coli* B**		
EMG31	unknown	Luria, Delbruck, 1943
ATCC11303	unknown	American Tissue Culture Collection
L. Gorini	harbours a P2 related cryptic prophage	source unknown-CGSC # 5713
Phages		
λpapa	wild type bacteriophage λ*rex*⁺	S.H. stock #271
λ*rexB*go293	λ*rexB*⁻-*rexA*⁺	Matz et al., 1982
λ*rexA*30A	λ*rexB*⁺-*rexA*⁻	Matz et al., 1982
λ*imm*434T	λ*rex*⁻ (immunity region of 434 substituted for λ)	S.H. stock #539
λ*cI*857Sam7	amber mutation of λ *S* holin	S.H. stock #759
λ*imm*434*cII*2002Sam7	immunity region of 434, *cII*, amber mutation in *S*	S.H. stock #873
ϕ80*imm*λ*cI*857	phage ϕ80 with λ immunity region; *rexA*⁺-*rexB*⁺	this study
ϕ80*imm*λ*cI*857*rexA*amQ	*rexB*⁺-*rexA*[CDL]	this study
ϕ80*imm*λ*cI*857*rexB*5A)	*rexB*⁻-*rexA*⁺	this study
T4D	wild type T4	from G. Mosig
T4*rIIA*	*rIIA*⁻ *rIIB*⁺	from G. Mosig
T4*rII*Δ1589	Δ(*rIIA-rIIB*)	from G. Mosig
T4*t*A3	amber mutation of *t* holin	from G. Mosig
Plasmids		
pTZ19R	P_{T7}-MCS-*lacZ'*	Pharmacia
pUC19	P_{Lac}-MCS-*lacZ'*	NEB
pHBRexB	P_{T7}-MCS-P_{Lit}-*rexB*-t_{imm} (pTZ19R)	Hayes et al., 1997
pRS7	P_{Lac}-MCS-*rexA*-*rexB*-t_{imm} (pUC19)	this study
pRS13	P_{Lac}-MCS-*rexA*-$t_{lacZ'}$ (pUC18)	this study
pRS14	P_{Lac}-MCS-P_{Lit}-*rexB*-t_{imm} (pUC19)	this study

Table 2. Rex and Protease Influence on Plaque Morphology

Host Cells	Plaque Morphology	
	T4*rII* [a]	T4D [a]
L. Gorini [*Eco* B]	r [b]	+
ATCC11303 [*Eco* B]	r	+
EMG31 [*Eco* B]	r	+
R594 [*Eco* K]	r	+
clpA::*kan*	nt	+
clpB::*kan*	nt	+
clpP::*kan*	nt	+
hflA::*kan*	nt	+
ssrA::*cat*	nt	? [c]
Δ*tsp*::*kan*	nt	r [d]
R594 (λ)	Rex exclusion [e]	+
R594 (λ*rexA*30A)	r [f]	+
R594 (λ*rexB*go293)	r	+
R594 (λ*imm*434T)	r	+
R594 [pTZ19R]	r	+
R594 [pHBRexB]	r [f]	? [g]
R594 [pUC19]	r	r
R594 [pRS14]	r [h]	r [h]

nt not tested

[a] Phage were streaked onto cell lawn overlays (approximately 100 phage) and incubated for 16 hours at 37°C. A "+" denotes LIN. An "r" denotes rapid lysis.

[b] r plaques are indicative of lysis inhibition, whereas r-type (rapid lysis) plaques show defined edges, indicative of phage inability to establish lysis inhibition.

[c] Majority of T4D plaques were very tiny, but appeared to have well defined edges. One percent of plaques were larger and visibly r-type. T4D relative e.o.p = 1.0.

[d] **Majority of T4D plaques were much smaller than .those seen on R594 and ranged in size. Larger plaques were clearly r-type.** T4D relative e.o.p.= 0.03.

[e] expression of λ *rexB*$^+$-*rexA*$^+$ prevents plating of T4*rII* due to the Rex exclusion phenotype.

[f] rapid lysis plaque was larger, but not as well defined as T4*rII* on L. Gorini *Eco* B.

[g] plaques could not be differentiated, exhibiting a morphology between r-type and r.
[h] plaques were large and exhibited a "halo" of cell lysis beyond the plaque boundary.

Table 3. RexB Plasmid Suppression of Holin Mutations.

Host Cells	λ*cI*857 Sam7 e.o.p. (39°C)	T4*tam*3 e.o.p. (37°C)
Y-mel [SupF]	1.0	1.0
MC4100 [Sup°]	$<1.9 \times 10^{-6}$	1.3×10^{-6}
MC4100 *clpP*::*kan* [a]	$<1.9 \times 10^{-6}$	1.8×10^{-6}
MC4100 Δ*tsp*	1.9×10^{-6}	$<1.2 \times 10^{-6}$
MC4100 *ssrA*::*kan*	2.0×10^{-6}	$<1.2 \times 10^{-6}$
MC4100 [pTZ19R] (*rex*$^-$)	3.5×10^{-6}	$<2.8 \times 10^{-7}$
MC4100 [pHBRexB] (*rexB*$^+$)	6.7×10^{-4} [b]	0.06 [b]
MC4100 [pUC19] (*rex*$^-$)	3.8×10^{-6}	7.5×10^{-4}
MC4100 [pRS7] (*rexA*$^+$-*rexB*$^+$)	$<1.9 \times 10^{-6}$ [c]	$<2.8 \times 10^{-7}$ [d]
MC4100 [pRS14] (*rexB*$^+$)	1.0	0.11

[a] insertion in *clpP* is polar on downstream *clpX*.
[b] plaques were pinpoint compared to those on SupF (Pm$^+$). Result is the average of two trials.
[c] λ*cI*857 (S$^+$) plated on MC4100[pRS7] with e.o.p. of 0.69
[d] T4 and T4*t* are sensitive to Rex exclusion encoded by multicopy *rexA-rexB* genes.

Table 4. Prophage Level RexB Suppression of λ *S* Holin Mutation.

Host Cells	Rex Phenotype	λ*imm434cII*2002 Sam7 e.o.p. (30°C)
Y-mel [SupF]	Rex^-	1.0
MC4100 [Sup°]	Rex^-	$<8.3 \times 10^{-7}$
MC4100 (ϕ80*imm*λ*cI*857) [a]	Rex^+	$<8.3 \times 10^{-7}$
MC4100(ϕ80*imm*λ*cI*857*rexA*amQ)	$RexA^-B^+$	2.1×10^{-4}
MC4100 (ϕ80*imm*λ*cI*857*rexB*5A)	$RexA^+B^-$	$<8.3 \times 10^{-7}$

[a] ϕ80*imm*λ hybrid phage were constructed by F. I assay (Hayes and Hayes, 1986) to prevent λ S^+ marker rescue from the prophage.

Figure 1

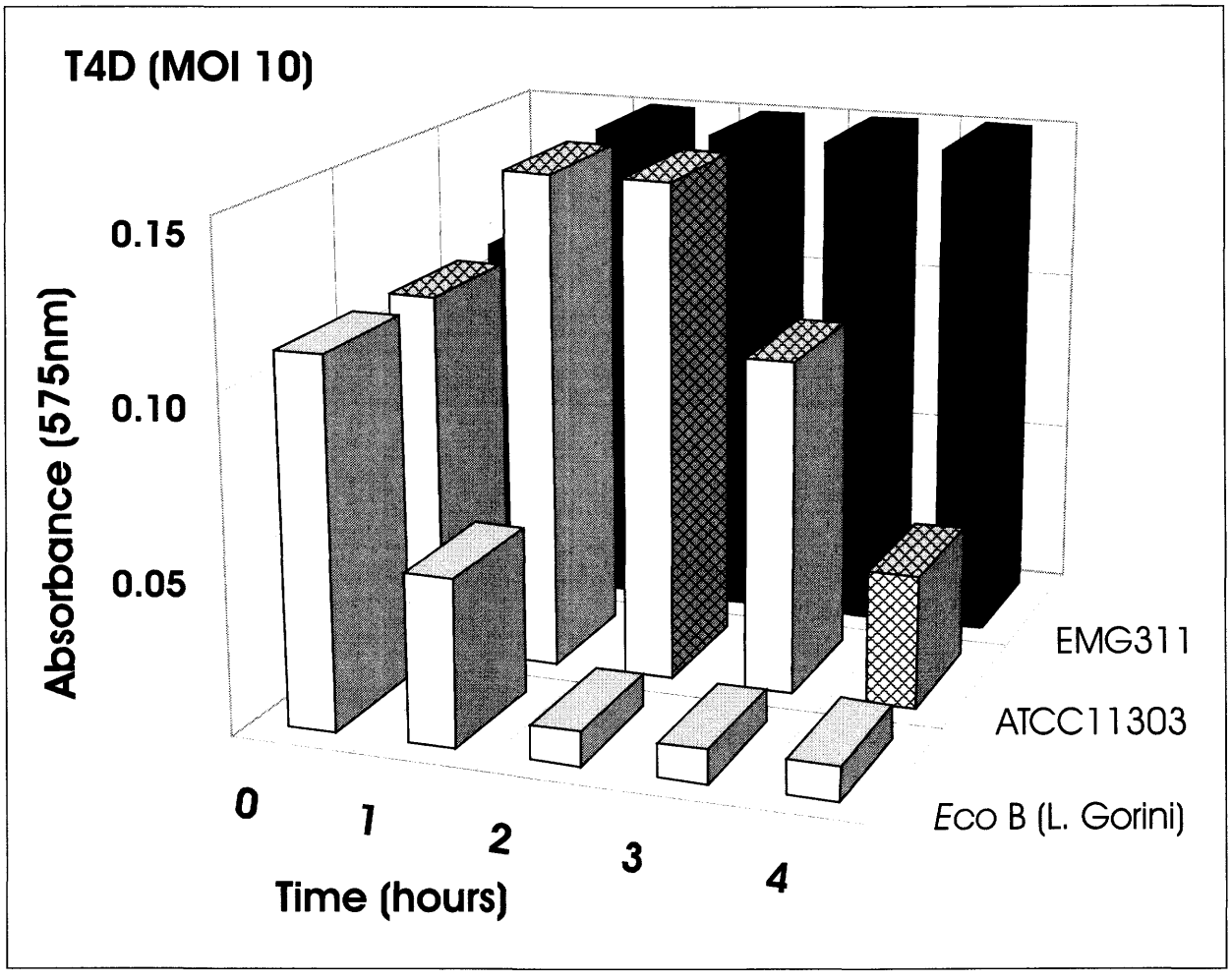
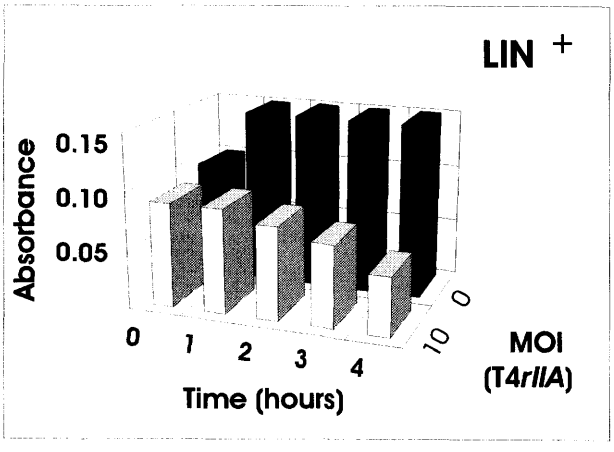
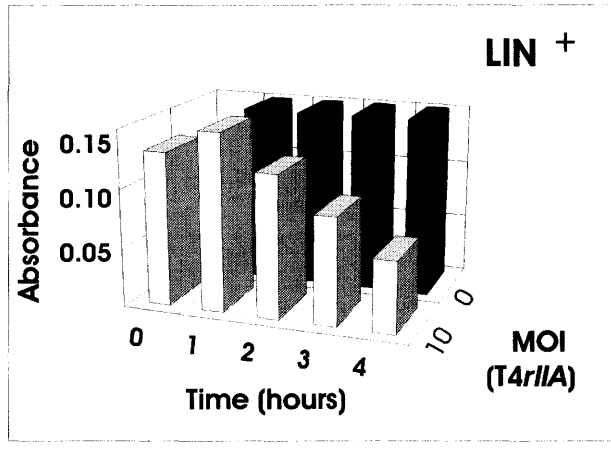

T4D (MOI 10)
Absorbance (575nm)
0.15
0.10
0.05
0
1
2
3
4
Time (hours)
EMG311
ATCC11303
Eco B (L. Gorini)

Figure 2

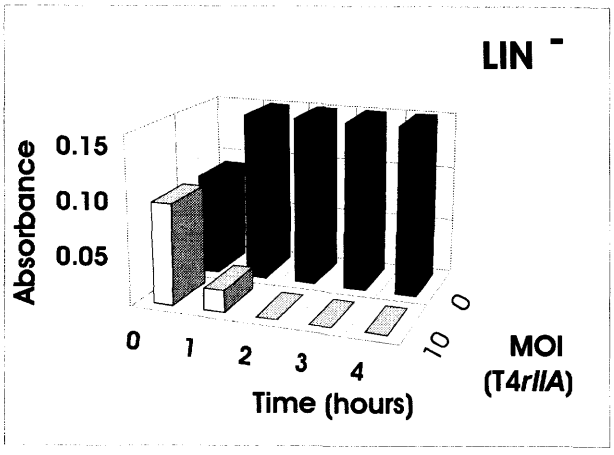
594



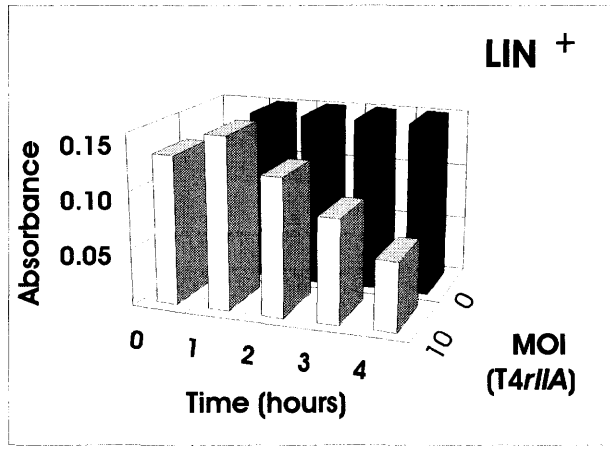
594(λ*imm434*T)



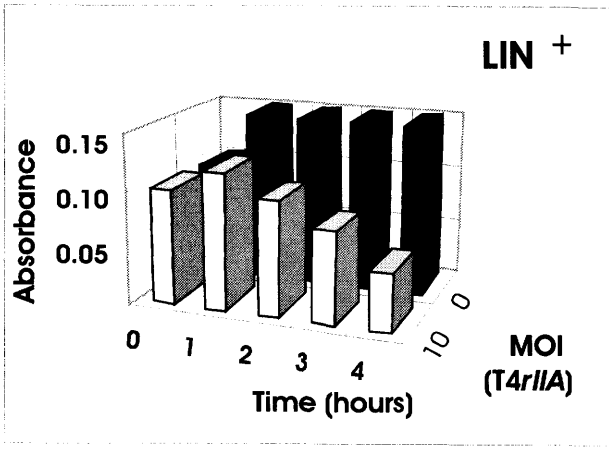
594(λ*rexA*30A)



594(λ*rexB*go293)



594[pTZ19R]



594[pHBRexB]

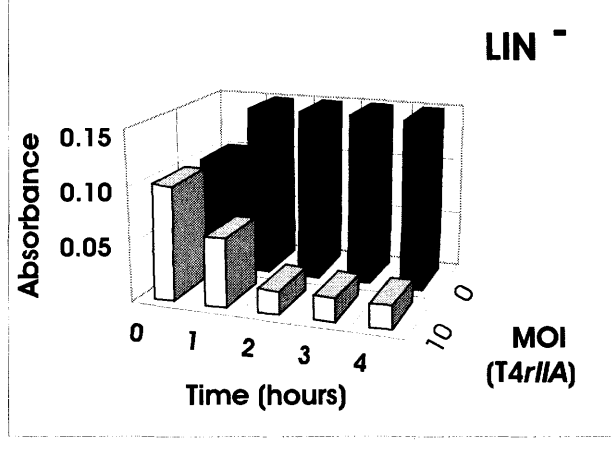


Figure 3

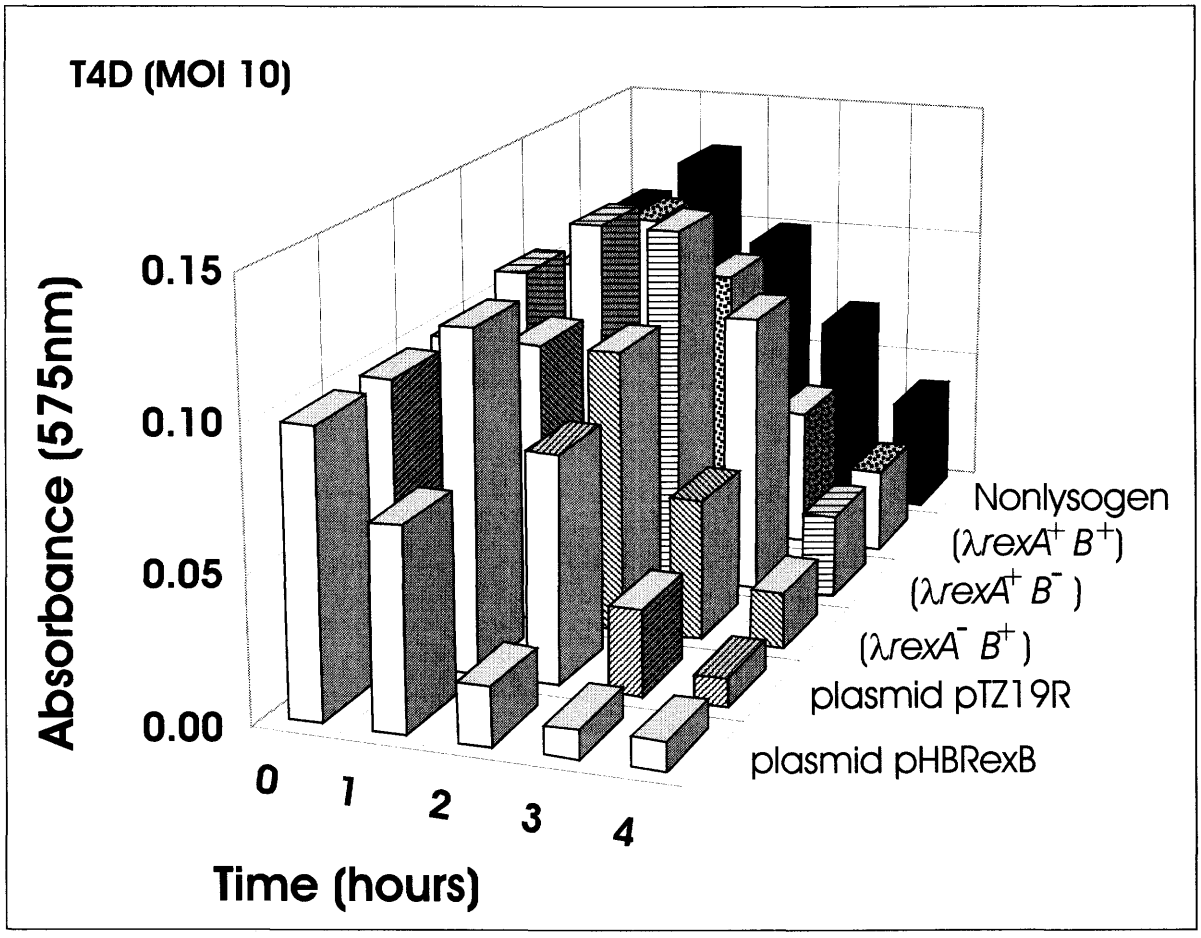
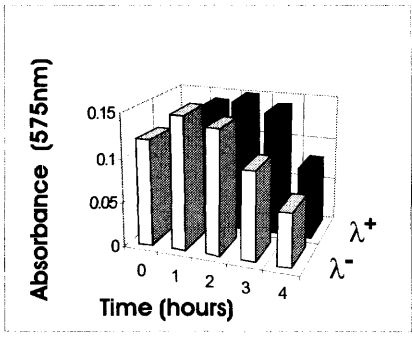
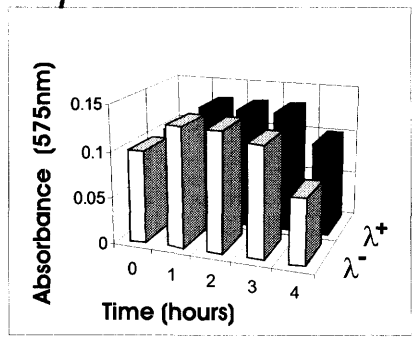

T4D (MOI 10)
Absorbance (575nm)
0.15
0.10
0.05
0.00
0
1
2
3
4
Time (hours)
Nonlysogen
(λrexA+ B+)
(λrexA+ B-)
(λrexA- B+)
plasmid pTZ19R
plasmid pHBRexB

Figure 4

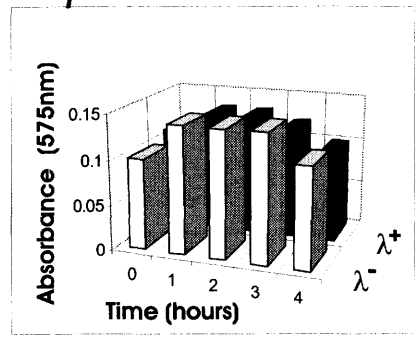
wt



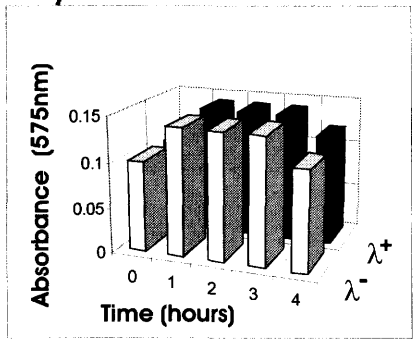
clpA⁻



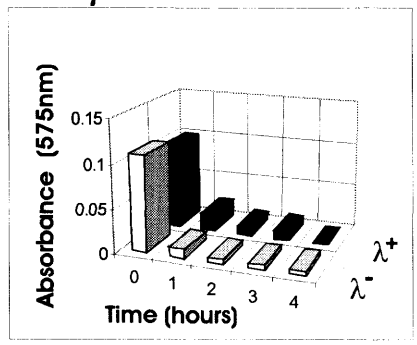
clpB⁻



clpP⁻



Δ*tsp*



ssrA⁻

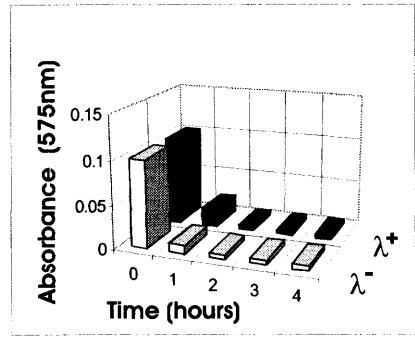
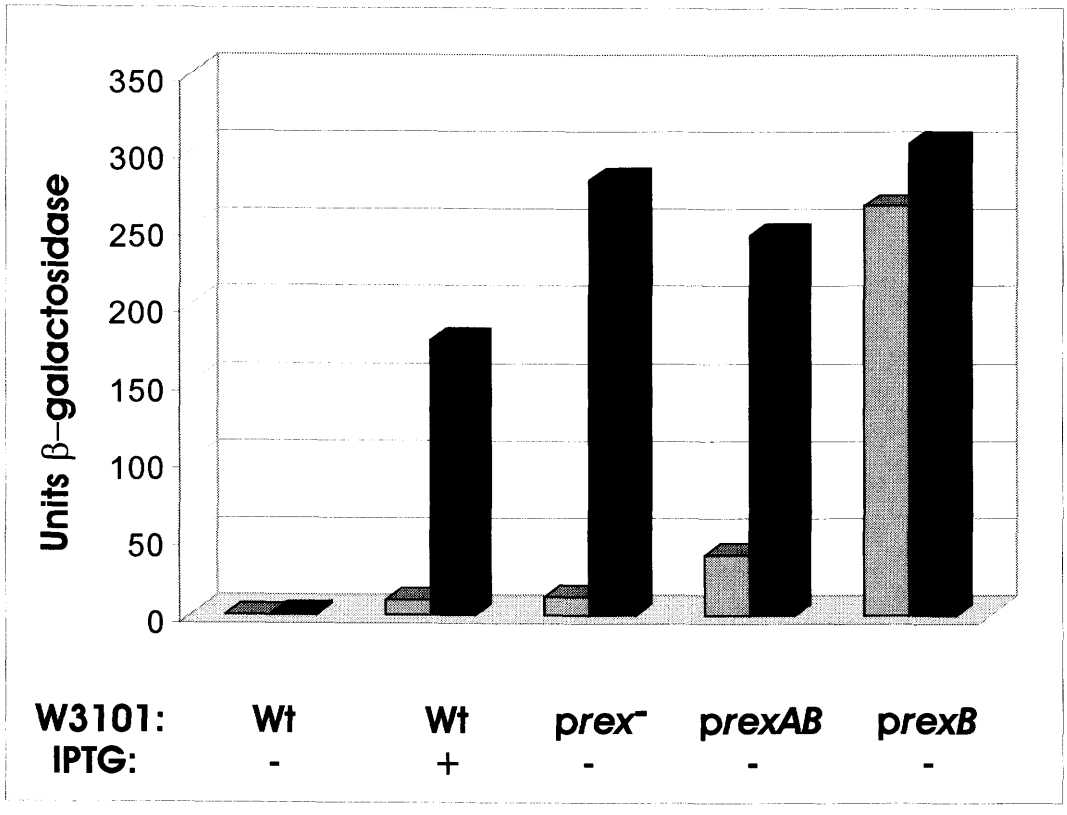


Figure 5

A


Units β–galactosidase
350
300
250
200
150
100
50
0
W3101: Wt Wt prex⁻ prexAB prexB
IPTG: - + - - -

B

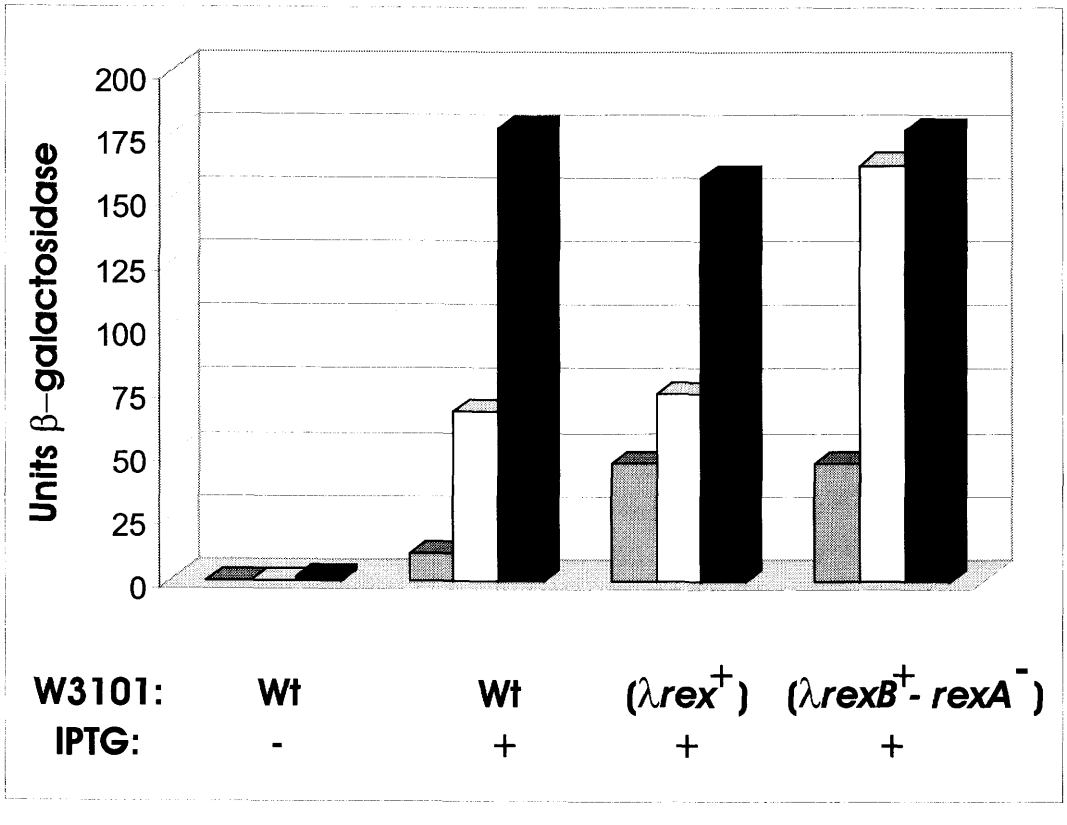

Units β–galactosidase
200
175
150
125
100
75
50
25
0
W3101: Wt Wt (λrex⁺) (λrexB⁺- rexA⁻)
IPTG: - + + +

Figure 6

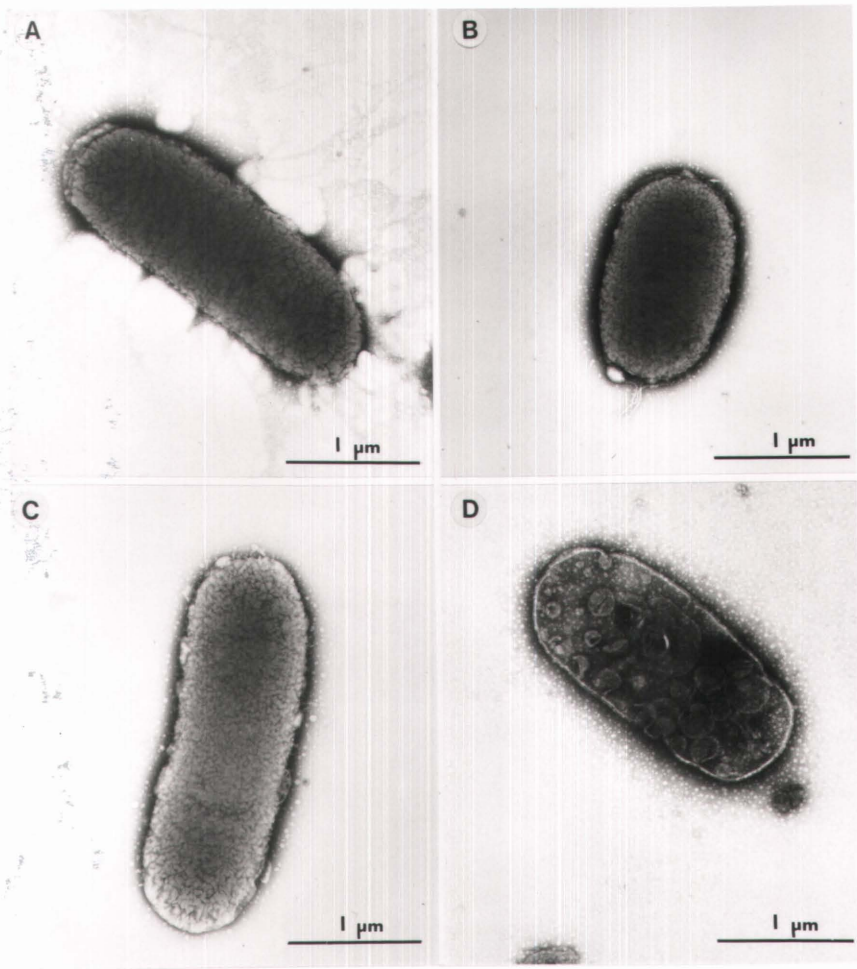
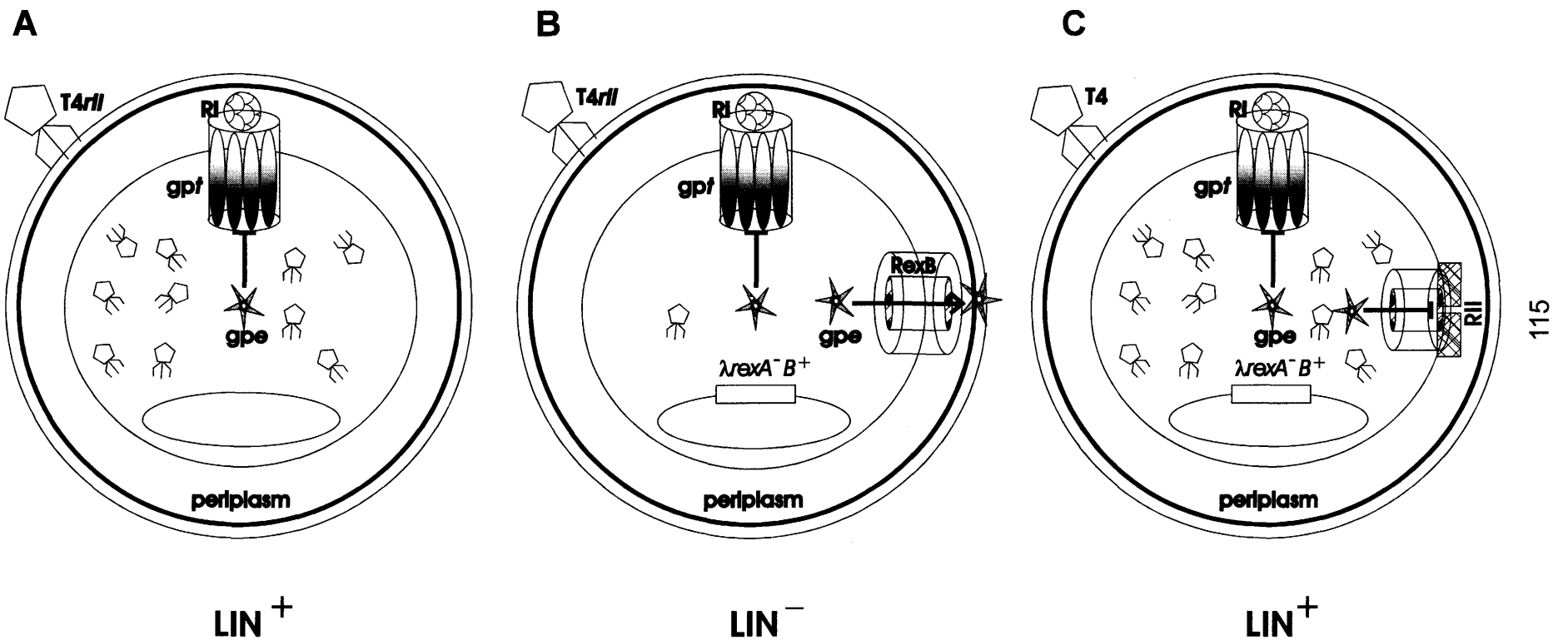
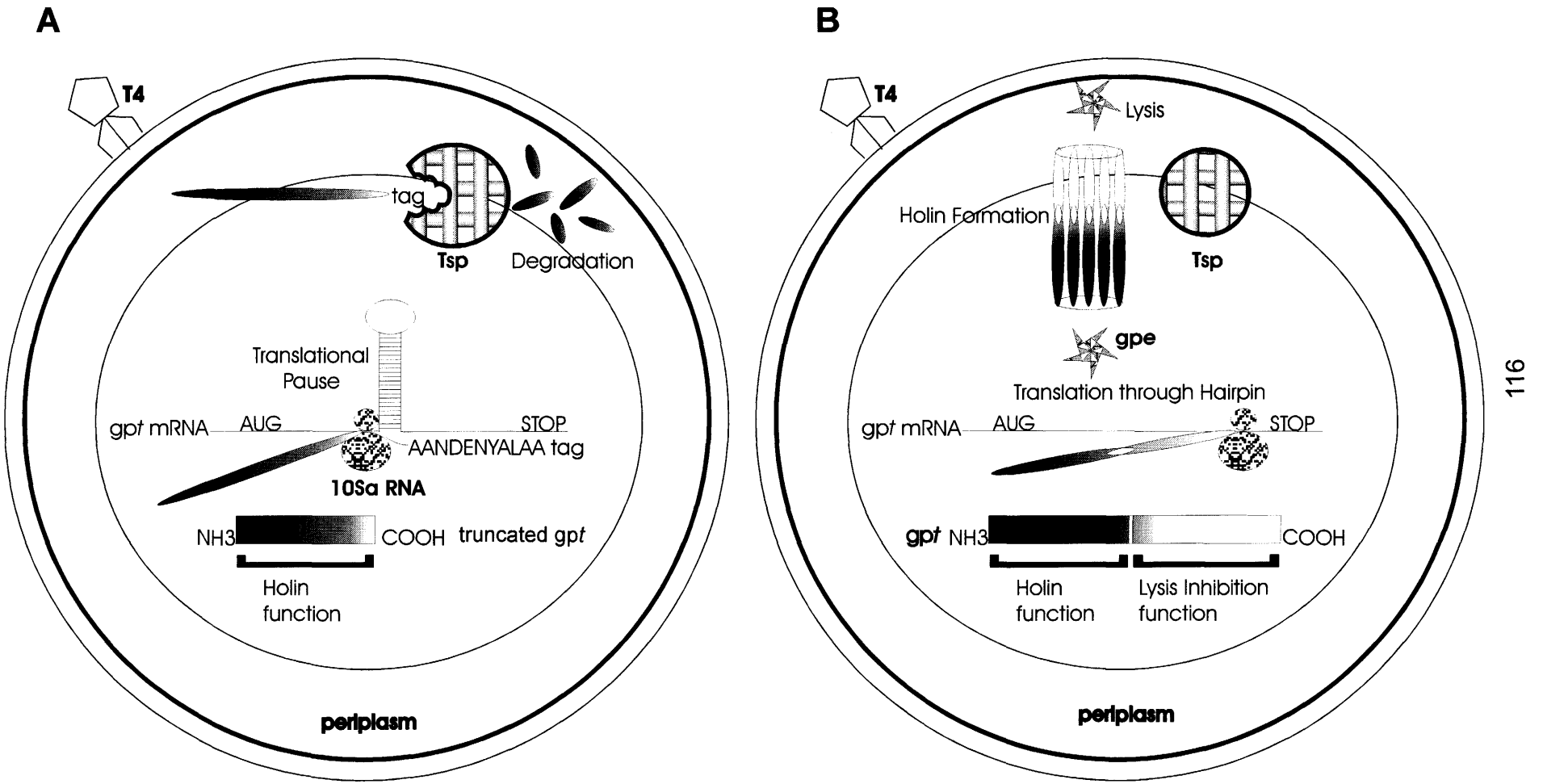


Figure 7


A
T4rII
RI
gpt
gpe
periplasm
LIN +
B
T4rII
RI
gpt
RexB
gpe
λrexA⁻B⁺
periplasm
LIN −
C
T4
RI
gpt
RII
gpe
λrexA⁻B⁺
periplasm
LIN+

115

Figure 8


A
T4
tag
Tsp
Degradation
Translational
Pause
gpt mRNA
AUG
STOP
AANDENYALAA tag
10Sa RNA
NH3
COOH
truncated gpt
Holin
function
periplasm
B
T4
Lysis
Holin Formation
Tsp
gpe
Translation through Hairpin
gpt mRNA
AUG
STOP
gpt NH3
COOH
Holin
function
Lysis Inhibition
function
periplasm

CHAPTER FIVE

Polarity in the *cI-rexA-rexB* Operon of Bacteriophage λ and The Temperature-sensitive Conditional Rex Phenotype.

Keywords: (bacteriophage λ; Rho-dependent transcriptional terminator; translational frameshift; repressor; Rex exclusion)

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Abbreviations: A, adenosine; A_{575} absorbance 575nm; aa, amino acids; bp, base pairs; Δ, deletion; G, guanosine; *lacZpO*, *lac* promoter-operator; N, any nucleoside; *O*, operator; *p* promoter; *t*, terminator; ( ), denotes prophage (lysogenic) state; e.o.p., efficiency of plating; db, downstream box on mRNA complementary to 16S rRNA; anti-db, sequence on 16S rRNA complementary to db on message; SD, Shine-Delgarno sequence; CFU, colony forming unit; PFU, plaque forming unit; RK, replicative killing.

NOTE: Transcription of λ DNA shown in Figures 1, 2, 3, and table 3 were performed by Dr. S. Hayes. Complementation data shown in table 2 was performed by Dr. S. Hayes.

5.1 Abstract

The *cI-rex* operon of bacteriophage λ is expressed from the p_M maintenance promoter of the prophage as p_M-*cI-rexA-rexB-*t_{imm} message and confers a T4***rII*** mutant phage **ex**clusion phenotype to the lysogen (**Rex** exclusion). Replication and excision defective, cryptic λ*cI*857[Ts]*cro*27 lysogens exhibit a conditional Rex[Ts] phenotype. At repressor-permissive temperatures for the *cI*[Ts]857 allele, *rex* expression from p_M confers full Rex activity. However, upon thermal inactivation of the repressor little or no Rex exclusion is observed, despite a much higher level of transcription from p_E. The same conditional Rex phenotype was observed in cells harbouring a low copy plasmid encoding a p_{Tet}-p_M-*cI*857-*rexA-rexB-*t_{imm} fragment, but not with a cI^+ derivative plasmid, that imparted a Rex^+ phenotype. Thermally derepressed λ*cI*[Ts]857*cro*27 lysogens exhibited powerful p_E transcription levels that abated dramatically toward the C-terminal of *rexA*, exerting a powerful polar effect on downstream *rexB*. Renaturation of CI857 following prophage induction does not significantly restore CI repressor activity suggesting that transcription from p_E may not result in functional CI production. Introduction of a *rho* mutation into our conditional Rex[Ts] strains partially suppressed Rex thermosensitivity, increasing activity at 43°C by up to 10^4 fold, while mutation of *hflA* to stabilize CII and heighten CII-dependent p_E transcription conferred only a slight increase. Partial suppression of the conditional Rex phenotype was also imparted by $ssrA^-$ and $clpP^-$ null mutations, which suggests that Rex may be subject to 10Sa RNA tagging and ClpP(X) degradation. We propose two possible models to account for *cI-rex* polarity and correlation between CI activity and Rex exclusion.

5.2 Introduction

A lambda prophage (λ) is maintained in the lysogenic state through the expression of λ gene *cI*, which encodes a repressor that blocks further λ transcription and lytic development (reviewed by Meyer et al., 1980). The CI repressor functions by binding to operator sites O_L and O_R overlapping promoters p_L and p_R (Fig. 1). The p_M promoter for *cI* also overlaps O_R. The normal binding of CI to O_R serves to block transcription from p_R and stimulate transcription from p_M (Heinemann and Spiegelman, 1970; Echols and Green, 1971; Reichardt, 1975; Meyer et al., 1980; Hawley and McClure, 1982; Li et al., 1994). The contiguous genes *cI-rexA-rexB* spanning λ bases 37,940-35,828 (Fig. 1) are transcribed in a repressed lysogenic cell from p_M (starting from base 37,940) through to terminator t_I (Hayes and Szybalski, 1973), renamed t_{imm} at 35,804 bp λ (Daniels et al., 1983; Hayes et al., 1997), without continuing through the intervening 220 bp region between *rexB* and p_L. The expression of *rexB-rexA* in a lysogenic prophage depends upon transcription initiated from p_M, and would therefore be controlled as *cI* by the autoregulatory influence of CI at p_M. The *rexB* gene also has an independent promoter, p_{Lit} (Hayes and Szybalski, 1973; Pirrotta et al., 1980; Landsmann et al., 1982; Hayes et al., 1997), Fig. 1. Some constitutive independent expression of *rexB* from p_{Lit} must occur in a lysogenic prophage, since a p_M^- prophage in double lysogens {[(λ *cI*857 p_M116)(λ *gal*8 *rex*$^-$ *cI*857 *cII*68)] or [(λ *cI*857 p_ME37)(λ *gal*8 *rex*$^-$*cI*857 *cII*68)]} can complement for *rexB* mutations, but not for *rexA* mutations (Matz et al., 1982).

Rex exclusion, encoded by genes *rexA* and *rexB* (Matz et al., 1982;

Landsman et al., 1982), can prevent the lytic growth of several bacteriophages that infect λ lysogens (Shinedling et al., 1987; Toothman and Herskowitz, 1980b). The *rexB* gene encodes a cytoplasmic membrane protein with the potential to form a transmembrane pore (Parma et al., 1992). According to the model of Parma et al. (1992), in the presence of an inducing signal, RexA interacts with RexB in such a way as to cause the pore to open, depolarizing and killing the cell. This exclusion system for preventing the proliferation of an infecting phage appears to recognize and respond to some aspect of the replication of the infecting phage (Toothman and Herskowitz, 1980c). Rex exclusion is sensitive to the stoichiometric balance between *rexA* and *rexB* proteins. If *rexA* is over-expressed, relative to *rexB*, growth arrest is triggered in the absence of a signal from an infecting phage (Snyder and McWilliams, 1989). Conversely, if *rexB* is over-expressed relative to *rexA*, then Rex exclusion is suppressed (Parma et al., 1992). The present ideas regarding *cI-rexA-rexB* regulation should not afford the over-expression of *rexA* relative to *rexB*. We further explore the regulation of *rexA-rexB* herein.

Transcriptional termination was observed within gene *cI* in the p_M-*cI-rexA-rexB-*t_{imm} operon. Both Meyer et al (1975) and Gussin et al (1987) reported premature termination of p_M-directed transcripts in vitro at ~300 nucleotides from the p_M start point. For convenience, this termination signal is shown as t_{CI} in Fig. 1. Gussin et al. (1987) showed *in vivo* that t_{CI} is *rho*-dependent and exerts a polar effect on downstream gene expression in the absence of *cI* translation.

Belfort (1978) followed the appearance of a 28.5 KDa Rex band (likely RexA) on acrylamide gels and concluded its synthesis paralleled that of the of CI

repressor. In a lysogen the synthesis of the band was dependent upon an intact p_M promoter, which could be repressed by Cro; whereas, the establishment mode of synthesis for both CI and the Rex band required promoter p_E activation by CII. The transcription from p_E (Fig. 1) is activated by the *cII* product of λ (Ho et al., 1983), and transcription levels from p_E can significantly exceed those from p_M (Reichardt and Kaiser, 1971; Hayes and Hayes, 1978, 1979). Astrachan and Miller (1972) showed earlier that Rex exclusion occurs in the absence of active CI repressor when *rex* is transcribed from the *cI* establishment promoter, p_E. Evidence that CI activity is not necessary for Rex exclusion was presented by Mark and Szybalski (1973), who found that loss of Rex exclusion following CI[Ts] prophage induction is slower than the immediate loss of repression. Evidence for the independence of Rex exclusion from CI repressor activity was provided by the demonstration that *rexA* and *rexB* expression from a multicopy plasmid in the absence of gene *cI* confers a Rex^+ phenotype to the cells. However, in this instance, the transcription of *rexA-rexB* was initiated from an inducible *lacZpO* promoter (Shinedling et al., 1987), on a multicopy plasmid, and thus would exceed *rexA-rexB* transcription from a prophage. Neither experiment (Mark and Szybalski, 1973; Shinedling et al., 1987) monitored the involvement of CI in *rexA-rexB* expression.

Rex exclusion was measured from six lysogenic cells with independently isolated replication defective [O^- or P^-] *cI*857[Ts] *cro*$^-$ *cII*$^+$ λ prophage deleted for genes *int-kil* and all late genes to the right of *ren* (Hayes et al., 1998). The mutants were selected for their ability to form CFU at 42°C where the defective prophage was constitutively derepressed. The temperature sensitive CI[Ts]857 repressor is

active at 30°C, but is quickly inactivated when the cells are shifted to 42°C. The prophage-induced cells grown at 42°C were shown to have *cII* gene activity (Hayes et al., 1998). Therefore, we expected p_M-*cI-rexA-rexB* expression at 30°C from these cells and p_E-*cI-rexA-rexB* transcription at 42°C. We assumed, based upon the Rex expression from p_E, seen in earlier studies by Astrachan and Miller (1972), that Rex expression at 42°C from the six strains would be equivalent or stronger than their Rex expression at 30°C. However, each of the six strains were Rex^+ at 30°C and Rex^- at 42°; ~10^6 fold loss of Rex activity compared to that at 30°C. (see also Table 1, this report). This observation led us to further examine the participation of active CI857 repressor in expression of the Rex^+ phenotype from the p_E-*cI-rexA-rexB-*t_{imm} message.

5.3 Materials and Methods

5.3.1 Bacterial strains, Bacteriophages and Plasmids.

Bacteriophages: phages λ*rexB*5A, λ *cI*857[Ts]*rexB*5A, λ*rexA*30A, and λ *cI*857[Ts] *rexA*Qam[allele301] preparations were from G. Gussin (GG) via W. Szybalski (WS), as was λ*rex*go293 [likely a *rexB* mutant since it mapped in the same interval left of *bio*16-3 endpoint as the *rex*5A mutation (Gussin and Peterson, 1972)].[1] Variant cI^+ *rex* phage were obtained by crossing λ*cI*sus14 by

[1] The *rex* mutants were obtained by Gussin and Peterson (1972) using strain W3350A(λ*N*am7am53*cI*857*O*Δ58) [=WNQ8] as the rex^+ parent. They then produced the λ *cI*857*rex* phage by marker rescue of imm^{λ} region of the WNQ8 *rex* mutants by infection with λimm^{434}.

the λ*rex cI*857 recombinants. We distinguished the *cI*[Ts] and *cI*$^{+}$ variants of λ*rexB*$^{-}$, and λ*rexA*$^{-}$ phage mutants by preparing lysogens and confirming thermolability. The mapping of these mutants was described by Gussin et al. (1973), Matz et al. (1982), and Landsmann et al. (1982). Phage λ*cI*72 was from our collection [lysate #510]. Phage λ*cII*68 was obtained from D. Wulff. T4[D] wt, T4*rIIA* [mutation of *rIIA* gene of T4] and T4*rII*Δ1589 [Δ spanning the *rIIA* and *rIIB* genes] were from G. Mosig.

Bacterial Strains: We utilized derivatives of *Eco* K strains : R594 F^{-} *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1 λ^{-}; W3350A F^{-} *lac*-3350 *galK*2 *galT*22 IN(*rrnD-rrnE*)1 λ^{-} (Bachmann, 1987), SA500 F^{-} *his*-87 *relA*1 *strA*181 *tsx*-83 λ^{-}. SA500(λ*bio*275*cI*857*cro*27*P*::IS*2*-100aΔ431) and the method for preparing λ lysogens, are described in Hayes et al. (1998). M72 F^{-} *lac*am *gal trp*am Sm^{R} Chl^{R} (chlorate) Su^{-} (λ*N*am7am53*cI*857ΔH1) was obtained as H. Greer strain M5158 (Greer, 1975) derived as a Chl^{R} derivative and then *gal*3 mutant of M72(λ*N*am7,53*cI*857) (H. Greer, personal communication) from WS. [The deletion ΔH1 removes prophage DNA between *cro-J*b of λ and through *chlA* of host.] SA500(λ*cI*857Δ297) [Δ*cII-J*b of λ through *chlA* host] is the S. Adhya strain SA297 (the parent strain was SA500, B. Bachmann, personal communication, 1990). M72(λ*N*am7am53*cI*857p_RX13) was from P. Brachet via WS. The approximate endpoints for ΔH1 and Δ297 are shown in Figure 1 (Dove et al., 1971). The strains C600 *suII*$^{+}$ (λ*N*am7am53*cI*857*cro*27*cII*2002) [phage was from L.F. Reichardt], FA22 *dnaB*22[Ts](λ*cI*857*cro*27), and W3350A(λ*cI*857*cro*27) were previously

described by Hayes and Hayes (1979). The marker *ilv*500::Tn*10* was moved from donor strain CAG18431 into recipient strain HD173 *rho*702[Ts] by P1vir transduction (both strains were obtained from the *E. coli* Genetic Stock Center at Yale University via M. Berlyn). The construct, HD173 *rho*702[Ts] *ilv*500::Tn*10*, was used as a donor strain to transfer tet^R into recipient Rho^+ strains M72(λ*N*am7am53*cI*857) and the RK^- mutants of SA500(λ*bio*10*cI*857*cro*27Δ431) and SA500(λ*bio*275*cI*857*cro*27Δ431) [shown in Table 4]. The potential *rho*702[Ts] *ilv*500::Tn*10* transductants were examined for retention of imm^{λ} by FI assay (Hayes and Hayes, 1986), for cI^+ genotype by cross-streaking with λ*cI*72 and λ*vir*, and for co-transduction (found to be ~90%) of *rho*702[Ts] *ilv*500::Tn*10*, using λ*N*am7am53*imm*434. The latter assay involved overlaying mixtures comprising 0.1 ml of culture cells for each potential transductant plus dilutions of λ*N*am7am53*imm*434 and 3 ml Tryptone top agar onto fresh Tryptone agar plates, incubating at 32°C overnight, and scoring for PFU. The e.o.p. for λ*N*am7am53*imm*434 on the Rho^+ strains was $3.5x10^{-7}$ (on W3350A, Su^o), $9x10^{-7}$ (on M72 F^- *lac*am *gal trp*am Sm^R Chl^R (chlorate) Su^o (λ*N*am7am53*cI*857ΔH1), and about 10^{-5} on the RK^- Nie strains. The e.o.p. was about 0.02 on Rho^- strains HD173 ilv500::Tn*10* (32°C) and the *rho*702[Ts] *ilv*500::Tn*10* transductants of M72(λ*N*am7am53*cI*857ΔH1) and the RK^- Nie strains. Protease mutant strains SG12045 (*clpA::kan*), SG22100 (*clpP::kan*--polar on *clpX*), X9368 (*hflA:kan*), and SG22069 (*ssrA::cat*) were received from S. Gottesman (Gottesman et al., 1998). Mutations were transduced into SA500(λ*bio*10*cI*857*cro*27Δ431*P*145c) and SA500(λ*bio*10*cI*857*cro*27Δ431*P*145d) conditional Rex[Ts] strains with P1vir.

Plasmids: pHB29 was described (Hayes et al., 1997). pACYC184 was from New England Biolabs (NEB). pRS1 was made by digestion of λwt (NEB) with *Bgl*II and ligation of λ fragment 38,103 bp through 35,711 bp (including genes p_M-*cI-rexA-rexB-*t_{imm}) into tet^R (*Bam*HI site at 1,869bp) of pACYC184 (NEB). pRS1 carries λ DNA with the orientation p_{Tet}-p_M-*cI-rexA-rexB-*t_{imm} (p_{Tet} promoter from pACYC184). pRS2 is identical to pRS1, with the exception that the λ fragment was from pCH1 (Hayes et al., 1997) and has the *cI*857[Ts] allele replacing cI^+ on pRS1. pUC18 was from (NEB). pRS4 was constructed by digestion of pRS2 with *Bst*YI, yielding λ fragment 38,103 bp through 35,711 bp and and ligation of λ fragment 38,103 bp through 35,711 bp (including genes p_M-*cI-rexA-rexB-*t_{imm}) into the MCS *lacZ'* of pUC18 (*Bam*HI site at 486 bp). pRS4 carries λ DNA with the orientation p_{lacZ}-p_M-*cI-rexA-rexB-*t_{imm} ($p_{lacZ'}$ promoter from pUC18).

5.3.2 Biological Assays for CI, CII, and Rex Exclusion.

The biological activities for CI repressor and for Rex exclusion encoded by prophage genes *rexA-rexB* was measured in culture cells grown between 30°C and 42°C as shown in Table 1. The e.o.p. for λ*cI*72 and T4*rIIA* phages was determined by dividing the titer on the assayed culture cells by the titer obtained in parallel on the permissive host cells R594 or W3350A. For each assay, the cultures were grown overnight at 30°C, diluted (0.1X) into fresh TB broth (10g Bacto Tryptone, 5g NaCl / liter) and grown 4-6 hr at the assay temperature. Assays were performed at the temperatures shown by first transferring culture aliquots (about 3 X 10^8 CFU) to heating blocks, adding 0.1 ml of dilutions of phage lysates for either λ*cI*72 or T4*rII*

[T4*rIIA* and T4*rII*Δ1589 gave equivalent results] to MOI's of 0.1 to 10^{-7}. The mixtures were incubated for about 5 min, 3 ml TB top agar (TB plus 6.5g Bacto agar/liter) was added and poured onto TB bottom agar plates (TB plus 11g Bacto agar and 1 mg thiamine·HCl / liter) pre-warmed to the assay temperature. The plates were incubated at the assay temperature for 16 hr. Phage T4 was used as a control for Rex exclusion, showing sensitivity of the cells to T4 plating.

5.3.3 Assay of p_E-p_M-cI-rexA-rexB-t_{Imm} and p_L Transcription from Lysogens.

The *l*-strand λ p_L and p_E-t_{imm} region transcription from repressed, induced, or induced and re-repressed prophage were assayed as previously described (Hayes and Hayes, 1979; Hayes et al., 1997). The procedure involves a subtractive prehybridization technique to remove nonspecific λ and *E. coli* RNA transcripts. The transcription per hybridization interval is relative to that for the corresponding non-induced prophage [0-time pulse]. In Fig. 1 and Table 3, transcription for induced cultures was normalized to that from the corresponding intervals (1 or 2) from the non-induced prophages [set equal to 1.0]. In Fig. 2, the transcription per 100 bp per interval was normalized to the non-induced [0-time] prophage transcription between marker t124 and p_M. The lysogenic cultures were pulse labeled with uridine 5-^{3}H for 1 minute. Transcription interval markers: 1 (*bio*30-7 to p_M) and 2 (*bio*3h-1 to *bio*30-7) for Figure 1 and Table 3; intervals A (rightward markers imm^{434} to imm^{21}), B (rightward *bio*t124 to imm^{434}), C (*bio*30-7 to *bio*t124), D (*bio*t75 to *bio*30-7), E (*bio*16-3 to *bio*t75) and F (t_{Imm} [where *bio*3h-1 was set equal to bp position for t_{Imm}] to *bio*16-3) for Figure 2. The transcription from p_L was assayed using an identical procedure as described (Hayes, 1979; Fig. 3), except that the results reflect the difference in the percentage of

input ^{3}H-RNA hybridizing to the *l*-strands for λ*cI* and λ*bio*3h-1 (to eliminate transcripts from promoters p_{Lit}, p_M, p_E, and p_O). The approximate bp position of each marker was noted by Daniels et al. (1983). Non-induced cultures were grown at 30°C to A_{575}=0.35, and labeled. The λ prophage was thermally induced by raising the culture temperature above 39°C to denature the temperature sensitive *cI*[Ts]857 repressor encoded by the λ prophage. Induced cultures were grown at 30°C to A_{575}=0.35, shaken at 60°C for 0.15 min, transferred to 41°C and labeled at indicated times following culture shift from 30° to 41°C. Cultures, where the prophage was induced and then re-repressed, were grown at 30°C to A_{575}=0.35 and then shaken at 60°C for 0.15 min and transferred to a 41°C shaking bath for 5, 10, or 15 min. The denatured CI857 repressor in these induced lysogenic cells was renatured by shaking the culture flask for 0.12 minutes in an ice bath and returning the flask to a 30°C bath. The cultures that were induced for 5, 10 or 15 minutes were labeled with uridine 5-^{3}H for 1 min respectively at 12.5, 14.5 and 20.5 min from time 0 (when initially shifted to 41°C). In parallel, cultures (not re-repressed) were induced and held at 41°C, and then labeled at 12.5, 14.5 and 20.5 min from time 0.

5.4 Results

5.4.1 Repressor Activity and Rex Exclusion.

We examined the influence of temperature dependent denaturation of CI857[Ts] repressor on the cellular Rex exclusion phenotype (Table 1), measuring in parallel the *imm*$^{\lambda}$ phenotype, dependent upon CI repressor activity. Both Rex exclusion and λ immunity to super-infecting *imm*$^{\lambda}$ phage was retained in lysogens

of λ wild type [*cI*$^+$] assayed at 30°C and 42°C (comparable to highest CI and Rex activities measured). The phenotypic activity of the CI[Ts]857 repressor (i.e., immunity or *imm*λ phenotype) from a λ*N*$^-$ *cI*857 ΔH1 prophage, which is deleted for p_E, (Fig. 1 map) was undistinguishable from the λ*cI*$^+$ lysogens at 30°C. However, the *imm*$^\lambda$ phenotype for CI857 declined by 50-fold between 30°C to 36°C, by more than a thousand-fold by 37°C, and virtually disappeared (i.e., was reduced >10^5-fold) at and above 38°C. The results in Table 1 for CI activity resemble the results for DNA binding activity of repressor by Mandal and Lieb (1976), who found that the CI857 and CI$^+$ repressors bound equivalently at 22°C, but only ~20% binding activity of CI857 remained by 26°C. They observed that the *cI*857 allele was the most heat-labile of the CI[Ts] repressors they examined, having little if any measurable DNA binding activity *in vitro* (their Figure 2) at or above 33°C.

Rex exclusion was assayed in parallel from the same cells used to measure CI activity (Table 1). Rex exclusion remained undiminished between 30°C through 38°C, but was powerfully attenuated above 39°C. This is a paradox. If *rex*A-*rexB* transcription is solely dependent upon CI binding at O_R for stimulating *cI-rexA-rexB* transcription from p_M, then how can an inactive CI857 repressor held between 36°-39°C stimulate *cI-rexA-rexB* transcription?

In independent studies with the λ*N*$^-$ *cI*857 ΔH1 prophage (Fig. 1A), the p_M-*cI-rexA-rexB* operon was transcribed at 41°C, albeit at a reduced level (Fig. 1A), for at least 20 minutes after thermal inactivation of the CI857 repressor. The transcription either arises from p_M in the absence of active CI857 at 42°C (i.e. slight constitutive transcription from p_M), or possibly from an adjacent, unidentified *E. coli*

promoter. p_M transcription was previously reported by Hayes and Hayes (1979) to be gene-dosage dependent in the absence of CI and Cro activities. Thus, why doesn't the Rex exclusion phenotype persist when *cI-rexA-rexB* transcription continues at 41°C from a *cro*⁻ λ*N*⁻ *cI*857 ΔH1 prophage, relieving the need for an active repressor to stimulate p_M-*cI-rexA-rexB* transcription?

We subsequently measured Rex exclusion from excision and replicative-killing defective (RK⁻) mutant cells (Hayes et al., 1998) with an integrated defective *cI*857 *cro*⁻ *cII*⁺ λ prophage. The prophages were deleted for genes *int-kil* and all late genes to the right of *ren*, and were also defective in λ replication initiation gene *P* (i.e., RK⁻ mutant 100a, Table 1). These cells, like those with the ΔH1 prophage strain, plate T4*rII* with an e.o.p. approaching 1 when incubated at 42°C; except that the defective prophage in these strains includes the p_E promoter, activated by λ gene CII. We found that upon shifting them from 30°C to 42°C the Rex exclusion phenotype was also lost; although, the cells were shown to complement for CII activity at 42°C (Table 2; see also Hayes et al., 1998). Furthermore, their ability to complement for CII at 42°C could be prevented by expression of p_O-promoted OOP antisense RNA from pHB29, which binds to the *cII* transcript. We expected CII-stimulated p_E-*cI-rexA-rexB* transcription at 42°C to impart equivalent, or stronger Rex activity at 42°C relative to 30°C, based upon the earlier studies by Astrachan and Miller (1972). The absence of Rex exclusion in the RK⁻ mutant strains at 42°C led us to re-examine the participation of the CI857 repressor in expression of the Rex⁺ phenotype. We constructed low copy number plasmids possessing λ fragments p_M-*cI*⁺-*rexA-rexB*-t_{imm} (pRS1), or p_M-*cI*[Ts]857-

rexA-rexB-t_{imm} (pRS2) downstream of a p_{Tet} promoter (pACYC184) to assay the involvement of the *cI* repressor in the conditional Rex[Ts] phenotype in the absence of any λ genes outside the *imm*$^{\lambda}$ fragment encoding CI^{+} and pRS2 encoding CI[Ts] (Table 1). Nonlysogenic cells transformed with the pRS2 plasmid also produce the conditional Rex[Ts] phenotype suggesting that p_E is not directly involved in abrogation of Rex activity.

5.4.2 Transcription of the cI-rexA-rexB Operon is Polar in the Absence of Functional Repressor.

The level of *cI-rexA-rexB* operon transcription (Fig. 1A) was compared in Fig 1B with strain M72(λ *Nam*7,53 *cI*857 p_RX13), which also exhibits the conditional Rex[Ts] phenotype, but carries a mutation in p_R preventing expression of the rightward p_R-*cro-cII* operon. The p_M-*cI-rexA-rexB* transcription was also divided into two intervals, p_M-proximal (N-terminal end of *cI*) and p_M-distal, and expressed as a percentage of non-induced transcription per interval. In both the ΔH1 and p_RX13 strains the thermal inactivation of CI857 resulted in an initial reduction in p_M-proximal transcription, with subsequent return to nearly the non-induced level. However, the p_M-distal transcription (including the C-terminal end of *cI* and that of downstream genes *rexA* and *rexB*) did not return at 41°C (or at best showed half the return exhibited by the p_M-proximal N-terminal end of *cI*.) This result parallels the previous observations of Meyer et al. (1975), and Gussin et al. (1987), who found that transcriptional polarity arises within *cI* in the absence of *cI* translation. We find here that the p_M-distal transcription is reduced relative to the p_M-proximal transcription when the repressor is inactive (compared to equivalent intervals for *cI-*

rexA-rexB transcription from a non-induced prophage where the repressor is active). In an analogous *cro*$^+$ lysogenic strain (Δ297, Fig. 1C), thermal derepression of the prophage resulted in significantly lower levels of *cI* transcription arising at 41°C. This can be explained by the Cro repressor binding to O_R and preventing transcription initiation from p_M. However, differential levels of *cI-rexA-rexB* operon transcription were still observed between the p_M-proximal and distal ends of the operon (relative to non-induced prophage) following thermal inactivation of the CI857 repressor at 41C°.

We compared the total transcription in intervals 1 and 2 (same as shown, Fig. 1) for the *cI*$^+$ lysogen W3350A(λ) at both 30°C and 41°C.[2] The average ratio, of transcription for interval-1/interval-2 for 7 cultures labeled at 30°C was 0.316, and was 0.310 for 7 equivalently labeled cultures at 41°C (data not shown). In an identical experiment with strain SA500(λ*cI*857Δ431), the average ratio of total transcription from interval 1/interval 2 was 0.270 at 30°C. These results suggest that lysogens with the active CI[Ts]857 repressor are about 15% more efficient [100(0.316-0.27)/0.316] than lysogens with the CI$^+$ repressor in transcribing the p_M-distal (interval 2) region of the *cI-rexA-rexB* operon.

5.4.3 Renaturation of Thermally Inactivated CI[Ts] Reverses the p_M-distal Polarity of p_M-cI-rexA-rexB Transcription Seen for Induced (CI-inactivated) Defective Prophage.

The CI857[Ts] repressor is inactivated in cells shifted from 30°C to 42°C,

[2] pulse labeling was for either 0.5, 1, 1.5, 2, 3, 4, or 5 minutes at either temperature.

and the inactivated CI857 repressor is readily reactivated upon shifting the cells back from 42°C to 30°C, even when the cells have spent 15 minutes at 42°C (Hayes and Szybalski, 1973), where repressor activity was measured by monitoring the induction or repression of p_L transcription regulated by CI binding to O_L operator site; see also Mandal and Lieb, (1976). We took advantage of this observation to determine if active CI857 could suppress the transcriptional termination accounting for reduced p_M-distal *cI-rexA-rexB* transcription (seen when CI857 is inactive), Table 3 (representative results for experiment shown Fig. 1B). Renaturation of the CI857 repressor in M72(λ *N*am7,53 *cI*857 p_R X13) cells after 5, 10 or 15 minutes of prophage induction at 41°C results in about a 3-fold [2.7-, 3.2-, 3.1-fold] stimulation of p_M-proximal transcription (bottom three lines, right column, interval 1, Table 3) and about 6-fold [4.3-, 7.4-, 5.7-fold] stimulation of p_M-distal *cI-rexA-rexB* transcription (same lines, column 6, interval 2). The unequal stimulation of the two intervals, two fold greater for the distal interval, supports the interpretation that active CI857 repressor can suppress the polar block on *cI-rexA-rexB* transcription occurring in the absence of active CI. The stimulation of downstream transcription (up to 7.4-fold) shown in Table 3 roughly agrees with the 10 fold repressor-dependent stimulation of *lacZ* reported by Maurer et al. (1980) and the stimulation of *cI* protein production reported by Reichardt (1975).

5.4.4 Restoring a Rex Exclusion Phenotype of Defective Prophage By Making Cells Rho⁻.

The *E. coli rho* product, functions as a hexamer, binding at the 5'-phosphoryl end of a growing mRNA chain, and moves unidirectionally toward the RNA

polymerase-DNA template complex. In the absence or interruption of mRNA translation, Rho dissociates the RNA polymerase from the DNA template, prematurely terminating the mRNA chain; termed polarity (Das, 1993). We asked if a *rho*702[Ts] mutation, which compromises Rho activity between 32-42°C (shown by cells ability to support plating by λNam7am53*imm*434, data not shown), could suppress the loss of Rex exclusion seen at 42°C for the RK⁻ mutants (Table 1; and Hayes et al., 1998). Substitution of *rho*702[Ts] for the *rho*⁺ allele in RK⁻ mutants 141e, 145c, and 145d resulted in a several thousand fold increase in Rex exclusion at either 40° or 43°C (Table 4). These results show that *rexB-rexA* expression in *cro*⁻ lysogens is regulated by a Rho-dependent transcriptional termination. The introduction of the *rho*702[Ts] allele into strain M72(λ*N*am7am53*cI*857ΔH1) caused the cells to die at 42°C. We attribute the observed cell killing to *rho*⁻ suppression of the *N*⁻ phenotype, where in the absence of Rho, the *N* product of λ is no longer required for the transcription initiated from promoter p_L to continue through the Rho-dependent t_L terminator(s): Rho⁻ suppression of t_L termination would allow the lethal expression of downstream gene *kil* (Greer, 1975) in the cryptic λ*N*am7am53*cI*857ΔH1 prophage. Several of the *rho*702 RK⁻ mutants were also compromised for viability to some extent at 43°C (notably mutant 141e). Since these strains are deleted for *kil*, the substitution of the *rho*702 allele may partially suppress the replicative-killing defect, trigger Rex-dependent cellular growth arrest (Snyder and McWilliams, 1989; Parma et al., 1992), or kill cells due to unregulated transcription.

5.4.5 Premature Termination of the p_E-cI-rexA-rexB-t_{imm} Transcript from

Thermally Induced Cro⁻ Lysogens.

We asked if premature transcriptional termination of the promoter-distal region of the *cI-rexA-rexB* operon was observed when the operon was transcribed from the CII-dependent establishment promoter, p_E.[3] The transcription per 100 bp per intervals A-F of the p_E-p_M-*cI-rexA-rexB*-t_{imm} operon was expressed relative to the level of p_M-proximal transcription (interval B) from the non-induced prophage, set equal to 1.0 (Fig. 2). Prior to prophage derepression: the level of transcription per 100 bp interval was constant across the five intervals representing the p_M-*cI-rexA-rexB* operon with no discernable polarity; there was no transcription from p_E (interval A); and the transcription from interval E was somewhat higher than for interval B.[4] The higher transcription in interval E is most likely explained by additive constitutive *rexB* transcription from p_{Lit2} (Hayes et al., 1997). Upon repressor inactivation (see 5 minute induction) transcription from p_M disappeared, while

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[3] previously, we examined *cI* transcription from more than thirty λ lysogens with *cI*857[Ts] prophage that were induced (derepressed) by shifting the cells from 30°C to 40°C (Hayes and Hayes, 1978, 1979; Hayes, 1979, Hayes, unpublished). Although *cII* should be expressed from p_R and in turn, CII should stimulate p_E transcription, *only induced cro*27-defective (*cII*-competent) prophage were shown to initiate CII-dependent p_E-*cI-rexA-rexB* (establishment) transcription. We never fully understood this observation, since *cII* is transcribed following the derepression of *cro*⁺ prophage. However, it was found that *oop* transcription was significantly depressed from an induced *cro*⁻ prophage (Hayes, 1978). Since then, OOP RNA was shown to act as an antisense regulator for CII expression (Krinke and Wulff, 1987).

[4] For non-induced prophage only the bases transcribed between p_M and left side of interval B were counted to determine the average transcription per 100 bp interval. For induced prophage, all bases within interval B were used for measuring transcription per 100 bp interval.

transcription from p_{Lit2} continued unchecked. By seven minutes after derepression some transcription from p_M was observed, which terminated, and p_{Lit2} transcription had ceased. Establishment transcription from p_E (interval A) arose between 7 to 12 minutes and continued strong thereafter. The initial burst of p_E-*cI-rexA-rexB* transcription (12 min.) per 100 bp per interval was relatively constant at about 5X the non-induced level for each of the six intervals. However, the transcription of intervals E and F, including primarily *rexB*, was significantly reduced thereafter when compared to transcription from intervals C-D, which was reduced compared to transcription from the p_E-proximal intervals A-B. These results indicate extreme polarity of the p_E-*cI-rexA-rexB* transcript and suggest that, either *cI*, or both *cI* and *rexA*, are transcribed by a factor of 10 or more above that of downstream gene *rexB*. This is a condition predicted by Snyder and McWilliams (1989) and Parma et al. (1992) to "trigger" Rex exclusion and cessation of cellular growth.

5.4.6 Influence of Proteases on Rex Exclusion Activity from the p_E-cI857-rexA-rexB-t_{imm} Message.

To determine whether we could increase Rex activity by stabilizing CII-dependent transcription from p_E, we transduced an *hflA::kan* mutation into our conditional Rex strains (Table 5). The *hflA*$^-$ null allele conferred a ~50 fold increase in Rex activity in our transductants (average for three mutants). This finding suggests that the polar effect exerted upon the p_E-*cI*857-*rexA-rexB-*t_{imm} message is only slightly relieved by increasing the level of transcription from p_E.

Transduction of the Hsp100 family chaperone *clpA*$^-$ null mutation did not appear to influence the conditional Rex phenotype, while introduction of a *clpP*$^-$

protease mutation restored Rex by up to five hundred fold at 42°C compared to the Clp$^+$ parent strains (Table 5). Since the *ssrA*-encoded 10Sa RNA tags peptides derived from damaged transcripts with an 11 a.a. degradation signal (Keiler et al., 1996; Karzai et al., 2000) recognized by ClpPA, and ClpPX proteases, we transduced an *ssrA*$^-$ mutation into the conditional Rex[Ts] strains to determine whether 10Sa RNA might be involved in targeting Rex for ClpP degradation. We found that the *ssrA*$^-$ mutation increased Rex activity by more than 500 fold (average of three independent mutants), at 42°C; resembling Rex activity imparted by the *clpP*$^-$ mutants. These findings suggest 10Sa RNA may post-transcriptionally regulate Rex expression in the conditional Rex strains at 42°C by targeting Rex for degradation by ClpP(X). We also constructed conditional Rex[Ts] strains possessing both the *rho*[Ts] *ssrA*$^-$, or *rho*[Ts] *clpP*$^-$ mutations, which exhibited full Rex activity at 30°C, but were killed upon incubation at temperatures above 39°C (data not shown).

5.4.7 Is the Exceptional Level of p_E-cI857 mRNA Synthesized at 42°C Translated Into CI Protein Which is Active at 30°C?

The derepression of a λ*cI*857*cro*27 prophage resulted in synthesis of p_E-*cI* mRNA 20 to 30$^+$ fold in excess of that produced from the p_M transcript from a repressed (non-induced) prophage where CI857 is active (Fig. 2). We attempted to determine whether the exceptional *cI*857 mRNA formation seen by 12 to 25 minutes after prophage induction was translated, yielding massive levels of CI857 repressor within the cell. Repressor activity was monitored by its ability, upon renaturation, to repress induced transcription from promoter p_L, as shown in the

schematic above Fig. 3. Repressor activity was monitored following the induction of λ*cI*857*cro*27 and λ*cI*857*cro*27*cII*2002 prophages. Previous studies have shown that p_E transcription from an induced *cI*857*cro*27 prophage was not detectable if the phage was made defective in *cII*, or if the host was made *dnaB*[Ts], inhibiting replication of the induced λ prophage (Hayes, 1979; Hayes and Hayes, 1979). We found that in the absence of any p_E-transcription, the CI857 repressor activity prior to shifting the cells to 41°C could be restored 5, 10, or 15 minutes following cellular incubation at 41°C, as seen by 20-30 fold reductions in p_L-transcription (columns 4-5, Fig. 3). The results in column 6, Fig. 3 show that prior to the synthesis of p_E-*cI*857 mRNA the existing CI857 repressor reduced p_L-transcription by 26.7 fold (21.12/0.79) upon renaturation; the data in column 6, Fig. 3, is for the same cells; time points as shown in Fig. 2. However, upon the commencement of p_E transcription, between 7 to 12 minutes following prophage induction, the level of renaturable repressor activity dropped by 10-fold compared to that prior to p_E-*cI*857 transcription initiation.

5.5 Discussion

Thermal inactivation of repressor activity in *N*⁻ *cI*857 ΔH1 prophage results in the severe diminishment of Rex exclusion. At temperatures permissive for CI[Ts]857 activity (30°C), Rex exclusion activity toward T4*rII* was indistinguishable from that of a CI^+ prophage. At culture temperatures above 39°C, where CI[Ts]857 activity is lost, Rex exclusion activity was also lost (> 10^5-fold reduction). However, *cI-rexA-rexB* transcription continued, albeit at a diminished rate from either p_M, or

from an adjacent *E.coli* promoter in these cells. A conditional Rex exclusion phenotype was also observed upon shifting cultures from 30 to 42°C with cryptic lysogens with a replication defective, *cI857cro27* prophage. For these strains, the thermal denaturation of the CI repressor results in gene *cII* expression, enabling p_E-*cI857-rexA-rexB* transcription. Thus, the results can be interpreted to suggest that CI repressor exerts a post transcriptional effect on the expression of the *cI-rexA-rexB* message. The temperature-dependent loss in Rex exclusion from the derepressed λ*N*⁻ *cI857* ΔH1 prophage (Table 1) resembles the *lacZ* expression curve [measured between 30° and 39°C] obtained by Maurer et al. (1980) for a p_M-*cI857-lacZ* gene fusion. Genes outside of the immunity region seem not to be involved in the conditional Rex phenotype since the conditional Rex phenotype was observed with nonlysogens carrying a low copy plasmid expressing the *cI857-rexA-rexB* from either p_M or p_{Tet} promoters.

We found that the *cI857-rexA-rexB* message was subject to strong polarity in the conditional Rex lysogens at temperatures that render the CI857 repressor completely inactive (Table 4). The p_M –distal message (terminal end of *cI* and downstream *rexA-rexB*) was significantly reduced as compared to the p_M-proximal message. These findings are in accordance with previous observations by Meyer et al. (1975), and Gussin et al. (1987), who noted that polarity occurs in *cI* in the absence of active *cI* translation. The level of polarity that we detected agrees with that exhibited by the *rho*-dependent termination event described by Gussin et al. (1987). We localized the terminator to about 260 bp (or more) downstream of p_M, assuming that transcription of the 5' proximal end of *cI* does not exceed the level

from a repressed prophage.

The reduction in distal transcription from the p_M-promoted *cI-rexA-rexB* operon may be greater than we have detected (Fig. 2), especially if *rexB* is constitutively transcribed from p_{Lit}. Any independent transcription originating from p_{Lit} will result in an overestimation of the downstream transcription that arises from p_M, and thus will obscure the effect of a polar block occurring within *cI-rexA-rexB* operon. The discrepancy between transcription immediately arising from p_M and the distal portion of the operon (Fig. 1), may therefore be an overly conservative estimate of the occurrence of transcriptional polarity.

We introduced a *rho*702[Ts] allele into our conditional Rex[Ts] strains to determine whether we could restore Rex at CI[Ts]857 non-permissive temperatures by reducing *rho*-dependent transcriptional termination, and alleviating polarity of the p_M-distal message (Table 4). Rex activity was stimulated (up to 10^4 fold) at either 40°C or 42°C (depending upon strain) in lysogens carrying the *rho*702 allele, compared to the isogenic *rho*$^+$ parent. All the *rho*702 transductants grew well enough to form an adequate cell lawn at 40°C (or above). The *rho*026 allele has been reported to prevent the growth of T4[wt] by severely delaying and reducing phage DNA replication (Stitt and Mosig, 1989). T4 plating efficiency has not previously been determined on the *rho*702[Ts] mutation. Our results support the inference that the Rex$^-$ phenotype observed in our conditional Rex strains is at least partially due to polarity within the *cI-rexA-rexB* transcript at temperatures that are non-permissive for the CI857 repressor.

We moved an *hflA*$^-$ mutation into our conditional Rex strains to increase CII

stability and p_E transcription. The mutation increased Rex activity only slightly at 42°C suggesting that the polar effect exerted upon the p_E-*cI*857-*rexA*-*rexB*-t_{imm} message is only partially relieved by increasing the level of transcription from p_E Table 5). However, since HflA was reported to merely modulate the activity of HflB, increasing CII stability in both its absence and over-production (Kihara et al., 1997), we have no idea what level of stability is conferred to CII in an *hflA*$^-$ background. Mutation of the Hsp100 chaperone *clpA* did not appear to influence the conditional Rex phenotype, while *clpP*$^-$ protease mutants exhibited a dramatic increase in Rex activity (>300 fold) at 42°C (Table 5). Conditional Rex[Ts] strains transduced with an *ssrA*$^-$ allele behaved similarly to ClpP$^-$ mutants, also exhibiting up to a 500 fold increase in Rex activity at 42°C. This finding suggests that Rex translation originating from the p_E-*cI*857-*rexA*-*rexB*-t_{imm} message may be tagged by 10Sa RNA and subject to ClpP-dependent proteolytic regulation.

The elevated levels of repressor transcription that we observed at 41°C from a thermally induced λ*cI*857*cro*$^-$ prophage (Fig. 2) may not directly correlate with the reduced levels of repressor antigen from induced λ*cI*857 lysogens that were observed by Reichardt and Kaiser (1971), and Reichardt (1975). They reported a reduction in CI antigen production following prolonged incubation at 42°C, from induced λ lysogens W3350A(λ *N*am7,53 *cI*857 p_RX13), W3350A(λ *N*am7,53 *cI*857*cro*27*O*$^-$), W3350A(λ *N*am7,53 *cI*857p_RX3*y*42), and M72*gal*(λ*N*am7,53*cI*857ΔHI). They argued that since the inactive repressor made at 42°C retains its antigenicity and is stable, the repressor antigen level in a λ*cI*857 lysogen grown at 30°C or 42°C will reflect the rate of *cI* gene expression (i.e., with

an inference to *cI* transcription). However, both results are compatible if the repressor were to exhibit a post-transcriptional regulatory effect on expression of the *cI-rexA-rexB* operon.

Mandal and Lieb (1976) assayed the *in vitro* DNA binding affinity for several alleles of *cI* between 22°C and 43°C. They found that the CI857 and CI^+ repressors bound equivalently at 22°C but not at higher temperatures. Only 20% DNA binding affinity remained by 26°C for the CI857 repressor, dropped about 100-fold between 33°C-36°C, and was undetectable above 36°C. Our biological assay for CI857 repressor activity essentially paralleled these results. It is inconceivable to us that at 37°C, where the CI857 repressor retains less than 0.1% of its superinfection exclusion activity observed at 30°C, it nevertheless, remains able to bind at O_R and stimulate enough transcription from p_M to provide for full Rex exclusion. Although the abrogation of Rex does agree with complete loss of CI857 activity at 41°C we noted no loss of Rex activity at 37°C and partial loss of Rex at 38°C and 39°C, where CI857 is completely labile. Thus, it is difficult to reconcile CI857 involvement in *rexA-rexB* expression solely in terms of p_M stimulation.

Meyer et al. (1980) suggested that p_M transcription (at sub-lysogenic repressor concentrations) continued even when the repressor had lost its ability to effectively prevent transcription from p_R. However, their data were obtained for a CI^+ repressor at an unreported temperature. Some level of constitutive transcription of *cI-rexA-rexB* arises from p_M in the absence of CI857 and Cro activity (Fig. 2; Hayes and Hayes, 1979). Thus a basal level of Rex expression should remain after CI857 activity is lost from an induced cro^- prophage. We reported

herein that Rex exclusion was lost above 39°C from induced *cI*857 *cro*⁻ replication - defective cryptic prophages. We expected that the conditions for Rex exclusion and repressor-dependent homoimmune exclusion would be equivalent and would depend (in the absence of p_E) upon an ability of the repressor to bind at O_R and stimulate transcription of *cI-rexA-rexB* from p_M. The CI857-dependent immunity and Rex exclusion correlated well in the conditional Rex strains between 30°C and 36°C. However, full Rex exclusion toward T4*rII* was seen between 37 and 39°C in the absence of activity for CI857 repressor. These divergent observations suggest to us that a post transcriptional activity of the CI repressor, or an activity of *cI-rexA-rexB* transcripts, can influence the expression of the *rexA-rexB* genes.

We previously examined *cI* transcription from more than thirty λ lysogens with *cI*857[Ts] prophage that were thermally induced by shifting the cells from 30°C to 40°C (Hayes and Hayes, 1978, 1979, Hayes, 1979, Hayes, unpublished). Contrary to all the rational expectations for *cII* expression from p_R and in turn, CII stimulation of p_E transcription, only induced *cro*27-defective (*cII*-competent) prophage were shown to initiate CII-dependent p_E-*cI-rexA-rexB* (establishment) transcription. However, it was found that *oop* transcription was significantly depressed from an induced *cro*⁻ prophage (Hayes, 1978). Since OOP RNA acts as an antisense regulator for CII expression (Krinke and Wulff, 1987), the suppression of *oop* transcription would relieve anti-sense regulation of the *cII* message from an induced *cro*⁻ prophage.

We envision two circumstances that would account for how λ prevents the synthesis of more repressor than needed during infection or prophage induction; 1)

active CI is required for its own efficient translation; or 2) the p_E-*cI* mRNA or a product made from it competed for CI activity. The conditional Rex[Ts] phenotype conferred by the low copy pRS2 (pACYC184-p_{Tet}-p_M-*cI857*-*rexA*-*rexB*-t_{imm}) plasmid suggests that the conditional Rex phenotype is not limited to our RK^- defective lysogens, and is not confounded by genes outside of the imm^{λ} region.

5.6 Conclusions

1.) Some p_M-*cI*-*rexA*-*rexB* transcription arose from an induced cryptic prophage held at 41°C where the CI857 prophage repressor was inactive. Transcription at the promoter proximal end of *cI* was higher than distal *cI*-*rexA*-*rexB* transcription, suggesting, in support of Gussin et al. (1987), that transcriptional polarity occurs downstream from p_M in the absence of functional repressor. Increased levels of *cI*-*rexA*-*rexB* transcription arising from p_E were also observed to be subject to polarity downstream from *cI*. Transcriptional polarity can account for the attenuation of downstream *rexA*-*rexB* expression.

2.) We found that transcription of the promoter distal region of the p_M-*cI*-*rexA*-*rexB* operon was stimulated upon renaturation of a thermally denatured CI857 repressor. The activity of renatured CI857 was measured by repression of induced p_L transcription. The restored CI857 repressor activity suppressed the polar block to distal *cI*-*rexA*-*rexB* transcription. However, very high levels of *cI* mRNA synthesis from p_E nullified the ability of renatured CI857 to block p_L transcription.

3.) Wild type levels of Rex exclusion toward T4*rII* plating occurred at 42°C from a cI^+ lysogen. A conditional Rex exclusion phenotype, i.e., loss of exclusion

at 40°C and above was observed for λ*cI*857*cro*27 lysogenic cells in which the cryptic prophage was also defective for phage replication and cell killing functions (enabling these cells to form colonies at 42°C).

4.) Full Rex exclusion activity was observed between 30 and 39°C for λ*cI*857*cro*27 lysogenic cells in which the cryptic prophage was also defective for phage replication and cell killing functions. In parallel, we measured repressor activity in these cells by their ability to prevent the plating of a homoimmune λ*cI* phage and found 2% activity at 36°C, 0.05% activity at 37°C, <0.0003% activity at 38°C and <0.0002% at 39°C, all relative to CI857 repressor activity at 30°C. These results suggest that *rexA-rexB* expression, as manifested by Rex exclusion, occurs between 36 to 39°C with minimal to virtually no CI857 activity (i.e., binding at O_R to activate p_M transcription).

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5.8 Figure Legends

Figure 1. Polarity of *cI-rexA-rexB* Transcription from Defective λ Lysogens Induced by Shifting Cells from 30° to 42°C.

Data/Figure provided by S.Hayes. The relative p_M proximal transcription (interval 1, solid lines), and distal transcription (interval 2, broken lines), from λ prophage was measured for cultures of lysogens shifted from 30°C to 41°C. The temperature sensitive CI857 repressor is active in non-induced prophage cultures grown at 30°C, and denatured when the cells are shifted to 41°C. Experiments are shown for lysogens: A, M72(λ*N*am7,53*cI*857ΔH1); B, M72(λ*N7N*53*cI*857p_Rx13); and C, SA500(λ*cI*857Δ297). These lysogens are incapable of *cI* establishment transcription from promoter p_E, which is deleted or inactive in the three strains shown. (The activation of transcription from p_E is dependent on the *cII* gene product, which is also deleted or not expressed.) The induced prophages in experiments A and B are defective for Cro, which is expressed from the induced

prophage in C. The map shows p_M-*cI-rexA-rexB* operon from bacteriophage λ. *cI-rexA-rexB* are transcribed from p_M to t_{imm} in a lysogenic prophage. Gene *rexB* has separate p_{Lit} promoters (Hayes, 1997). Promoters p_R and p_L initiate transcription immediately upon phage infection or prophage induction and are repressed in a lysogen by the DNA binding of CI at the operator sites O_R and O_L (not shown), which overlap with p_R and p_L. The square and triangle within gene *cI*, and circle within *rexA*, represent slippery heptamer sequences affording the possibility for translational frameshifting (Atkins et al., 1990; Weiss et al., 1990; Tsuchihashi an Brown, 1992; Hayes et al., 1998; Hayes and Bull, 1999). t_{imm} is a terminator for transcription of the *rexB-rexA-cI* operon transcribed from p_M or p_E (Hayes and Szybalski, 1973; Hayes and Hayes, 1979; Landsmann et al., 1982; Hayes et al., 1997). t_{CI} is a potential rho-dependent terminator described by Gussin et al. (1987).

Figure 2. Polarity in the p_E-*cI-rexA-rexB* Transcript from Induced λ*cI*[Ts]857*cro*27 Prophage.

Data/Figure provided by S. Hayes. The kinetics of the p_M transcription (starting in interval B from non-induced prophage, time 0) and p_E transcription (starting in interval A from a prophage induced between 7 and 12 minutes) were measured from non-induced and induced prophage in the lysogen W3350A(λ*cI*857*cro*27). The transcription for six regions (intervals A – F) of the p_E-*cI-rexA-rexB*-t_{imm} operon were averaged per 100 bp per interval and then compared to the reference level of transcription per 100 bp in interval B (0-time). Two versions of the kinetic data (qualitative in top figure, quantitative below) are shown, with averaging of the results for 21 and 25 min induced samples shown in bottom line.

Figure 3. Induction and Repression of p_L Transcription from Induced λ*cI*[Ts]857*cro*27 Prophage.

Data/Figure provided by S. Hayes. The strains employed include, Column 4: C600 *sul*I$^+$ (λ*N*am7,53*cI*857*cro*27*cII*2002) [λ-Replication$^+$; mutation *cII*2002 confers a defect in repressor establishment transcription from p_E]; Column 5: FA22 *dnaB*22[Ts](λ*cI*857*cro*27) [λ-Replication$^-$, *cII*$^+$]; and Column 6: W3350A(λ*cI*857*cro*27) [λ-Replication$^+$, *cII*$^+$]. The transcription between t_{Imm} and p_E for non-induced and induced prophage conditions for these strains was previously described (Hayes and Hayes, 1979). The rex alleles *rexB*5A, *rexA*amQ[allele 301] and *rexA*30Awere mapped by Matz et al. (1982).

Table 1. The Conditional Rex Exclusion Phenotype.

Host Cells	Temp (°C)	T4*rIIA* e.o.p.	λ*cI*72 e.o.p.
R594	30	1.0	1.0
	42	1.0	1.0
R594(λ)	30	$<10^{-6}$ [a]	$<10^{-6}$ [a]
	42	$<10^{-6}$	$<10^{-6}$
R594(λ*cI*857*rexA*amQ[301])	30	1.0	$<10^{-6}$
R594(λ*cI*857*rexB*5A)	30	1.0	$<10^{-6}$
R594 [pACYC184]	30	1.0	0.68
	40	1.0	0.92
	42	1.0	0.92
R594 [pRS1] (p_{Tet}-p_M-*cI*$^+$-*rexA*-*rexB*-t_{imm})	30	$<10^{-6}$	$<10^{-6}$
	40	$<10^{-6}$	$<10^{-6}$
	42	$<10^{-6}$	$<10^{-6}$
R594 [pRS2] (p_{Tet}-p_M-*cI*857-*rexA*-*rexB*-t_{imm})	30	$<10^{-6}$	$<10^{-6}$
	40	6.1×10^{-5}	0.05
	42	0.18	0.25
M72(λ*N*am7am53*cI*857ΔH1)	30	$<10^{-6}$	$<10^{-6}$
	36	$<10^{-6}$	5.0×10^{-5}
	37	$<10^{-6}$	1.7×10^{-3}
	38	$<10^{-6}$ [c]	0.33 [c]
	39	variable	0.5
	42	1.0	1.0
SA500(λ*bio*275*cI*857*P*100a::IS2Δ431) [b]	30	$<10^{-6}$	$<10^{-6}$
	42	0.01	---[d]

[a] The $<10^{-6}$-fold inhibition of plating of λ*cI*72 or T4*rII* represents the typical inhibitory levels shown by infected lysogens with a single prophage copy. The absence of CI or Rex exclusion is shown as the level of inhibition approaches 1, which is equivalent to the plating efficiency of either λ*cI*72 or T4*rII* on W3350A.

[b] The ΔH1 deletion = Δ*cro* – *Jb*2

[c] Values for 8 determinations at 38°C

[d] These strains exhibit nonimmune exclusion (Nie) phenotype at 42°C, preventing plating by imm^{λ} and imm^{434} phages (Hayes et al., 1998).

Table 2. Assaying for CII Activity in RK⁻ Nonimmune Exclusion Mutant Strains at 42°C.

Host Cells	λ*cII*68 e.o.p (42°C) [a]	Plaque Morphology (42°C) [b]
Nonlysogens	1.0	Clear
SA500(λ*bio*275*cI*857[Ts]*cro*27Δ431) RK⁻ mutant:		
*P*100a::IS2	0.03	Turbid
*P*100a::IS2 [pHB29]	0.03	Clear [c]

[a] The host cells exhibit the Nie (nonimmune exclusion) phenotype when shifted to 42°C, reducing plating efficiency by lambdoid phage (Hayes et al., 1998). The values are approximate.

[b] Complementation by induced defective prophage for CII activity. Turbid plaques indicate that the *cI* repressor is being made via CII stimulating p_E transcription of *cI*. Clear plaques indicate the absence of complementation for *cI* transcription from p_E, or normal plaque phenotype on nonlysogens W3350A, R594 or SA500.

[c] The OOP RNA expression from plasmid pHB29 (Hayes et al., 1997) serves as an antisense inhibitor of prophage *cII* gene expression (Krinke and Wulff, 1987).

Data provided by S. Hayes.

Table 3. Suppression of p_M – distal *cI-rexA-rexB* Transcription Termination by Active CI. [a]

Induction and renaturation scheme		mRNA pulse labeling time (final minute)	Relative p_M-t_{imm} transcription per measured interval		Stimulation of transcription upon CI renaturation (active renatured CI / induced inactive CI interval	
min. at 41°C	min. at 30°C		1 p_M-proximal	2 p_M-distal	1 p_M-proximal	2 p_M-distal
Non-induced (active CI)			1.00	1.00	---	---
			(0.0068)	(0.0406)		
Induced (inactive CI)						
13		13	0.85	0.45	---	---
15		15	0.93	0.37	---	---
21		21	1.08	0.49	---	---
Induced and renatured (inactive CI to active CI)						
5	5-13	13	2.26	1.94	2.7	4.3
10	10-15	15	3.01	2.73	3.2	7.4
15	15-21	21	3.35	2.78	3.1	5.7

[a] The results are for Exp. B, Fig. 1, and show the effect of renaturing the CI857 repressor on p_M-*cI-rexA-rexB* transcription from induced defective λ*N*am7,53*c*I[Ts]ΔH1 prophage.

Data provided by S. Hayes.

Table 4. Influence of Rho on Rex Exclusion Phenotypic Activity in RK⁻ Nonimmune Exclusion Strains.

Host Cells	T4*rIIA* e.o.p.			T4D e.o.p.
	30°C	40°C	42°C [a]	42°C [a]
R594	1.0	1.0	1.0	1.0
R594(λ)	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$	0.61
HD173 (*rho*702[Ts])	1.0	1.0	1.0	0.66
M72(λ*N*am7am53*c*I857ΔH1)	$<10^{-6}$	0.3	1.0	0.49
M72(λ*N*am7am53*c*I857ΔH1)*rho*702[Ts]	$<10^{-6}$	0.3	c.d. [b]	c.d.
SA500(λ*bio*10*c*I857*cro*27Δ431) RK⁻ mutants:				
O141e::IS2	$<10^{-6}$	0.4	0.8	n.d.
O141e::IS2 *rho*702[Ts] [c]	$<10^{-6}$	$1.0X10^{-5}$	c.d. [b]	c.d. [b]
*P*145c::IS2	$<10^{-6}$	0.001	0.1	0.71
*P*145c::IS2 *rho*702[Ts]	$<10^{-6}$	$<10^{-5}$	$5X10^{-4}$	n.d.
*P*am145d	$<10^{-6}$	0.005	0.2	1.0
*P*am145d *rho*702[Ts] [d]	$<10^{-6}$	$<10^{-5}$	$<10^{-4}$	0.79

n.d. not done

[a] incubation temperature varied from 42-43°C.

[b] e.o.p. could not be determined because of extensive killing of host cell lawn at 42°C.

[c] results are the average from two independent *rho*702[Ts] transductants.

[d] results are the average from four independent *rho*702[Ts] transductants.

Table 5. Influence of Protease and 10Sa RNA Tagging Mutations on the Conditional Rex Exclusion Phenotype.

Host Cells	T4*rIIA* e.o.p.	
	30°C	**42°C**
SA500(λ*bio*10*cI*857*cro*27Δ431) RK⁻ mutants:		
*P*145c::IS2	$<10^{-6}$	0.09
*P*145c::IS2 *clpA*	$<10^{-6}$	0.07
*P*145c::IS2 *clpP* [a]	$<10^{-6}$	n.d.
*P*145c::IS2 *hflA*	$<10^{-6}$	0.003 [b]
*P*145c::IS2 *ssrA*	$<10^{-6}$	7.4×10^{-5}
*P*am145d	$<10^{-6}$	0.06
*P*am145d *clpA*	$<10^{-6}$	0.13
*P*am145d *clpP* [a]	$<10^{-6}$	7.0×10^{-4}
*P*am145d *hflA*	$<10^{-6}$	0.003
*P*am145d *ssrA*	$<10^{-6}$	2.4×10^{-4}

n.d. not done.

[a] *clpP*::*kan* insertion exerts polarity on downstream *clpX*.

[b] extensive killing of host cell lawn at 42°C.

Figure 1

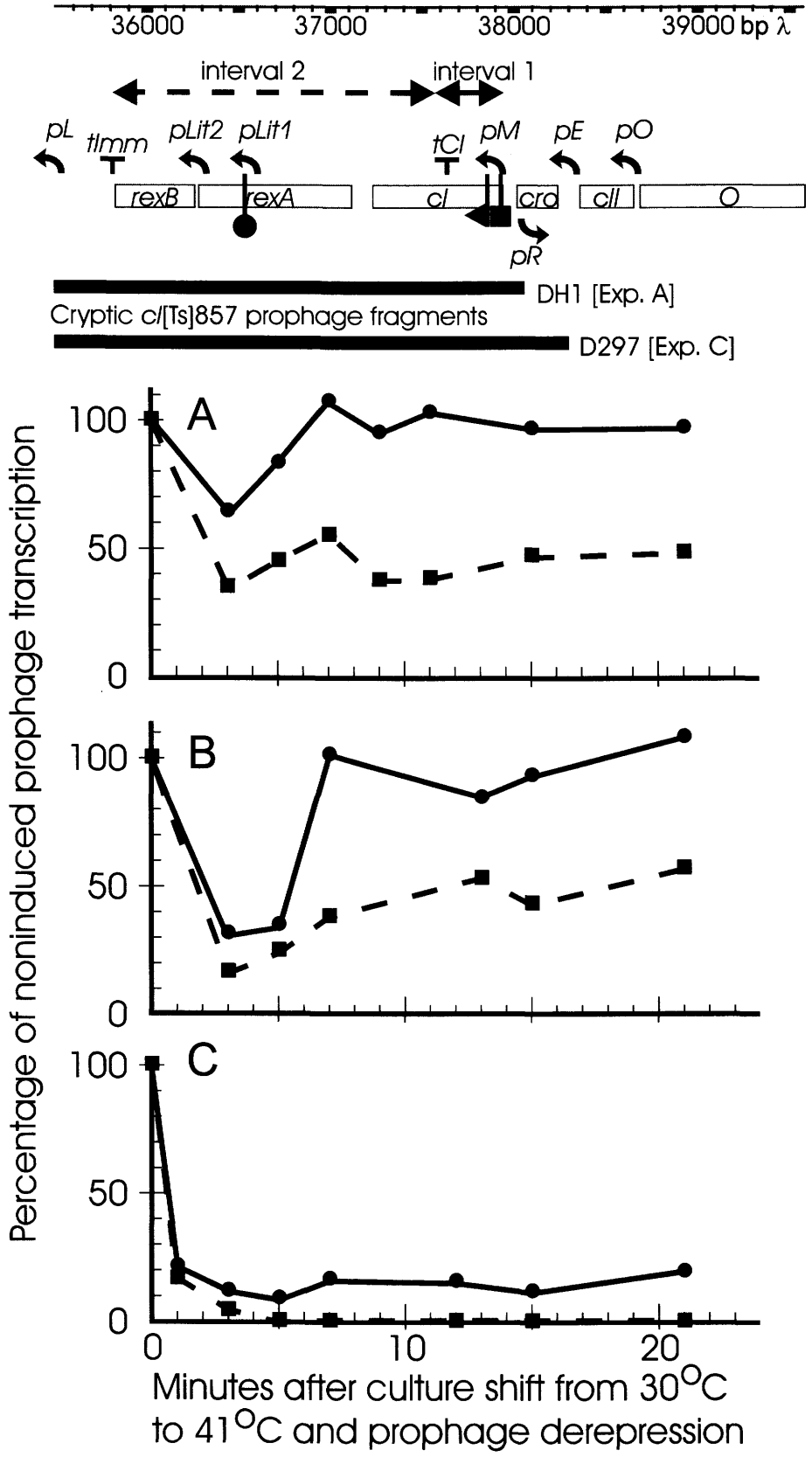
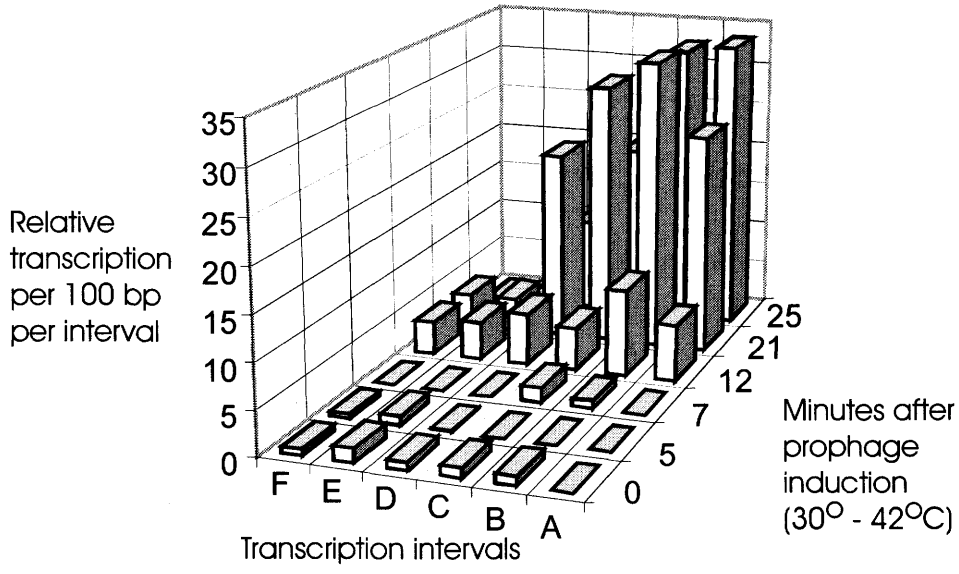

36000
37000
38000
39000 bp λ
interval 2
interval 1
pL
tIIIm
pLit2
pLit1
tCI
pM
pE
pO
rexB
rexA
cI
cro
cII
O
pR
DH1 [Exp. A]
Cryptic cI[Ts]857 prophage fragments
D297 [Exp. C]
Percentage of noninduced prophage transcription
100
50
0
A
B
C
0
10
20
Minutes after culture shift from 30°C
to 41°C and prophage derepression

Figure 2


Relative transcription per 100 bp per interval
35
30
25
20
15
10
5
0
F
E
D
C
B
A
Transcription intervals
25
21
12
7
5
0
Minutes after prophage induction (30° - 42°C)

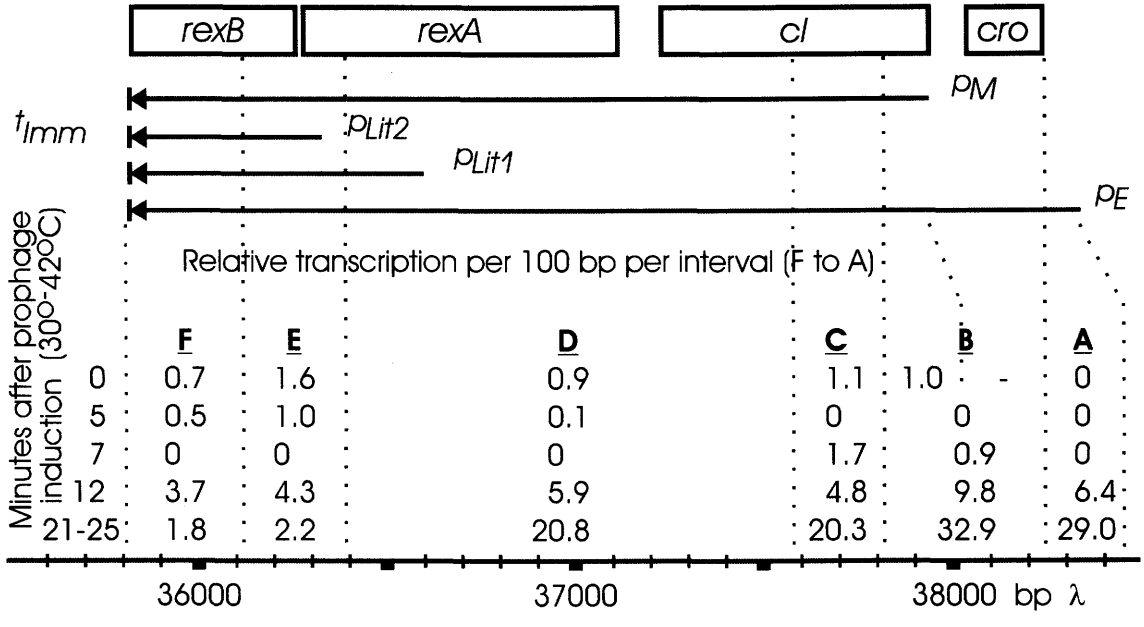
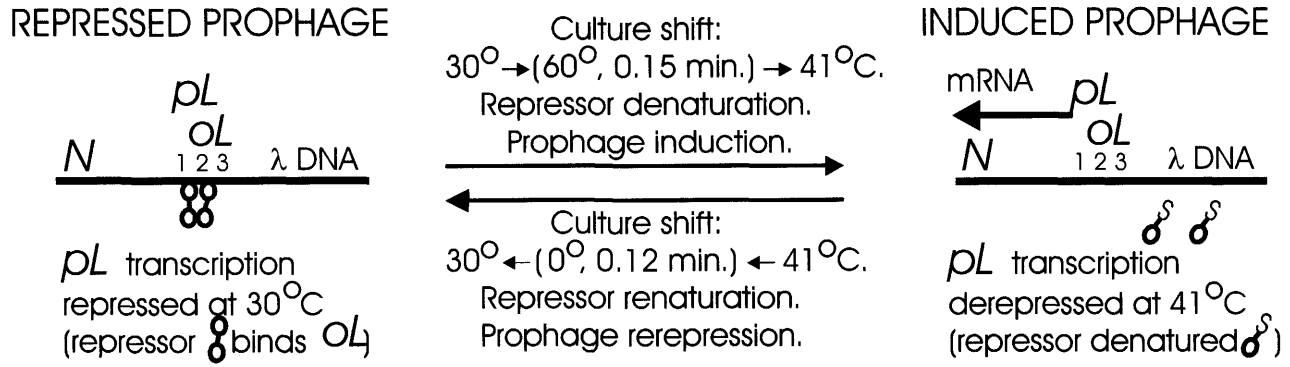

rexB
rexA
cI
cro
t_{Imm}
p_{Lit2}
p_{Lit1}
p_M
p_E
Relative transcription per 100 bp per interval (F to A)
Minutes after prophage induction (30°-42°C)
F E D C B A
0 0.7 1.6 0.9 1.1 1.0 - 0
5 0.5 1.0 0.1 0 0 0
7 0 0 0 1.7 0.9 0
12 3.7 4.3 5.9 4.8 9.8 6.4
21-25 1.8 2.2 20.8 20.3 32.9 29.0
36000
37000
38000 bp λ

Figure 3



Rerepression of derepressed *pL* transcription following repressor renaturation.

Induction and renaturation scheme		mRNA pulse labeling time (final minute)	REPRESSION FACTOR *pL* transcription from induced prophage: (induction / induction, then renaturation)		
			Relevant phenotype of λcI857cro27 prophage		
min. at 41°C	min. at 30°C		*cII*2002 Replication$^+$	*cII*$^+$ Replication$^-$	*cII*$^+$ Replication$^+$
5	5-13	13	28.0 (6.15/0.22)	30.3 (6.06/0.20)	26.7 (21.12/0.79)
10	10-15	15	26.4 (17.68/0.67)	29.2 (6.72/0.23)	2.8 (16.57/5.88)
15	15-21	21	21.5 (18.04/0.84)	19.7 (6.90/0.35)	2.2 (13.77/6.37)

CHAPTER SIX

Over-expression of *rexA* Suppresses T4*rII* Exclusion in *E. coli* K(λ) Lysogens

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Key words: bacteriophage lambda (λ); P_M-*cI-rexA-rexB*-t_{imm} operon; Rex exclusion phenotype; conditional Rex exclusion phenotype, prophage induction.

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Note: Data in Figure 2 was provided by S. Hayes.

6.1 Abstract

The Rex exclusion phenotype is a property of lambda lysogens of *E. coli*. The expression of genes *rexA-rexB* from the prophage excludes plating of an infecting T4*rII* phage mutant. Cryptic lambda prophages, defective in replication initiation, deleted for *int-kil,* lysis and structural genes *ninA-J*, and regulated by a *cI*[Ts]857 repressor, exhibited a conditional Rex exclusion phenotype: The lysogens were Rex^+ at 30° and Rex^- at 40°. Wild type λ lysogens, regulated by a cI^+ repressor, retained the Rex^+ exclusion phenotype between 30° and 43°. We measured transcription of the *cI-rexA-rexB* operon from promoters P_M and P_E, i.e., both before and after thermal induction of a λ*cI*[Ts]*cro* prophage. No polarity was observed for the P_M transcript, but polarity was observed within the >20-fold amplified P_E-*cI-rexA-rexB* transcript, with greatly reduced transcription of the distal *rexB* interval relative to promoter proximal *cI-rexA* transcription. We examined whether increased *rexA* to *rexB* expression was able to abrogate the Rex exclusion phenotype and could explain the conditional Rex phenotype exhibited by the cryptic lambda lysogens. Our results were consistent with this hypothesis.

6.2 Introduction

The ability of a repressed lambda lysogen of *E. coli* (*Eco* K) to inhibit the plating of T4*rII* mutants, termed the Rex (*rII* exclusion) exclusion phenotype (Benzer, 1955) is encoded as part of the P_M-*cI-rexA-rexB*-t_{imm} operon expressed from a repressed λ prophage (Matz et al., 1982; Hayes et al., 1997). In the stable λ lysogen, the CI repressor binds the leftward (O_L) and the rightward (O_R) operators, simultaneously blocking transcription from P_R, and stimulating low-level *cI-rexA-rexB* transcription from the P_M promoter (Heinemann and Spiegelman, 1970; Echols and Green, 1971; Reichardt, 1975; Meyer et al., 1980; Hawley and McClure, 1982; Li and Susskind, 1994). Upon induction of a lambda lysogen, *cII* is expressed from the P_R rightward promoter, stimulating transcription of the establishment message from P_E through the *cI-rexA-rexB* operon (Echols and Green, 1971; see Fig. 1). Late immunity transcription also occurs from the late immunity promoter P_{Lit1} within the C-terminal of *rexA*, independently expressing high levels of *rexB* in the absence of any measurable *cI,* or *rexA* message (Hayes and Szybalski, 1973; Hayes et al., 1997).

Cryptic lambda prophages, defective in replication initiation, deleted for *int-kil,* lysis and structural genes *ninA-J*, and regulated by a *cI*[Ts]857 repressor, were shown to exhibit a conditional Rex exclusion phenotype (CdI-Rex): The lysogens were Rex^+ at 30° and Rex^- at 40° (Hayes et al., 1998). Levels of *cI-rexA-rexB* transcription from repressed and induced λ*cI*857[Ts]*cro*$^+$ prophage were described by Hayes et al. (1997). Upon prophage induction, the transcription from P_M was repressed by Cro, the transcription of *rexB* from P_{Lit1} (Fig. 1A) was stimulated; and

no transcription was detected from P_E.

The stoichiometric balance in *rexA-rexB* gene expression may play an important role in the regulation of the Rex exclusion phenotype. The disruption of RexA:RexB stoichiometry may serve to "activate" or trigger cellular manifestations described as Rex exclusion phenotypes. T4*rII* infection of a λ lysogen may produce this distortion in Rex:RexB stoichiometry resulting in the loss of membrane potential, proton motive force and cellular ATP (Colowick and Colowick, 1983; Parma et al., 1992). Several lines of evidence suggest that the Rex exclusion phenotype is governed by RexA:RexB stoichiometery. Snyder and McWilliams (1989) noted that plasmid over-expression of *rexA* relative to *rexB* results in the cessation of host macromolecular synthesis in the absence of phage infection. The over-expression of *rexB* has been previously documented to suppress T4*rII* exclusion in a λ lysogen (Parma et al., 1992). In addition, the over-expression of *rexA-rexB* from a multicopy plasmid excludes both T4 and T4*rII* (Shinedling et al., 1987). However, since the over-expression of *rexA* in a λ lysogen is presumed to result in a lethal membrane depolarization event, the influence of expressing multiple copies of *rexA* in a λ *rex*$^+$ lysogen on Rex exclusion has not been examined. We previously reported that approximately half of Rex$^+$ lysogens remain viable following infection with T4*rII* (MOI 10), but are temporarily arrested for growth (Slavcev and Hayes, 2002). We show elsewhere that Rex$^+$ lysogens transformed with multicopy *rexA* plasmids exhibit 11-20% viability, and a similar prolonged growth arrest (Slavcev and Hayes, unpublished).

The RexA protein has yet to be characterized, and shares no significant

amino acid sequence similarity with any other proteins. Amino acid analysis predicts that RexA is a hydrophilic protein and thus probably resides within the cytoplasm. RexB is a very hydrophobic protein and has been localized to the inner membrane of the host by *phoA* fusion analysis (Parma et al., 1992). RexB traverses the membrane five times, possesses smalll hydrophilic stretches that adhere to the "plus inside" amino acid rule (von Heine, 1986) for transmembrane proteins, and shares sequence similarities with several ion channels. The requirement of monovalent Na^+ ions in the external medium for expression of the Rex phenotype and its inhibition by divalent ions such as Mg^{2+}, or Ca^{2+} is consistent with RexB participating in ion regulation (Garen, 1961; Ames and Ames, 1965; Brock, 1965).

In this study we sought to identify the mechanism of the Cdl-Rex phenotype: 1) We measure transcription of the *cI-rexA-rexB* operon before and after thermal induction of a λ*cI*[Ts]*cro* prophage 2) We examine the effect of disrupting RexA:RexB stoichiometry on Rex exclusion activity in λ lysogens by transforming them with *rexA*, or *rexB* plasmids.

6.3 Materials and Methods

6.3.1 Cells and Phage.

We utilized derivatives of *Eco* K strains of *E.coli*, namely, W3350A: F^- *lac*-3350 *galK2 galT22* IN(*rrnD-rrnE*)1 λ^- (Bachmann, B., 1987); R594: F^- *lac*-3350 *galK2 galT22 rpsL*179 IN(*rrnD-rrnE*)1 λ^- (Bachmann, B., 1987); TC600 *supE*44 (Hayes collection) from C600 (Bachmann, B., 1987) and JM101 F' *traD*36 $lacI^q$

Δ(*lacZ*)M15 *proA*$^{+}$*B*$^{+}$ / *supE thi* Δ(*lac-proAB*) λ^{-}. Conditional Rex strains: SA500(λ*bio*275*cI*857*cro*27Δ431*P*101b) was described by Hayes et al.(1998); SA500(*bio*275*cI*857Δ431rep$^{\lambda-}$ Y870-3) was prepared identically to SA500(λ*bio*275*cI*857*cro*27Δ431*P*101b) except the isolate was selected as a replication defective isolate of *cro*$^{+}$ Y836 at 42°C (Hayes and Hayes, 1986, and unpublished). The marker *ilv*500::Tn*10* was moved from donor strain CAG18431 into recipient strain HD173 *rho*702[Ts] by P1 transduction (both strains were obtained from the *E. coli* Genetic Stock Center at Yale University via M. Berlyn). The construct, HD173 *rho*702[Ts] *ilv*500::Tn*10*, was used as a donor strain to transfer the Tn*10* (*tet*^{R}) marker into recipient Rho^{+} strains SA500(λ*bio*275*cI*857*cro*27Δ431*P*101b) and SA500(λ*bio*275*cI*857Δ431 rep$^{\lambda-}$ Y870-3). The potential *rho*702[Ts] *ilv*500::Tn*10* transductants were examined for retention of *imm*λ by F.I. assay (Hayes and Hayes, 1986), for *cI*$^{+}$ genotype by cross-streaking with λ*cI*72 and λvir, and for co-transduction (found to be ~90%) of *rho*702[Ts] with *ilv*500::Tn*10* marker. Presence of the *rho*702[Ts] allele was screened by the acquired ability to allow λ*N*am7am53i*mm*434 plaque formation. The latter assay involved overlaying onto fresh Tryptone agar plates mixtures comprising 0.1 ml of culture cells for each potential transductant, plus dilutions of λ*N*am7am53i*mm*434, and 3 ml tryptone top agar; incubating plates at 32°C overnight, and scoring for PFU. The efficiency of plating (e.o.p.) for λ*N*am7am53i*mm*434 on the Rho^{+} strains was 3.5x10^{-7} (on W3350A, Su^{o}), and about 10^{-5} on the RK^{-} Nie strains at 32°C. The e.o.p. was about 0.02 on Rho^{-} strains HD173 *ilv*500::Tn*10* (32°C) and on the *rho*702[Ts] *ilv*500::Tn*10*

transductants of the RK^- conditional Rex strain. Lysogens of R594 and W3350A were made using λ phages previously described (Hayes et al., 1998; Slavcev and Hayes, 2002), including λ, λ*cI*857, λ*cI*857*rexB*5A and λ*cI*857*rexA*amQ lysogens of R594, and a λ*cI*857*cro*27. Wild-type bacteriophage λ was from laboratory stock # 271; λ*cI*857*rexA*amQ[301], λ*cI*857*rexB*5A (initially G. Gussin via W. Szybalski) and λ *cI*857*cro*27 phages were from our collection. The T4 phages were obtained from G. Mosig and include T4*rIIA* (point mutation in *rIIA* gene of T4), T4*rII*Δ1589 (deletion spanning the *rIIA* and *rIIB* genes), and T4D.

6.3.2 Plasmids.

A map of plasmid constructs used in this study is shown in Fig. 1. Plasmid pRS2 was constructed by digesting pCH1 (Hayes et al., 1997) with *Bgl*II and ligating λ DNA 38,103 bp through 35,711 bp (Daniels et al., 1983) into pACYC184 tet^R, digested with *Bam*HI at 1,869 bp. pRS2 carries λ P_M-*cI*857-*rexA*-*rexB*-t_{imm} downstream from promoter for tet^R. pRS10 was constructed as follows. Primers *im*3 (5' AAGTCGACAGTGAGTTGTATCTATTT 3') and *im*5 (5' TTGGATCCTTAACGTATGAGCACAAA 3') were used to amplify λ sequences 35,731 bp – 37,948 bp from pRS2; the PCR amplified fragment of λ gene interval *cI*857-*rexA*-*rexB*-t_{imm} was digested at the ends with *Bam*HI and *Sal*I and ligated into pACYC184 double digested with *Bam*HI and *Sal*I at 1,869 bp and 2,145 bp respectively. pRS10 carries λ *cI*857-*rexA*-*rexB*-t_{imm} downstream of promoter for tet^R. pUC18 and pUC19 were obtained from New England Biolabs (NEB). pRS7 was constructed by digesting pRS2 with *Mfe*I and ligating λ DNA 35,764bp – 37,186 bp into the MCS of pUC19, digested at 396 bp with *Eco*RI. Inserts were

screened by blue/white colony formation on IPTG + X-gal plates in JM101. pRS7 carries the λ *rexA-rexB-*t_{imm} downstream of the promoter for *lacZ'*. pRS13 was constructed as follows. Primers *im*3 (5' AAGTCGACAGTGAGTTGTATCTATTT 3') and *im*5 (5' TTGGATCCTTAACGTATGAGCACAAA 3') were used to amplify λ sequences 35,731 bp – 37,948 bp from pRS2. The PCR amplified fragment was double digested with *Mfe*I (λ37,186 bp) and *Ase*I (λ 36,276 bp) yielding λ fragment 37,186 bp – 36,276 bp; and this was ligated into pUC18 double digested with *Nde*I (at 183 bp within *lacZ'*) and *Eco*RI (at 396 bp within the MCS). In pRS13 *rexA* is downstream of the promoter for *lacZ'*. pRS14 was made by *Hin*dIII digestion of pRS7 removing λ DNA 36,895 bp – 37,186 bp, with one cut within the *Hin*dIII site in the MCS of pRS7, and religation. pRS14 carries λ DNA 35,764 bp – 36,895 bp corresponding to genes *rexA*[partial Δ]-*rexB-*t_{imm}. The *rexB* insert for pRS14, which includes the low-level constitutive P_{Lit2} promoter (Hayes and Szybalski, 1973), is downstream of the promoter for *lacZ'*. Plasmid pRS4 was constructed by digesting pCH1 with *Bgl*II and ligating λ DNA 38,103 bp through 35,711 bp into pUC18, digested with *Bam*HI in MCS at 486 bp. Plasmid pRS4 carries λ P_M-*cI*857-*rexA-rexB-*t_{imm} downstream from the promoter for *lacZ'*. pRS11 was constructed by digesting pRS4 with *Mfe*I to remove λ DNA 37,186bp – 35,764bp. pRS11 carries λ P_M-*cI*857 downstream of the promoter for *lacZ'*. pRλ*cI*857 was constructed as follows. pRS11 was double digested with *Sal*I and *Eco*RI yielding the chimeric fragment *Eco*RI-MCS-λ*cro-*P_R-P_M-*cI*857-*Sal*I (22 bp pUC18 MCS – 970 bp λDNA – 12 bp MCS). The fragment was ligated into pBR322 double digested with *Ava*I and *Eco*RI at 1,429 bp and 4, 359 bp respectively. pRλ*lacZ'* was constructed by double

digesting pUC19 with *Sma*I and *Aat*II at 412 bp and 2,617 bp respectively and ligating the *lacZ'-*$t_{lacZ'}$ fragment into pRλ*cI*[Ts]857double digested with *Sma*I and *Aat*II in the MCS generating the chimeric gene interval t_{lacZ}-*lacZ'* MCS*cro*-P_R-O_R-P_M-*cI*857. Inserts were screened by blue colony formation on IPTG + X-gal plates in JM101 conferred by the in-frame λ*cro-lacZ'* fusion. pRS5 was constructed by digesting λDNA (NEB) with *Mfe*I and ligating λ DNA 35,764 bp – 37,186 bp into pUC19 digested with *Eco*RI at 396 bp in the MCS. pRS7 carries λ t_{imm}-*rexB-rexA* downstream of the promoter for *lacZ'*. pRS6 was constructed in the same manner as pRS7 with the exception that λ*cI*857*rexA*amQ DNA was digested with *Mfe*I and pRS6 carries λ t_{imm}-*rexB-rexA*amQ downstream of the promoter for *lacZ'*. pRS5 was constructed in the same manner as pRS6 and pRS7 with the exception that λ*cI*857*rexB*5A was digested with *Mfe*I and pRS5 carries λ t_{imm}-*rexB*5A-*rexA* downstream of the promoter for *lacZ'*. pRS15 was constructed by double digesting pRS7 with *Aat*II and *Sma*I and ligating the chimeric t_{lacZ}-*lacZ'- rexB-rexA* fragment into pRλ*lacZ'* double digested with *Aat*II and *Sma*I. Inserts were screened by blue/white colony formation on IPTG + X-gal plates in JM101. pRS15 carries λ *rexB-rexA* genes downstream of the CI[Ts]857-regulated λ promoter P_R, which is repressed at 30°C and induced at 35-40°C. Temperature regulation of downstream *rexA-rexB* genes was confirmed by T4*rII* and λ*cI*72 on R594[pRS15] at 25, 30, 34, 37 and 40°C (see Fig. 1 legend). pRS16 was constructed in the same manner as pRS15 except that pRS6 was digested with *Aat*II and *Sma*I. pRS16 carries λ *rexB-rexA*amQ genes downstream of the λ P_R promoter. pRS17 was constructed in the same manner as pRS15 and pRS16 except that pRS5 was digested with *Aat*II and

*Sma*I. pRS17 carries the λ *rexB*5A-*rexA* genes downstream of the λ P_R promoter. pRS18 was constructed as follows. Primers *im*3 (5' AAGTCGACAGTGAGTTGTATCTATTT 3') and *im*5 (5' TTGGATCCTTAACGTATGAGCACAAA 3') were used to amplify λ sequences 35,731 bp – 37,948 bp from λ*cI*857*rexA*amQ. The amplified fragment of λ gene interval *cI*857-*rexA*amQ-*rexB*-t_{imm} was digested at the ends with *Bam*HI and *Sal*I and ligated into pACYC184 double digested with *Bam*HI and *Sal*I at 1,869 bp and 2,145 bp respectively. pRS18 carries λ *cI*857-*rexA*-*rexB*-t_{imm} downstream of the promoter for tet^R. pRS19 was constructed in the same manner as pRS18 except that primers *im*3 (5' AAGTCGACAGTGAGTTGTATCTATTT 3') and *im*5 (5' TTGGATCCTTAACGTATGAGCACAAA 3') were used to amplify λ sequences 35,731 bp – 37,948 bp from λ*cI*857*rexB*5A. pRS19 carries the λ *cI*857-*rexA*-*rexB*5A-t_{imm} downstream of the promoter for tet^R. The orientation of the λ DNA inserts within plasmids was confirmed by restriction pattern analysis following *Hin*dIII digestion.

6.3.3 Measuring P_E-P_M-cI-rexA-rexB-t_{Imm} Transcription.

A multi-step liquid DNA-[H^3]RNA Hybridization technique was used to measure λ RNA transcription and has been previously described (Hayes and Szybalski, 1973; Hayes, 1979; Hayes et al., 1997).[1]

---

[1] Single colony isolates of the λ lysogenic strains were grown in 20 ml cultures at 30°C. Phage λ transcription was induced by raising the culture temperature to 41°C, causing thermolability of the CI857[Ts] repressor. The procedures and medium used for pulse-labeling with [5-^{3}H]uridine, and extracting the [^{3}H]RNA is described by Hayes and

6.3.4 Biological Assays for Rex Exclusion.

Rex exclusion phenotypic activity encoded by λ prophage genes *rexA-rexB* was measured in lysogenic cells grown between 30 and 42°C (see Table 1). The e.o.p. for each phage was determined by dividing the e.o.p. of T4*rII* on the assayed host cells by the titer obtained in parallel on the permissive host cells R594. For each assay, the cultures were grown overnight at 30°C, diluted (0.1X) into fresh TB broth (10g Bacto Tryptone, 5g NaCl / liter) and grown 4-6 hr at the assay temperature. Assays were performed at various temperatures by first transferring culture aliquots (about 3 X 10^8 CFU) to a heated water bath, adding 0.1 ml of dilutions of phage lysates of either T4*rIIA*, or T4*rII*Δ1589. Three ml of TB top agar (TB plus 6.5g Bacto agar/liter) was added and the mixtures were poured onto TB

Szybalski (1973). A final volume of 0.9 ml to 1 ml of [^{3}H]RNA was extracted per culture. To assay the low levels of λ RNA synthesis (0.001% - 0.1% of total *E. coli* plus λ [^{3}H]RNA input counts to a hybridization) a two-step hybridization protocol was used. In the first "prehybridization" step, 12-15 μg of DNA from a λ/hybrid l-strand was annealed (65°C for 4.5 hours) to either 0.3 or 0.45 ml of the extracted [^{3}H]RNA from the tested culture plus 0.5 ml 1/2XSSC, 2% phenol. The major RNA transcribed rightward form the λ r-strand P_R-*Q-R-A-Jb*, λ l-strand *att*-P_L, and from *E.coli* are eliminated by this technique, while retaining the l-strand *cI-P* transcripts, which bind to the phage strand. In the second step, the *cI-P* [^{3}H]RNA was eluted from the λ strand used for prehybridization and was hybridized (67°C for 4.5 hours) to DNA strands from different λ/hybrid phage (0.4 ml eluted [^{3}H]RNA, 0.2 ml 1/2XSSC, 2% phenol, 3-5 μg λDNA strand in about 0.02 ml of 5M CsCl). This technique retains only the RNA from a selected region of the *cI-P* interval. Next, a sham hybridization was employed, where the normally added λ DNA strand was replaced by 0.02 ml of a saturated CsCl solution.

bottom agar plates (TB plus 11g Bacto agar and 1 mg thiamine·HCl / liter) pre-warmed to the assay temperature. The plates were incubated inverted at the assay temperature for 16 hr. Phage T4D [wt] infections were used as a control for Rex exclusion to show that host cells remained sensitive to T4.

6.4 Results

6.4.1 Polarity in the P_E-cI-rexA-rexB-t_{imm} Operon.

Rex phenotypic activity was measured in SA500 *E. coli* K-12 cells possessing a cryptic λ prophage defective for λ replication initiation and prophage excision (Hayes et al., 1998). The cryptic prophages were deleted for genes *int-kil* and all late genes to the right of *ren*, and defective in λ replication initiation gene *P*. The RK^- mutant cells plate with an e.o.p. near 1.0 at both 30°C and 42°C. When the cells were shifted from 30°C to 42°C the Rex exclusion phenotype was abrogated and T4*rII* plating was enabled (Table 1). This conditional Rex exclusion phenotype (Cdl-Rex) cannot be due to temperature sensitivity of the *rexA*, or *rexB* genes since cI^+ λ lysogens retain full Rex activity at 30°C and 43°C (Table 1). The transcription of the *cI-rexA-rexB* operon was measured from repressed and induced lysogen W3350A(λ*cI*857*cro*27). At 30°C the active CI857 repressor represses P_R and P_L transcription and stimulates low-level transcription of the *cI-rexA-rexB* message from P_M (Heinemann and Spiegelman, 1970; Echols and Green, 1971; Reichardt, 1975; Meyer et al., 1980; Hawley and McClure, 1982; Li and Susskind, 1994). We noted that the amount of transcription originating from P_M at 30°C in noninduced W3350A(λ*cI*857*cro*27) lysogens was constant per 100 bp interval

throughout the *cI-rexA-rexB* operon (Fig. 2). Shifting the lysogen to 41°C renders the CI857 repressor inactive and relieves repression of the λ P_R promoter leading to *cII* expression and CII-stimulated transcription of the P_E-*rexA-rexB-t*$_{imm}$ operon (Echols and Green, 1971). Transcription from P_E is further stimulated by the defective *cro* repressor allele, which relieves repression of the P_R promoter and amplifies *cII* expression (Hayes and Hayes, 1978; see Fig. 1 λ map). Establishment transcription from P_E arose between 7 to 12 minutes (data not shown) following thermal induction of W3350(λ*cI*857*cro*27) and was maximal between 20-25 minutes. The transcription of intervals D-E and C-D, including primarily *rexB*, was significantly reduced when compared to transcription from the P_E-proximal intervals B-C and A-B. These results show polarity in the P_E-*cI-rexA-rexB* transcript in thermally induced W3350A(λ*cI*857*cro*27) lysogens. The transcriptional profile of *cI* and *rexA* from a thermally induced W3350(λ*cI*857*cro*27) lysogen suggests that they would be over-expressed (if mRNA is translated) compared to downstream *rexB* gene.

We sought to determine whether the loss of the Rex exclusion phenotype seen upon shifting λ CdI-Rex lysogens to 42°C was due to transcriptional polarity within the *cI-rexA-rexB* operon. The temperature-sensitive *rho*702 mutation was transduced into λ CdI-Rex lysogens (Table 1). We found that loss of Rho activity at 42°C restored Rex exclusion of T4*rII* by more than 10^4 fold in the 101b *rho*702[Ts] strain, but conferred no increase in Rex exclusion activity by the isogenic Cro^+ Y870 strain. The *rho*702[Ts] allele was not found to compromise T4D plating at either the permissive (data not shown), or non-permissive temperature (Table 1).

Our data indicate that the loss of Rex exclusion seen upon shifting the 101b λ*cI*[Ts]*cro* lysogen to 42°C is related to the observed polarity within the P_E-*cI-rexA-rexB-*t_{imm} message in induced λ*cI*[Ts]*cro* lysogens, since loss of Rho activity in 101b*rho*[Ts] at 42°C can restore Rex exclusion activity at this temperature by $>10^4$ fold. If this effect of polarity is translated into protein, then CI and RexA would be in abundance compared to RexB and could disrupt functional Rex stoichiometry. In contrast, the Rex$^-$ phenotype exhibited by the Y870 Cro$^+$ derivative at 42°C, is independent of Rho activity and most likely not imparted by polarity within the *cI-rexA-rexB* operon upon thermal induction of the lysogen.

6.4.2 Influence of rex Stoichiometry on T4rII Exclusion.

The Rex phenotype was assayed in R594 and R594(λ) cells that were transformed with multicopy *rexA*, *rexB*, or *rexA-rexB* plasmids (Table 2). The transformed R594 nonlysogenic culture cells with p*rexA-rexB* were Rex$^+$ and cells with p*rexA*, or p*rexB* did not exclude T4*rII*. R594(λ) cells transformed with the p*rexB* plasmid allowed T4*rII* to generate plaques (e.o.p.=0.58), while neither the p*rexA-rexB* plasmid, nor the control pUC19 plasmid influenced T4*rII* plating efficiency in the lysogenic cells (e.o.p. $<5.8 \times 10^{-6}$). R594(λ) cells transformed with p*rexA* were found to have lost the Rex exclusion phenotype (i.e. T4*rII* plated with an efficiency of 0.21). We examined if the Rex$^-$ phenotype of the R594(λ)[p*rexA*] and R594(λ)[p*rexB*] isolates was due to host mutation rather than plasmid expression. We isolated colonies spontaneously cured of either p*rexA*, or p*rexB* and tested these isolates again for Rex exclusion. We found that Rex exclusion phenotype was restored in all isolated colonies spontaneously cured of either

p*rexA*, or p*rexB* (all T4*rII* e.o.p's were <6X10^{-6}).

We transformed nonlysogenic cells, *rexA*$^{+}$-*rexB*$^{+}$ lysogens and *rexA*$^{-}$-*rexB*$^{+}$ *rexA*$^{+}$-*r exB*$^{-}$ derivatives with low-copy number plasmids expressing *cI*857-*rexA*$^{+}$*rexB*$^{+}$ (pRS10), *cI*857-*rexA*amQ-*rexB*$^{+}$ (pRS18), or *cI*857-*rexA*$^{+}$-*rexB*5A (pRS19) and assayed the Rex exclusion phenotype by measuring T4*rII* plating efficiency on transformed cell cultures at 30°C (Table 3). The pRS10 *rexA*$^{+}$-*rexB*$^{+}$ plasmid imparted a Rex^{+} phenotype to R594 nonlysogenic and lysogenic cells. The pRS18 *rexA*$^{-}$-*rexB*$^{+}$ plasmid complemented the R594(λ*cI*857*rexB*5A) *rexB* mutation and had no effect on the nonlysogenic, or λ*rexA*$^{+}$-*rexB*$^{+}$, or λ*rexA*$^{-}$-*rexB*$^{+}$ lysogenic derivatives. The pRS19 *rexA*$^{+}$-*rexB*$^{-}$ plasmid complemented for the R594(λ*cI*857*rexA*amQ) *rexA* mutation and attenuated Rex exclusion activity of R594(λ*cI*857) by greater than 50 fold. The pRS19 plasmid exerted no effect on Rex exclusion in R594 nonlysogenic and λ*cI*857*rexB*5A lysogenic derivatives although we noted that T4*rII* plaques formed on R594[pRS18] were tiny, suggesting that R594 may confer low-level suppression of the *rexA*amQ mutation.

We constructed temperature inducible p*rexA*$^{+}$-*rexB*$^{+}$ (pRS15), p*rexA*$^{+}$-*rexB*$^{+}$ (pRS16) and p*rexA*$^{+}$-*rexB*5A (pRS17) plasmids with *rexA-rexB* genes downstream from the P_RO_R operator (Fig. 1). In these constructs P_R transcription is regulated by the CI[Ts]857 repressor. We assayed the effect of these plasmids on the Rex exclusion phenotype under repressed and induced conditions in nonlysogenic R594 cells and R594 cells transformed with the *cI*[Ts]857-*rexA*$^{+}$-*rexB*$^{+}$ (pRS10) plasmid (Table 4). The *rex*$^{+}$ R594[pRS10] cells transformed with the p*rexA* plasmid exhibited the Rex exclusion phenotype when repressed for *rexA* gene expression

at 30°C (Table 4). However, when the cells were shifted to 34°C (slight derepression of *rexA*) pin-point T4*rII* plaques were visible on the cell lawn. At 37°C (partial derepression of *rexA*) T4*rII* formed slightly larger asymmetrical plaques (e.o.p.=0.06), and by 40°C plating was further augmented with complete loss of Rex exclusion phenotype. Very similar plating results were obtained on R594[pRS10] cells carrying the p*rexB* plasmid, raised from 30 to 40°C (Table 4). In contrast, R594[pRS10] cells transformed with p*rexA*$^+$-*rexB*$^+$, or parent pRλ*lacZ'* plasmid control retained full Rex exclusion activity at each of the assayed temperatures. We noted that T4*rII* formed large plaques on the control R594[p*rexA*$^+$-*rexB*5A] cells at 30-34°C and small plaques at 37°-40°C, suggesting that some very slight suppression for RexB activity arose from the multicopy *rexB*5A allele.

In summary, disrupting the stoichiometric *rexA*:*rexB* balance by expressing an excess of either from a multicopy plasmid in *rexA*$^+$-*rexB*$^+$ cells abrogates the Rex exclusion phenotype.

6.5 Discussion

It is essential to the viability of λ phage to encode a means by which it may escape its own exclusion system. The *ren* and *red* (*bet gam*) genes enable λ to escape the Rex exclusion phenotype (Toothman and Herskowitz 1980b; Toothman and Herskowitz 1980c). Expression of *oop* RNA on a multicopy plasmid also attenuates the Rex exclusion phenotype (Hayes et al., 1997). Parma et al. (1992) suggested that upon induction of a λ prophage, late immunity transcription arising

from P_{Lit1} in the C-terminal of *rexA* (Hayes and Szybalski, 1973) may stimulate *rexB* over-expression and confer a Rex$^-$ environment to allow phage growth. The noted levels of *cI-rexA-rexB* transcription from an induced λ*cI*[Ts] Cro$^+$ defective prophage confirmed that P_E transcription levels of *cI-rexA* are minimal, while *rexB* expression from P_{Lit1} is dramatically stimulated in the thermally induced prophage (Hayes and Hayes, 1978; Hayes et al., 1997). Here, we measured transcription of the *cI-rexA-rexB* operon in a repressed, or induced λ*cI*857*cro*27 prophage. Comparison of transcription levels standardized per 100bp interval suggests that *cI-rexA* transcription is about 20 fold higher than downstream *rexB* expression by twenty minutes following prophage derepression (data not shown).

We argue that truncating the *cI-rexA-rexB* transcript from derepressed λ*cI*[Ts]*cro*$^-$ lysogens is responsible for the conditional Rex[Ts] phenotype (CdI-Rex) observed with Δ(*int-kil*) *cI*[Ts]*cro*$^-$ replication defective λ lysogens. Our argument is based on the observation that introducing a *rho*[Ts] allele into the *cro*$^-$ CDL-Rex lysogen restored Rex exclusion phenotypic activity by more than 10^4 fold at the induced temperature, but had no noticeable effect on Rex exclusion in the isogenic *cro*$^+$ strain. We have not measured protein levels of RexA and RexB to be able to declare with certainty that RexA is being over-expressed with respect to RexB in the *cro*$^-$ lysogen. However, the loss of Rex exclusion phenotype correlates with the unequal transcription through *cI-rexA-rexB* from P_E. Furthermore, we also noted that T4*rII* forms small asymmetrical plaques on the induced defective Δ(*int-kil*) *cI*[Ts]*cro*$^-$ rep$^-$ λ lysogen at 42°C that are identical in morphology to those formed on wild type λ lysogenic cells with an added multicopy plasmid constitutively

expressing *rexA*. No polarity of the P_M transcript was observed from a repressed λ*cro*$^-$ prophage assayed at 30°C. Hayes and Bull (1999) reported the presence of three slippery heptamers (Tsuchihashi and Brown, 1992) residing within the immunity region of λ. They proposed that -1 frameshifting at the two AAAAAAG heptamer sites within *cI* and at a third site within *rexA* was somehow inhibited by functional CI repressor. In the absence of functional CI, active frameshifting at the heptamer sites would truncate CI and RexA translation and exert polarity on downstream *rexB* expression.

Will the imbalance in *rexA-rexB* transcription from thermally induced λ*cI*[Ts]*cro* prophage, caused by polarity in P_E transcript, translate into a protein imbalance? According to the Parma et al. (1992) model, such an imbalance should trigger an activation of RexB and result in host cell death. Yet, the λ*cI*[Ts]*cro* lysogens that exhibit the CdI-Rex phenotype retain essentially full viability when grown at 42°C, although they were delayed for colony formation at this temperature. We previously found that about half of the λ lysogens infected with T4*rII* at MOI 10 (presumably triggering the Rex exclusion phenotype) survived infection, albeit they were strongly arrested for growth, only forming colonies after 30+ hours incubation at 37°C (Slavcev and Hayes, 2002). We found that Rex$^+$ lysogens transformed with multicopy *rexA* plasmids exhibited 11-20% survival and showed a similar pattern of prolonged growth arrest (Chapter 3). Our results on arrested growth agree with the observations by Snyder and McWilliams (1989), who showed that plasmid over-expression of *rexA*, relative to *rexB* in non-lysogens results in cessation of cellular macromolecular synthesis and growth arrest (cellular

viability data was not presented). How does the over-expression of RexA to RexB, a condition that has been documented to compromise cellular growth (Snyder et al., 1989; Parma et al., 1992; Slavcev and Hayes, 2002) also perturb T4*rII* exclusion? Although the loss of cellular membrane potential, proton motive force (Parma et al., 1992) and ATP (Colowick and Colowick, 1983) has been documented following RexA, or T4*rII* "triggering" of the Rex exclusion phenotype, the effects of prolonged *rexA* over-expression in a λ lysogen recovering from these Rex-mediated cellular manifestations has not been examined.

In contrast to cryptic λ*cI*[Ts]*cro* lysogens, we have shown that the conditional Rex exclusion phenotype exhibited by Δ(*int-kil*) Cro$^+$ *cI*[Ts] rep$^-$ λ lysogens could not be suppressed by introducing a *rho*[Ts] mutation. T4*rII* formed large r-type (rapid lysis) plaques on the induced Cro$^+$ Δ(*int-kil*) *cI*[Ts]rep$^-$ λ lysogen at 42°C that were identical in morphology to those formed on wild type λ lysogenic cells with an added multicopy plasmid constitutively expressing *rexB*.. Hayes et al. (1997) reported that *rexB* is expressed from P_{Lit1} in a thermally induced Cro$^+$ λ*cI*[Ts] lysogen and that P_E- *cI-rexA* transcription was repressed by Cro (Hayes et al., 1997). In combination these observations indicate that upon induction of a Cro$^+$ prophage *cI-rexA-rexB* expression is not subject to polarity and if translated would result in the over-expression of RexB to RexA; a condition that abrogates the Rex exclusion phenotype. We reproduced the results of Parma et al. (1992) showing that multicopy *rexB* over-expression in *rexA*$^+$-*rexB*$^+$ λ lysogens results in the loss of T4*rII* exclusion. Based on this finding they proposed that P_{Lit} over-expression of *rexB* by an induced λ (Cro$^+$) prophage provides the distortion of Rex stoichiometry

necessary for λ to escape its own Rex exclusion system.

Our results suggest to us that the induction of Cro^+ or Cro^- prophage results in the disruption of Rex stoichiometry, thereby suppressing the Rex exclusion phenotype. An induced λ*cro*$^+$ prophage is not subject to polarity and may over-express *rexB* relative to *rexA*, escaping exclusion (Parma et al., 1992; Hayes et al., 1997). In contrast, thermal Induction of a λ*cro*$^-$ prophage results in powerful polarity within the *cI-rexA-rexB* operon that favours over-expression of *rexA* relative to *rexB*, suppressing the Rex exclusion phenotype. This hypothesis is supported by our finding that a multicopy *rexA* plasmid completely abolishes Rex activity in a λ*rex*$^+$ lysogen.

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6.7 Figure Legends

Figure 1. Plasmids Used in This Study.

A. λ Gene Regulation. **B.** Constitutive Expression Plasmids. **C.** Temperature Inducible Expression Plasmids. Temperature-sensitive CI857 repressor activity of pRλ*lacZ'*, pRS15, pRS16 and pRS17 was determined by e.o.p. of λ*cI*72 at 30°C, $<2.4X10^{-6}$; 34°C, $<2.4X10^{-6}$; 37°C, $<2.4X10^{-6}$; and 40°C, 0.27-0.34. Temperature controlled *rexA-rexB* gene expression from the λ P_R promoter of pRS15 was

determined by e.o.p. of T4*rII*Δ1589 at: 25°C, 0.57; 30°C, 0.25; 34°C, $8.5X10^{-3}$; 37°C, $<1.1X10^{-6}$; and 40°C, $<1.1X10^{6}$.

Figure 2. Measuring Transcription of the λ *cI-rexA-rexB* Operon from the P_M and P_E Promoters. (Hayes and Hayes, 1979; Hayes et al., 1997).

Points were plotted based upon the estimated map position for *bio* substitutions (Daniels et al., 1983). Markers: **A**, *bio*t124; **B**, *bio*30-7; **C**, *bio*t75; **D**, *bio*16-3; **E**, *bio*3h-1 set equal to t_{imm} (Landsman et al., 1982; Daniels et al., 1983; Hayes et al., 1997) as identified (Hayes and Szybalski, 1973). The transcription for the five regions (intervals A – E) of the P_E-*cI-rexA-rexB*-t_{Imm} operon was normalized per 100 bp in the intervals and then compared to the reference level of transcription per 100 bp in interval D (0-time). The results are for repressed (30°C) and induced (41°C) prophage λ*cI*857[Ts]*cro*27 in host W3350A.

Table 1. The Influence of Rho on the Conditional Rex Exclusion Phenotype.

Host Cells	T4*rIIA* e.o.p		T4D e.o.p.
	30°C	**42-43°C**	**42-43°C**
R594	1.0	1.0	1.0
R594(λ)	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$
R594(λ*cI*[Ts]857)	$<10^{-6}$	c.d. [a]	c.d. [a]
HD173 *rho*[Ts]702	1.0	1.0	0.66
SA500(λ*bio*275*cI*[Ts]857Δ431) RK^- mutant:			
Y870-3	$<10^{-6}$	1.0	1.0
Y870-3 *rho*[Ts]702 [b]	$<10^{-6}$	0.60	0.74
SA500(λ*bio*275*cI*[Ts]857*cro*27Δ431) RK^- mutant:			
*P*101b	$<10^{-6}$	0.30	0.96
*P*101b *rho*[Ts]702 [b]	$<10^{-6}$	$3X10^{-5}$	0.60

[a] cell death. Thermal induction of prophage results in cell lysis preventing formation of cell lawn for enumeration of PFU.

[b] The results for e.o.p.'s are the average from independent assays with two *rho*[Ts]702 transductants that both formed adequate cell lawns at 42-43°C.

Table 2. Influence of Multicopy *rexA, rexB* and *rexA-rexB* Expression on Rex Exclusion Phenotypic Activity.

Host Cells	Host *rex* Genotype	Plasmid	Plasmid *rex* Genotype	T4*rII*Δ1589 e.o.p. (37°C)
R594	*rex*$^-$	---	---	1.0
R594	*rex*$^-$	pUC19 [a]	*rex*$^-$	1.0
R594	*rex*$^-$	pRS7	*rexA*$^+$-*rexB*$^+$	$<6X10^{-6}$
R594	*rex*$^-$	pRS13	*rexA*$^+$	0.76
R594	*rex*$^-$	pRS14	*rexB*$^+$	1.0 [b]
R594(λ) [c]	*rexA*$^+$-*rexB*$^+$	---	---	$<6X10^{-6}$
R594(λ)	*rexA*$^+$-*rexB*$^+$	pUC19	*rex*$^-$	$<6X10^{-6}$
R594(λ)	*rexA*$^+$-*rexB*$^+$	pRS7	*rexA*$^+$-*rexB*$^+$	$<6X10^{-6}$
R594(λ)	*rexA*$^+$-*rexB*$^+$	pRS13	*rexA*$^+$	0.21[d]
R594(λ)	*rexA*$^+$-*rexB*$^+$	pRS14	*rexB*$^+$	0.58 [b]

[a] the multicopy pUC19 plasmid is the parent of pRS7 and pRS14. pUC18 is the parent of pRS13.
[b] T4*rII* generated large r-type (rapid lysis plaques) on R594[pRS14] and R594(λ)[pRS14].
[c] the minimal e.o.p. was recorded on host R594(λ) and two colonies from Amp^S R594(λ) lysogens cured of plasmid pRS13 and pRS14.
[d] T4*rII* generated tiny plaques asymmetrical plaques on R594(λ)[pRS13].

Table 3. Influence of Low Copy *rexA*, *rexB* and *rexA-rexB* Expression on Rex Exclusion Phenotypic Activity.

Host Cells	Plasmid	Plasmid *rex* Genotype	T4*rII*Δ1589 e.o.p. (30°C)
R594	---	---	1.0
R594	pACYC184	*rex*$^-$	1.0
R594	pRS10	*c*I857-*rexA*$^+$-*rexB*$^+$	$<3X10^{-7}$
R594	pRS18	*c*I857-*rexA*$^-$-*rexB*$^+$	0.20 [a]
R594	pRS19	*c*I857-*rexA*$^+$-*rexB*$^-$	0.09 [a]
R594(λ*c*I857)	---	---	$<3X10^{-7}$
R594(λ*c*I857)	pACYC184	*rex*$^-$	$<3X10^{-7}$
R594(λ*c*I857)	pRS10	*c*I857-*rexA*$^+$-*rexB*$^+$	$<3X10^{-7}$
R594(λ*c*I857)	pRS18	*c*I857-*rexA*$^-$-*rexB*$^+$	$<3X10^{-7}$
R594(λ*c*I857)	pRS19	*c*I857-*rexA*$^+$-*rexB*$^-$	$2.6X10^{-5}$
R594(λ*c*I857*rexA*amQ)	---	---	0.30
R594(λ*c*I857*rexA*amQ)	pACYC184	*rex*$^-$	0.67
R594(λ*c*I857*rexA*amQ)	pRS10	*c*I857-*rexA*$^+$-*rexB*$^+$	$<3X10^{-7}$
R594(λ*c*I857*rexA*amQ)	pRS18	*c*I857-*rexA*$^-$-*rexB*$^+$	0.15 [a]
R594(λ*c*I857*rexA*amQ)	pRS19	*c*I857-*rexA*$^+$-*rexB*$^-$	$1.8X10^{-5}$
R594(λ*c*I857*rexB*5A)	---	---	0.50
R594(λ*c*I857*rexB*5A)	pACYC184	*rex*$^-$	0.60
R594(λ*c*I857*rexB*5A)	pRS10	*c*I857-*rexA*$^+$-*rexB*$^+$	$<3X10^{-7}$
R594(λ*c*I857*rexB*5A)	pRS18	*c*I857-*rexA*$^-$-*rexB*$^+$	$<3X10^{-7}$
R594(λ*c*I857*rexB*5A)	pRS19	*c*I857-*rexA*$^+$-*rexB*$^-$	0.22

[a] plaques were tiny.

Table 4. Influence of Multicopy Inducible *rexA*, *rexB* and *rexA-rexB* Expression on Rex Exclusion Phenotypic Activity.

R594[plasmids] [a]	Inducible Plasmid *rex* Genotype	T4*rII*Δ1589 e.o.p.			
		30°C	34°C	37°C	40°C
Controls:					
---	---	1.0	1.0	1.0	1.0
pRS10 {*cI857rex*$^+$}	---	$<2X10^{-6}$	$<2X10^{-6}$	$<2X10^{-6}$	$<2X10^{-6}$
pRλ*lacZ'*	*rex*$^-$	0.40	0.61	0.57	0.70
pRS15	*rexA*$^+$-*rexB*$^+$	0.25 [b]	$9X10^{-3}$ [b]	$<2X10^{-6}$	$<9X10^{-7}$
pRS16	*rexA*$^-$-*rexB*$^+$	1.0	1.0	1.0	1.0
pRS17	*rexA*$^+$-*rexB*$^-$	0.74	0.62	1.0	0.46 [b]
pRS10+pRλ*lacZ'*	*rex*$^-$	$<2X10^{-6}$	$<2X10^{-6}$	$<2X10^{-6}$	$<2X10^{-6}$
pRS10+pRS15	*rexA*$^+$-*rexB*$^+$	$<2X10^{-6}$	$<2X10^{-6}$	$<2X10^{-6}$	$<2X10^{-6}$
Experiment:					
pRS10+pRS16	*rexA*$^-$-*rexB*$^+$	$<2X10^{-6}$	0.04	0.19	0.47
pRS10+pRS17	*rexA*$^+$-*rexB*$^-$	$<2X10^{-6}$	0.01	0.06	0.40

[a] Transformants of *E. coli* strain R594.
[b] plaques were tiny.

Figure 1

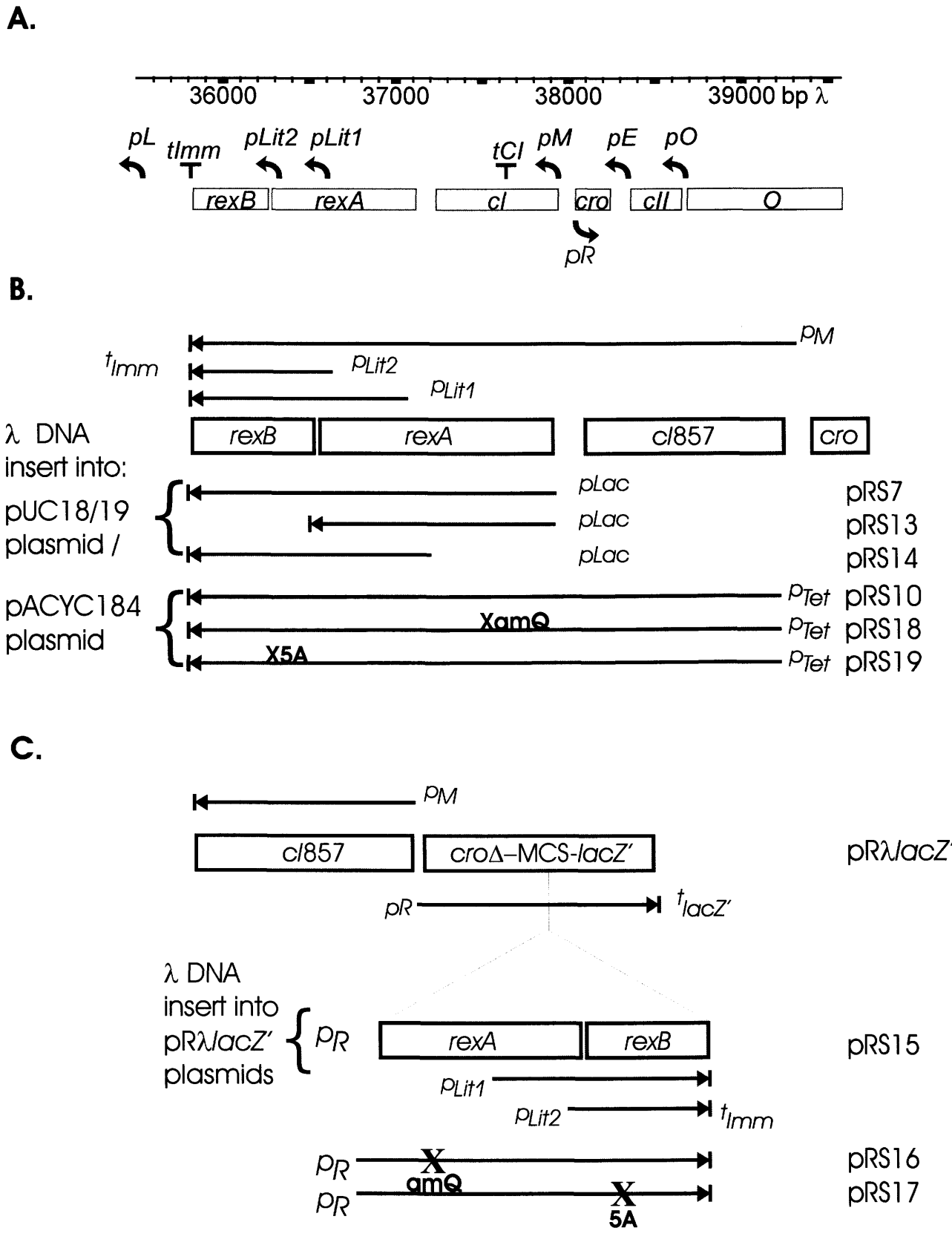
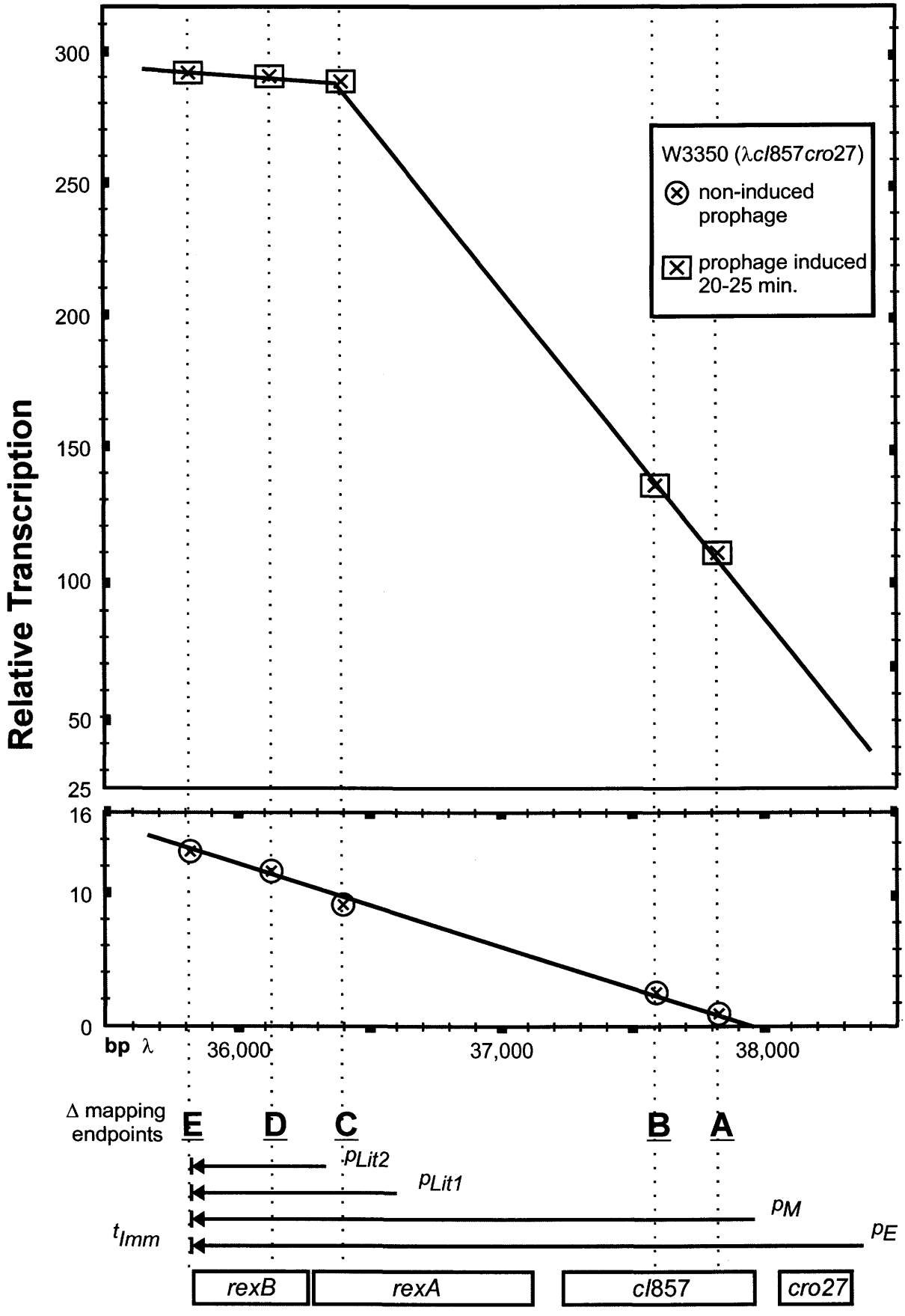

A.
36000
37000
38000
39000 bp λ
pL
tImm
pLit2
pLit1
tCI
pM
pE
pO
rexB
rexA
cI
cro
cII
O
pR
B.
pM
tImm
pLit2
pLit1
λ DNA
insert into:
rexB
rexA
cI857
cro
pUC18/19
plasmid /
pLac
pRS7
pLac
pRS13
pLac
pRS14
pACYC184
plasmid
pTet
pRS10
XamQ
pTet
pRS18
X5A
pTet
pRS19
C.
pM
cI857
croΔ–MCS-lacZ′
pRλlacZ′
pR
tlacZ′
λ DNA
insert into
pRλlacZ′
plasmids
pR
rexA
rexB
pRS15
pLit1
pLit2
tImm
pR
amQ
pRS16
pR
5A
pRS17

Figure 2


Relative Transcription
300
250
200
150
100
50
25
16
10
0
W3350 (λcI857cro27)
non-induced prophage
prophage induced 20-25 min.
bp λ
36,000
37,000
38,000
Δ mapping endpoints
E
D
C
B
A
pLit2
pLit1
pM
pE
tImm
rexB
rexA
cI857
cro27

CHAPTER SEVEN

Bacteriophage λ Repressor Allelic Modulation of the Rex Exclusion Phenotype.

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Key words: bacteriophage lambda (λ); CI repressor; Rex exclusion phenotype; *cI-rexA-rexB* operon; bacteriophage λspi156*nin*5; *cI*[Ts]857; *cI*[Ts]2

Running title: Lambda Repressor Allelic Modulation of the Rex Exclusion Phenotype.

7.1 Abstract

Bacteriophage λ Δ(*red-gam*) Δ*ren* mutants are sensitive to the Rex exclusion phenotype encoded by λ genes *rexA, rexB* expressed from a repressed prophage. We show that the sensitivity of these λ mutants is modulated by the *cI* repressor allele of the prophage. λspi156*nin*5 forms plaques with 10^5 fold higher efficiency on a cI^+-$rexA^+$-$rexB^+$ lysogen than on *cI*[Ts]857, or *cI*[Ts2] derivatives. The enhancement in Rex exclusion exhibited by the *cI*[Ts] lysogens is suppressed by complementation with a cI^+ plasmid.

7.2 Bacteriophage λ Repressor Allelic Modulation of the Rex Exclusion Phenotype.

The Rex exclusion system exhibited by cells lysogenic for bacteriophage λ restricts the growth of *rII* mutants of phage T4, as well as various derivatives of T5, T7 and λ (Benzer, 1955; Molineaux, 1991). The Rex phenotype is encoded by the *rexA-rexB* genes (Matz et al., 1982), which are co-expressed with the λ *cI* repressor from the P_M-*rexA-rexB*-t_{imm} operon in an established prophage (Landsmann et al., 1982). RexB protein is proposed to form an inner membrane pore that is opened upon direct interaction with at least two RexA proteins (Parma et al., 1992). According to this model phage infection somehow results in the disruption of Rex stoichiometry, favouring RexA, leading to the activation of the RexB pore and causing a lethal membrane depolarization event. We have shown that the *rex* genes function to protect lysogenic cells against T4*rII* infection rather than sacrifice the host, termed Rex-centric mutualism (Slavcev and Hayes, 2002). Rex-mediated cessation of macromolecular synthesis (Snyder and McWilliams, 1989) and growth arrest (Slavcev and Hayes, 2002) can also be induced by plasmid over-expression of *rexA*, relative to *rexB*, in the absence of infection. Lambdoid phages mutated for *ren* or *red* (*exo bet*) genes are sensitive to Rex exclusion to varying degrees (Toothman and Herskowitz, 1980b) and the restriction of phage growth is augmented by the presence of the *cI*[Ts]857 repressor allele (Toothman and Herskowitz, 1980a). However, the *rexB-rexA* genes can be expressed from a multicopy plasmid lacking *cI* repressor and confer a Rex exclusion phenotype (Shinedling et al., 1987). The modulation of the Rex phenotype by CI remains

unexplained.

We found that λspi156*nin*5 phage was as sensitive to Rex exclusion (expressed by a λ*cI*857 lysogen) as the indicator phage T4*rII*. In λspi156*nin*5, gene *ren* is removed by nin5 deletion and *exo-bet-gam* are removed by the spi156 substitution. Using λspi156*nin*5 we investigated repressor modulation of Rex activity, asking: i) whether *cI*[Ts]857 allelic augmentation of Rex activity was dominant, or recessive to cI^+, and ii) whether repressor modulation of Rex activity was solely dependent upon the *cI*857-*rexA-rexB* genes encoded by the immunity region of repressed λ*cI*857 prophage.

We used derivatives of *Eco* K strains of *E.coli* K-12: R594 F^- *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1 λ^- (Bachmann, 1987) to prepare lysogens. λ wild type, λ*imm*434*nin*5, λ*cI*[Ts]857 and λ*cI*[Ts]2 were from our stocks [#271, #28', #142 and #711 respectively]; λspi156*nin*5 was from G. Smith (1975); λ*rexB*5A, λ*cI*857[Ts]*rexB*5A, λ*rexA*30A, and λ*cI*857[Ts]*rexA*Qam[allele301] preparations were from G. Gussin via W. Szybalski, as was λ*rex*go293 [likely a *rexB* mutant since it mapped (Gussin and Peterson, 1972) in the same interval left of *bio*16-3 endpoint within *rexB* (Daniels et al., 1983) as the *rex*5A mutation] [1]. Variant cI^+ rex^- phages were obtained by crossing λ*cI*sus14 with the λ*rex cI*857 recombinants and selecting for plaques at 42°C. We distinguished the *cI*[Ts] and cI^+ variants of λ$rexB^-$

[1] The *rex* mutants were obtained by Gussin and Peterson (1972) using strain W3350A(λ*N*am7am53*cI*857*O*Δ58) [=WNQ8] as the rex^+ parent. WNQ8 was then mutagenized to give rex^- derivatives. They produced the λ *cI*857*rex* phage by marker rescue of the imm^{λ} region of the WNQ8 *rex* mutants by infection with λimm^{434}.

, and λ*rexA*$^-$ phage mutants by preparing lysogens and confirming thermoinducibility. The mapping of the *rex*$^-$ mutants was described by Gussin and Peterson (1972), Gussin et al. (1973) and Landsmann et al. (1982). Phage T4*rII*Δ1589 (deletion fusing the *rIIA* and *rIIB* genes that confers a RIIB$^+$ phenotype) was obtained from G. Mosig.

Plasmid pACYC184 was from New England Biolabs. pRS1 was constructed by digesting λwt (NEB) with *Bgl*II and ligating λ DNA 38,103 bp through 35,711 bp into pACYC184 *tet*^{R}, digested with *Bam*HI at 1,869 bp. pRS1 carries λ P_M-*cI*$^+$-*rexA*-*rexB*-t_{imm} downstream from promoter for *tet*^{R}. pRS2 was constructed by digesting pCH1 (Hayes et al., 1997) with *Bgl*II and ligating λ DNA 38,103 bp through 35,711 bp into pACYC184 *tet*^{R}, digested with *Bam*HI at 1,869 bp. pRS2 carries λ P_M-*cI*857-*rexA*-*rexB*-t_{imm} downstream from promoter for *tet*^{R}. pRS3 was constructed by digestion of pRS2 with *Mfe*I, removing λ DNA 35,764 bp through 37,186 bp, then religating. pRS3 carries λ P_M-*cI*$^+$ downstream from the promoter for *tet*^{R} from pACYC184. pUC18 was from New England Biolabs. pRS4 was constructed by digestion pRS2 with *Bst*YI, yielding λ fragment 38,103 bp through 35,711 bp and and ligation of λ fragment 38,103 bp through 35,711 bp (including genes P_M-*cI*-*rexA*-*rexB*-t_{imm}) into the MCS *lacZ'* of pUC18 (*Bam*HI site at 486 bp). pRS4 carries λ DNA with the orientation P_{lacZ}-P_M-*cI*-*rexA*-*rexB*-t_{imm} ($P_{lacZ'}$ promoter from pUC18). pRS11 was constructed by digestion of pRS4 with *Mfe*I, removing λ DNA 35,764 bp through 37,186 bp, then religating. DNA was extracted from λ wild type, and the λspi156*nin*5 mutant and the region upstream of λ gene *O* was sequenced by the protocol described with Sequenase kit (version 2.0, United States Biochemical

Corp.). In essence, 1-5 ug of each phage DNA was mixed with 1 pmol primer R21 (37,756 5'-TCTGCCACATTACGCTCC-OH 38,737 from λ O gene) plus Sequenase buffer from kit and made up to 10 ul with H_2O. The mixture was heated at 94°C for 5 min to denature λ DNA and then chilled on ice for 5 min to enable primer hybridization. Labeling mix was added including 0.1M dithiothreitol, labeling nucleotide mix from kit, αP^{32}ATP and Sequenase II. The mixture was incubated 3 min at room temperature and added into four termination mixes (ddGTP, ddATP, ddTTP, ddCTP). The solution was incubated 5 min at 37°C, 4 μl stop solution (kit) was added, heated 5 min at 94°C and run on a 6% acrylamide gels.

Rex exclusion phenotypic activity of lysogenic culture cells with various *cI* alleles was measured. (Table 1). The relative e.o.p. for λspi156*nin*5, λ*imm*434*nin*5 and T4*rII*Δ1589 phages was determined by dividing the titer on the assayed culture cells by the titer obtained in parallel on the permissive host cells R594. For each assay, the cultures were grown overnight at 30°C. Assays were performed at 35°C by first transferring 0.1 ml culture aliquots (1-2 X 10^8 CFU) to a 35°C water bath, then adding 0.1 ml of 10^1 to 10^6 fold dilutions of phage lysates: λspi156*nin*5, λ*imm*434*nin*5, or T4*rII*Δ1589. The mixtures were held for about 5 min and then 3 ml TB top agar (TB plus 6.5g Bacto agar/liter) was added and mixture poured onto TB bottom agar plates (TB plus 11g Bacto agar and 1 mg thiamine·HCl / liter) pre-warmed to the assay temperature. The plates were incubated at the assay temperature for 20 hours.

Phage λspi156*nin*5 (Smith, 1975) is insensitive to CI repression due to the replacement of λ genes *int* through *cII* with *E. coli* DNA, but it remains sensitive to

the Rex exclusion phenotype (Hayes and Hayes, 1986). We sequenced 202 bp of λspi156*nin*5 which revealed that the right substitution endpoint falls between the λ *cII* and *oop* genes. Theoretically, λ phages deleted for the rightward promoter P_R will be defective in plaque formation. The adjacent *E. coli* sequence was localized to the *moaA* chlorate resistance gene mapping at 17.6 min. on the chromosome, with the left end-point representing base 333 downstream from the ATG start. According to the DNA packaging restrictions of λΔ*nin*5, the spi156 substitution could carry up to 0.2 min (about 1.3 X 10^4 bp) of *E. coli* DNA including the *bioA-D-uvrB-moaA* genes. The substituting DNA must provide an alternate promoter for P_R, in order to enable expression of the downstream λ replication genes *O-P*. The *E. coli* sequence bg/AE000181.1/AE000181 reveals both sigma-70 and sigma-54 promoters within 170 bp of the *moaA* AUG translational start site (see Fig. 1B legend) , one or both of which may transcribe the downstream λ genes. One of the *moaA* promoters is modulated by the product of *modE* (McNicholas et al., 1997).

The influence of CI repressor on Rex phenotypic activity is shown in Table 1. The sensitivity of λspi156*nin*5, λ*imm*434nin5 and T4*rII* to the Rex exclusion phenotype is shown by comparing the results in lines 13 and 14 (*cI* [Ts] Rex^+ prophages) with lines 17 and 18 (*cI* [Ts] Rex^- prophages). When the *cI* [Ts] alleles were replaced by cI^+ (line 8) the Rex exclusion phenotype toward lambdoid phages λspi156*nin*5 and λ*imm*434nin5 was lost. Nevertheless, the cI^+ lysogen retained the classically defined Rex exclusion phenotype toward T4*rII* (showing that the cI^+ lysogen was $rexA^+$ $rexB^+$). Next, we sought to determine the effect of mixing cI^+ and *cI* [Ts] repressor alleles on the Rex exclusion phenotype. Plasmid pRS3

encoding the *cI*$^+$ allele was added to a *cI*857 [Ts] prophage (line 16), and plasmid pRS11 encoding the *cI*857 [Ts] allele was added to a *cI*$^+$ prophage (line 10). The addition of *cI*$^+$ suppressed the inhibition of plating for λspi156*nin*5 and λ*imm*434*nin*5 on a *cI*857 [Ts] lysogen (line 14). Whereas, the addition of *cI*857 [Ts] allele on plasmid (line 10) did not alter plating efficiency seen on a *cI*$^+$ lysogen (line 8). We conclude that a *cI*$^+$ allele is dominant to a *cI*857 [Ts] allele and can suppress the Rex exclusion of lambdoid phage plating. We observed that nonlysogenic cells possessing a low-copy plasmid encoding the λ P_M-*cI*857-*rexA*-*rexB*-t_{imm} region (line 4) did not show Rex exclusion toward lambdoid phages as found for *cI* [Ts] lysogens (lines 13,14). Therefore, we conclude that the observed Rex exclusion of lambdoid phages shown by *cI* [Ts] prophages requires, in addition to *rexA*-*rexB* function(s), the expression of a λ DNA sequence lying outside of the P_M-*cI*857-*rexA*-*rexB*-t_{imm} gene interval (line 4) from the prophage genome.

Due to the poor growth of λspi156*nin5* on nonlysogenic cells at 30°C and 40°C, we conducted plating experiments at 35°C where both the λ*cI*[Ts]857 and λ*cI*[Ts]2 lysogenic culture cells maintained high plating efficiency and could form ordinary cell lawns. In agreement with the results of Toothman and Herskowitz, (1980a, 1980b), we found that λ phages mutated for the *ren* and *red* genes varied in their sensitivity to Rex exclusion. Our results showed: i) Rex exclusion of λspi156*nin5* requires that the λ lysogenic host possess intact *rexA*-*rexB* genes; ii) augmentation of Rex exclusion activity by the *cI*[Ts]857 allele is repressed by *cI*$^+$; and iii) *cI*857 augmentation of Rex exclusion activity requires, in addition to *rexA*-*rexB* function(s), the expression of a λ DNA sequence lying outside of the P_M-

cI857-rexA-rexB-t_{imm} gene interval from the prophage genome.

We propose the following model to account for the observed CI allelic modulation of Rex activity. Leaky transcription from P_R arising from λ*cI*[Ts] results in some expression of *cro-cII-O-P* at 35°C (Bull, 1995), but should not in cI^+ lysogens. The expression of *cII* at 35°C could stimulate CII-dependent transcription of *cI-rexA-rexB* from the P_E promoter (Echols and Green, 1971; Eisen et al., 1970), thus increasing the level of *cI*[Ts]-*rex* expression/activity. This hypothesis may also account for why λ*cI*[Ts]857 and λ*cI*[Ts]2 lysogens are viable at 35°C, even though the DNA binding capacity of CI857 is too low to measure (Mandal and Lieb, 1976). Over-expression of *rexA-rexB* genes from a multicopy plasmid has been reported to augment Rex exclusion activity, restricting growth of λ*nin*5 and λ*ren*51 derivatives, but not λ (Ren^+) wild type (Shinedling et al., 1987). How the *ren* and *red* genes of λ confer protection to the phage against Rex exclusion remains to be determined.

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7.4 Figure Legend

Figure 1. Substitution endpoint in λspi156*nin*5. Data/Figure provided by S. Hayes. The *nin*5 deletion (not shown) removes about 5.5% λ including part of *ren*, downstream from *P*, thus conferring phage sensitivity to Rex exclusion. Restriction mapping (not shown) revealed that the left arm of λ and λspi156*nin*5 were equivalent, with the *left* substitution end-point lying in a 408 bp region between *Hin*dIII site at 27,479 bp λ and *Ava*I site at 27,887 bp λ, straddling the *att*λ site at 27,731 bp λ; and the *right* substitution endpoint between AvaI cut site at 38,214 and BglII site at 38,814. (In the drawings λ DNA is indicated by heavy solid line, and *E. coli* DNA by dashed line.) The precise sequenced right substitution endpoint (to the left of λ base 38,558) is shown to fall within the distal end of *cII*, and just downstream of *oop*. The adjacent *E. coli* sequence was localized to the *moaA* gene, with the left end-point representing base 333 downstream from the ATG start. The small bases positioned above bases shown represent nucleotides present in the *E. coli* gene bank *moaA* sequence that did not appear as bands on our gels. The effects of the observed base changes on *moa* gene function was not determined.

Table 1. CI Repressor Modulation of Rex Exclusion Activity.

Host cells[a]	Efficiency of Plating Phage [b]		
	λspi156*nin*5[c]	λ*imm*434*nin*5	T4*rII*Δ1589
1. Nonlysogen R594	1.0	1.0	1.0
2. [pACYC184]	0.9	1.0	1.0
3. [pRS1] {*cI*$^+$-*rexA*$^+$-*rexB*$^+$}	0.1	0.4	$<10^{-6}$
4. [pRS2] {*c*I857-*rexA*$^+$-*rexB*$^+$}	0.2	0.3	$<10^{-6}$
5. [pRS3] {*cI*$^+$}	0.2	n.d.	1.0
6. [pRS11] {*c*I857}	0.1	n.d.	1.0
7. (λ *imm*434)	0.4	n.d.	1.0
8. (λ *cI*$^+$)	0.1	0.5	$<10^{-6}$
9. (λ *cI*$^+$) [pUC18]	0.1	0.7	$<10^{-6}$
10. (λ *cI*$^+$) [pRS11]	0.2	0.6	$<10^{-6}$
11. (λ*rexB*5A *cI*$^+$)	0.3	0.3	0.6
12. (λ*rexA*30A *cI*$^+$)	0.2	0.4	0.5
13. (λ*c*I[Ts]2)	$<10^{-6}$	0.006	$<10^{-6}$
14. (λ*c*I[Ts]857)	$<10^{-6}$	0.003	$<10^{-6}$
15. (λ*c*I[Ts]857) [pACYC184]	4.5×10^{-6}	0.9	$<10^{-6}$
16. (λ*c*I[Ts]857) [pRS3]	0.1	0.7	$<10^{-6}$
17. (λ*rexB*5A *c*I857)	0.9	0.3	0.5
18. (λ*rexA*amQ[301] *c*I[Ts]857)	0.6	0.2	0.3

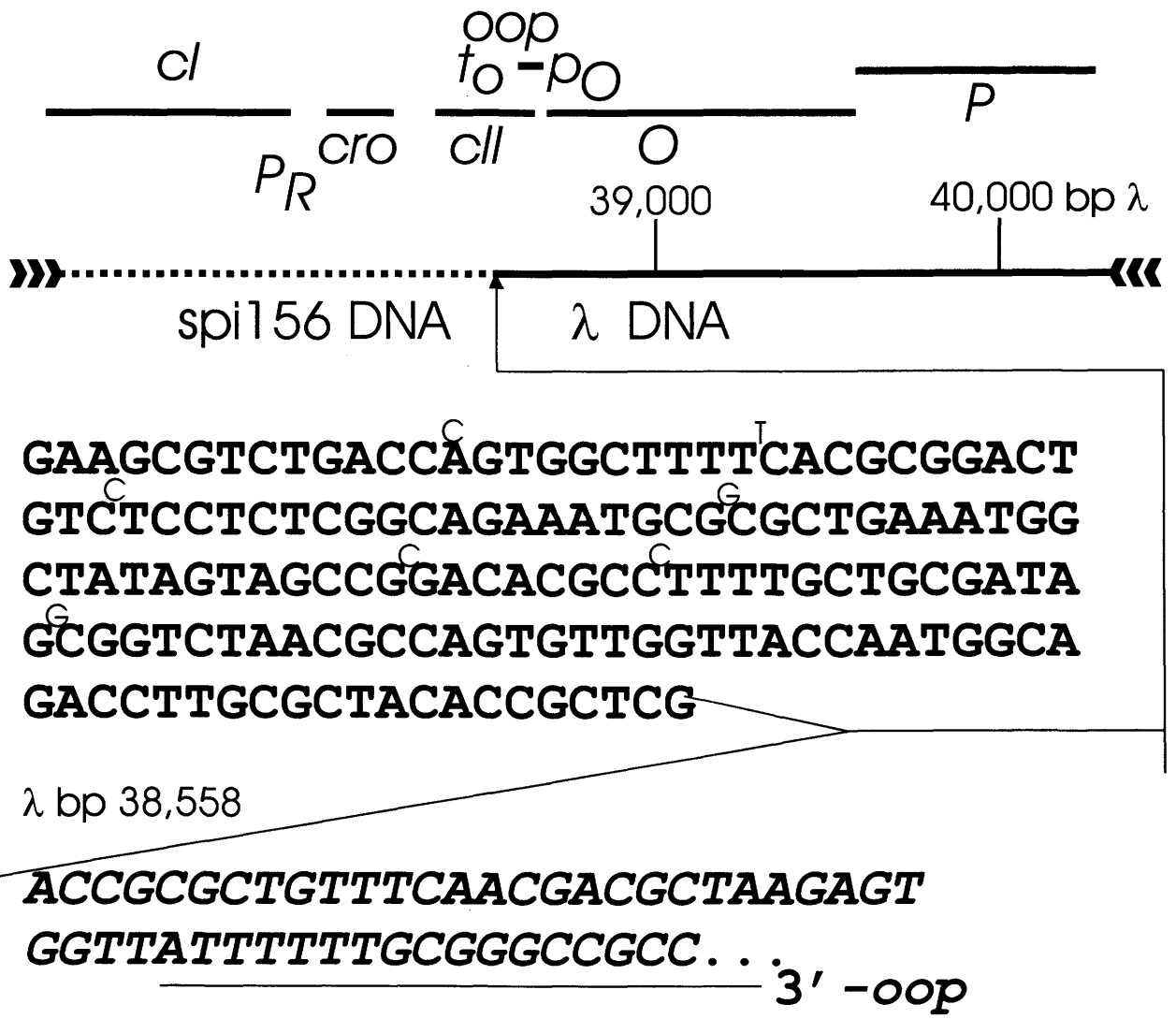
Plasmids are denoted by square brackets "[ ]"; plasmid inserts are denoted by squiggly brackets "{ }"; prophages are denoted by parentheses "( )".
n.d. not done.

[a] Strain R594 was used as nonlysogen and host for making lysogens.

[b] The efficiency of plating (e.o.p.) is expressed relative to PFU on host strain R594 at 35°C.

[c] Phage λspi156*nin*5 forms tiny, irregular plaques, which appeared larger and circular on the R594(λ*rexB*go293), R594(λ*rexB*5A *c*I857) and R594(λ*rexA*amQ[301] *c*I857) hosts.

Figure 1


cI
oop
t_O - p_O
P
P_R
cro
cII
O
39,000
40,000 bp λ
spi156 DNA
λ DNA
GAAGCGTCTGACCAGTGGCTTTTCACGCGGACT
GTCTCCTCTCGGCAGAAATGCGCGCTGAAATGG
CTATAGTAGCCGGACACGCCTTTTGCTGCGATA
GCGGTCTAACGCCAGTGTTGGTTACCAATGGCA
GACCTTGCGCTACACCGCTCG
λ bp 38,558
ACCGCGCTGTTTCAACGACGCTAAGAGT
GGTTATTTTTTGCGGGCCGCC...
3′ -oop

CHAPTER EIGHT

Conclusions and Future Directions

8.1 Rex-Centric Mutualism and Rex Exclusion Phenotype

Bacteriophage λ confers about 50% viability to a lysogenized host against T4*rII* infection at MOI 10, termed Rex-centric mutualism (Slavcev and Hayes, 2002). This protection is mediated by the *rexA-rexB* genes, expressed from the prophage and requires intact host *rpoS* stationary phase sigma factor (Chap. 3, Table 1). To ensure that T4*rII* titers remain identical for Rex^+ and Rex^- cell infections, viability experiments could be repeated using a T4*rII23*am double mutant. Using a replication defective *rII* mutant would ensure that no viable phage production occurs following infection of Rex^- strains. However, the onset of Rex exclusion has been noted to coincide with the initiation of T4 replication and thus, mutation of the replication capacity of T4*rII* may subvert the exclusion phenotype. The actual adsorption rates of T4 to *E. coli* K-12 strains is mediated by the availability of OmpC on the outer membrane, which was not determined for the salt conditions we utilized. To circumvent this problem, infections should be reproduced in *E. coli* B strains that adsorb T4 to LPS much more efficiently than to OmpC.

Infection of log-phase rex^+ λ lysogenic cells with T4*rII*, or transformation of nonlysogenic cells with a multicopy rex^+ (pHBRex) plasmid imparts changes in cellular morphology that resemble *E. coli* cells that are in or are entering, stationary phase. These cells: 1) are arrested for cell growth, 2) exhibit cellular aggregation, 3) are contracted is size, and 4) express flagellar structures (Chap. 3, Fig's. 2,3). Infection of a rex^+ lysogen with T4*rII* also results in the loss of cellular membrane

potential and loss of proton motive force (Parma et al., 1992) and energy (Colowick and Colowick, 1983). Collectively, these results suggest that the Rex system is activated, or triggered, but it is unclear whether these Rex-mediated cellular manifestations (Rex-centric mutualism) are also required for the Rex phage exclusion phenotype.

We argue that the λ *rex* genes temporarily shunt cells into a physiological state resembling stationary phase. The significance of this change in cell physiology is that T4-infection of cells in a post-exponential growth phase are inhibited for cell lysis and do not support the growth of T4 (Delbruck, 1940; Heden, 1951). I showed an involvement of *rpoS* in Rex-centric mutualism, but I was not able to show that σ^S participated in the Rex exclusion phenotype (Chap. 3, Table 4). These findings suggest that Rex exclusion is not conferred solely by a cellular shift to a stationary phase-like state, but the protective effect of Rex requires expression of stationary phase genes. Other workers preliminary data also suggest that stationary phase cells may remain viable for several days following T4 infection. It is quite possible that RpoS is required for cellular recovery following the triggering of a Rex exclusion phenotypic event, although the two phenotypes may be mutually exclusive.

Several questions remain to be answered regarding Rex-centric mutualism and the Rex exclusion phenotype:

1) Do the *rex* genes shunt cells into stationary phase?

Although host RNA and DNA synthesis are reported to cease following T4*rII* infection of a λ Rex^+ lysogen, growth arrest is temporary and is reported to

resume 15 minutes after initial infection (Sauerbier et al., 1969). Ideally, since an appreciable level of lysogenic cell viability was found following T4*rII* infection, a microchip array of all host genes expressed following infection and "activation" of the *rex* genes could resolve whether a number of stationary phase genes participate in Rex-centric mutualism. Alternately, a simple plasmid construct placing *lacZ* under a stationary phase promoter can be used to test for β-galactosidase activity in a Δ*lacZ* *rex*$^+$ lysogenic host following infection of T4*rII*.

2) What genes impart the cellular manifestations seen following T4*rII* infection of *rex*$^+$ lysogenic cells and are they required for the Rex phenotypic exclusion of phage?

Colowick and Colowick (1983) reported that the *rex* genes impart a loss of cellular ATP following infection with T4*rII*. This drop in cellular energy was shown to require the host *unc* (ATPase) genes. However, they reported that the *rex*$^+$ lysogenic *unc* mutants retained the Rex exclusion phenotype. In addition, we have shown that *fliC*, encoding the structural component of flagella (flagellin) encodes the flagellar structures secreted by T4*rII* infected *rex*$^+$ lysogenic cells and is not involved in the Rex exclusion of T4*rII* (Chap. 3, section 4.3; Fig's. 2,3). The genes responsible for the contracted cellular morphology and cellular aggregation of T4*rII* infected *rex*$^+$ lysogenic cells remain to be identified. The *bolA* gene confers a spherical morphology to stationary phase *E. coli* cells (Lange and Hengge-Aronis, 1991), and thus may also be involved in Rex-mediated contraction of cell size . This hypothesis

can be tested easily by transduction of a *bolA*::Tn*10* mutation into *rex*$^+$ lysogenic cells. The *bolA* mutants can then also be tested for retention of Rex exclusion phenotypic activity.

3) Can *rexA-rexB* over-expression confer protection to host cells against T4[wt] infection?

The Rex system has been criticized as being artificial, since T4*rII* mutants are not normally found in nature. However, the level of *rex* gene expression in naturally occurring strains of *E.coli* cells lysogenized by lambda is unknown and the over-expression of *rex* genes restricts growth of not only Rex-sensitive mutants, but also wild type phage such as T4, T5, T6, T7 (Shinedling et al, 1987). However, it remains to be tested whether the exclusion of T4 by *rexA-rexB* over-expression is accompanied by the Rex-centric mutualism phenotype. This question can be answered by assaying the viability of host cells carrying a multicopy *rexA-rexB* plasmid following infection by T4.

Determining whether Rex-centric mutualism and the classical Rex exclusion phenotype (Benzer, 1955) are in fact synonymous will require learning if host mutations that suppress Rex-centric mutualism can also abrogate the Rex exclusion phenotype. The isolation of Rex$^-$ host mutants has proven to be a very difficult task since *rexA* and *rexB* mutants would also be collected. Rolfe and Campbell (1977) reported that the *tolA, tolB, tolP* genes, required for colicin sensitivity and filamentous phage infection, are required for Rex exclusion phenotypic activity. We tested a *rex*$^+$ λ lysogenic *tolB*2 mutant (strain A593 from *E. coli* Genetic Stock Centre), but did not note a Rex$^-$ phenotype. However, we were

also unable to confirm colicin E2 resistance in this strain.

To isolate host mutations that block Rex exclusion and also maintain a clean genetic background, the *rex* genes could be inserted into a transposon mutagenesis cassette on a suicide plasmid and transposed along with the antibiotic resistance marker. CFU that exhibit a Rex^- phenotype may arise due to the insertion of the transposon into a host gene involved in Rex exclusion. Using primers to the known transposon sequence, the interrupted gene can then be sequenced and identified. This approach is limited to isolating nonessential host genes required to support Rex exclusion.

8.2 RexA:RexB Stoichiometry and Rex Activation

Stoichiometric balance between the Rex proteins is essential to maintaining a Rex^+ phenotype. We have reproduced the previously reported esults of Parma et al. (1992) who showed that the over-expression of *rexB* to *rexA* abrogates Rex phenotypic exclusion of T4*rII*. In contrast, Snyder et al. (1989) showed that Rex-mediated cessation of macromolecular synthesis can be induced by over-expressing *rexA* relative to *rexB* and they suggested that infection of a rex^+ lysogen by T4*rII* results in RexA over-expression and "activates" the Rex exclusion phenotype. Parma et al. (1992) proposed that two or more cytosolic RexA proteins interact directly with RexB thereby activating the putative RexB pore and causing the loss of cellular membrane potential.

Apart from phenotypes reported herein (Chap's. 3,4,6) very little is known about RexA. No significant homologies to other proteins, or gene sequences are

apparent, but the primary structure suggests that RexA is hydrophilic and thus may reside in the cytoplasm. We have shown that *rexA* over-expression in the presence of RexB causes a prolonged growth arrest (Chap. 3, Tables 2,3) and abrogates Rex exclusion phenotypic activity (Chap. 6, Tables 2,4). In addition, we have shown that over-expression of *rexB* relative to *rexA* also abrogates Rex phenotypic exclusion (Chap. 6, Tables 2,4), and that RexB overexpression confers the translocation of cytoplasmic protein to the periplasm and can suppress holin mutations (Chap. 4, Tables 3,4). We showed that these activities of RexB are inhibited by RexA. Schoulaker-Schwarz et al. (1991) and Engelberg-Kulka et al. (1998) showed that RexB inhibits Hsp100 ClpPX and ClpPA protease activity. However, it is unknown whether RexB inhibits ClpPX, ClpPA activity by direct interaction, or by serving as a substrate for degradation.

Based on previous findings and the results obtained herein, we propose this model to explain the mechanism of the Rex exclusion phenotype:

Infection of a Rex^+ lambda lysogen by T4*rII* somehow results in the disruption of RexA:RexB stoichiometry in the cell, resulting in an elevated RexA:RexB ratio, which forms a RexB-$(RexA)_n$ complex. RexB-$(RexA)_n$ forms a channel spanning the inner and outer membranes of the host and causes a disruption of the ionic balance across the inner membrane and the loss of proton motive force. The ionic osmotic imbalance stabilizes stationary phase sigma factor σ^S, stimulating the transcription of stationary phase genes. The RIIA, RIIB proteins of T4 wild type interact with RexB to inhibit formation of the active RexB-$(RexA)_n$ complex, thus suppressing the Rex exclusion phenotype.

This model makes several assumptions that need to be verified.

1) Do the RexA and RexB proteins interact?

Although the potential interactions can be tested by genetic assays such as SIP (selectively infecting phage) technology (Spada et al., 1997), or by a yeast/bacterial two hybrid assay (Chien et al., 1991), the question of RexA:RexB stoichiometry could be investigated by radioimmunoprecipitation assay (RIPA). Employing antibodies against RexA, and RexB, we could determine where RexA resides in the host by testing different cellular fractions of Rex^+ lambda lysogens before and after infection by T4*rII*. This assay would determine whether RexB interacts with RexA, both before and after infection of a Rex^+ host by T4*rII*.

2) Is the Rex Exclusion Phenotype activated?

Infection of a lambda Rex^+ lysogen by T4*rII* confers the following changes in cellular morphology and physiology: cellular contraction, cellular aggregation, secretion of flagellar structures, and loss of membrane potential. These phenotypes support a model where Rex is triggered, or activated. Parma et al. (1992) proposed that RexA functions as a "sensor" for phage infection, but it was unclear how activation was accomplished. RexA over-expression relative to RexB results in the cessation of macromolecular synthesis (Snyder and McWilliams, 1989) and prolonged growth arrest identical to that seen upon infection of a rex^+ lambda lysogen by T4*rII* (Slavcev et al., 2002). These finding suggest that an imbalance in RexA:RexB stoichiometry serves as the

trigger of Rex-mediated cellular manifestations rather than formation of a chemically modified "activated" form of RexA induced by T4*rII* infection as intimated by Snyder and McWilliams (1989). Chemical modifications of RexA, or RexB such as phosphorylation can be assayed by using radiolabeled ^{32}P and testing the incorporation of host phosphorous into RexA, or RexB before and after infection of a *rex*$^{+}$ lambda lysogen with T4*rII*. RexA or RexB may also be subject to proteolytic processing. This question can be tested by Western Blot using anti-RexB, and anti-RexA antibodies. One would assess the gel mobility of RexA and RexB found in non-infected and T4*rII*-infected lambda lysogens. This technique could also be used to determine whether RexB is a substrate of ClpPX and ClpPA proteases by assaying RexB degradation in wild type and *clpP* mutant cells.

3) Do the RIIA, RIIB proteins of T4 interact with RexA and/or RexB?

We noted that the *rII* genes of T4 reverse the inhibition of the T4 lysis inhibition (LIN) phenotype by *rexB* in a gene dosage-dependent manner (Chap. 4, Fig. 3). How the RII proteins inhibit this activity of RexB remains unexplored. Our model proposes that the RII proteins suppress Rex exclusion phenotypic activity by interacting with RexB and interfering with RexB function.

The RIIA plus RIIB proteins of T4 are believed to be toxic to *E. coli*. Thus, potential interactions between the RIIA and RIIB proteins and between the RII proteins and Rex proteins remains to be determined. However, using a conditional *rIIA-rIIB* expression system in *E. coli*, or a two-hybrid system in yeast may circumvent this problem. Furthermore, the potential interaction

between RexB and the RII proteins may relieve RII toxicity to the cell. If the *rII* genes could be cloned into a cell carrying a multicopy *rexB* plasmid, this would argue that RexB inhibits RII activity, possibly through direct interaction. A biochemical approach should also be employed to assay potential protein interactions. Anti-RexA, anti-RexB, anti-RIIA and anti-RIIB antibodies can be generated by passage of the purified protein through a rabbit and collecting the polyclonal antibodies. Using anti-RexA, anti-RexB, anti-RIIA and anti-RIIB antibodies a Western blot assay, or RIPA can be conducted to test for protein-protein interaction(s) between the RIIA and RIIB proteins, and between the RII proteins and RexA, or RexB. The RII proteins and RexB localize to the inner membrane of the cell (Weintraub and Frankel, 1972; Takacs and Rosenbusch, 1975; Parma et al., 1992). Thus, the membrane fraction of the host cell would need to be isolated in order to test for potential interactions between these proteins.

4) Does infection of a lambda Rex^+ lysogen by T4*rII* result in the over-expression of RexA relative to RexB?

Parma et al. (1992) proposed that RexA over-expression results in the activation of the putative RexB pore and this event in turn activated the Rex exclusion phenotype. They suggested that T4*rII* somehow induces the disruption in RexA:RexB stoichiometry. We noted that Rex^+ lambda lysogens infected by T4*rII* at MOI 10 exhibited similar cellular viabilities to Rex^+ lysogens transformed with a multicopy *rexA* plasmid (Chap. 3, Table 3). In addition, both these treatments conferred a prolonged growth arrest to the

cells (Slavcev and Hayes, 2002). These findings strongly suggest that T4*rII* infection of a lambda *rex*$^+$ lysogen disrupts RexA:RexB stoichiometry resulting in an elevated RexA:RexB ratio. To test this hypothesis, radioimmunoprecipitation of total cellular protein with anti-RexA and anti-RexB antibodies can be employed. If RexA and RexB interact, then by co-precipitation, the active RexA, RexB or RexA-RexB stoichiometric complex form(s) might be revealed by analysis of RexB:RexA band intensity upon running precipitated proteins on a gel. In order to determine whether T4*rII* induces a distortion of RexA:RexB protein stoichiometry the assay needs to be compared with T4-infected cells and noninfected cells.

8.3 The Conditional Rex Exclusion Phenotype

The *cI-rex* operon of bacteriophage λ is expressed from the P_M maintenance promoter of the prophage as P_M-*cI-rexA-rexB-*t_{imm} message and confers the Rex exclusion phenotype to the lysogen. We noted that replication and excision defective, cryptic λ*cI*857[Ts]*cro*27 lysogens exhibited a conditional Rex[Ts] phenotype. At repressor-permissive temperatures for the *cI*[Ts]857 allele, *rexA-rexB* expression from P_M conferred full Rex activity (Chap. 5, Table 1). However, upon thermal inactivation of the repressor little or no Rex exclusion was observed, despite a much higher level of transcription from P_E. The same conditional Rex phenotype was observed in cells harbouring a low copy plasmid encoding a P_{Tet}-P_M-*cI*857-*rexA-rexB-*t_{imm} fragment, but not with a *cI*$^+$ derivative plasmid, that imparted a Rex$^+$ phenotype. A testable model was generated by Hayes and Bull

(1999) in order to explain these findings:

1) Is CI repressor involved in the translation of the *cI-rexA* message?

Hayes and Bull (1999) postulated that the lambda CI repressor is involved in the translation of its own message as well as that of *rexA*. They proposed that CI prevents -1 frameshifts at three potential slippery heptamer (AAAAAAG) sites in *cI* and downstream *rexA*. To test this hypothesis constructs can be made that fuse *gfp* (green fluorescence protein) in all three frames to λ DNA following each of the -1 frameshift sites. Cells that fluoresce under UV light indicate in-frame translation of downstream *gfp*. This experiment can be conducted in conjunction with an inducible *cI* plasmid to determine if CI is indeed involved in preventing frameshifting at the slippery heptamers.

The Rex exclusion phenotype has proven to be one of the most enigmatic phenomena in the history of genetics. This work represents our endeavour to understand the mystery of the bacteriophage lambda Rex exclusion phenotype and attests to the intricate complexity and exquisite elegance of phage genetic design. To work with phage is to develop an insurmountable respect for the nature of these organisms that deservedly have achieved reproductive success unrivaled by that of any other life form.

8.4 References

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Appendix 1. Bacterial and Bacteriophage Strains

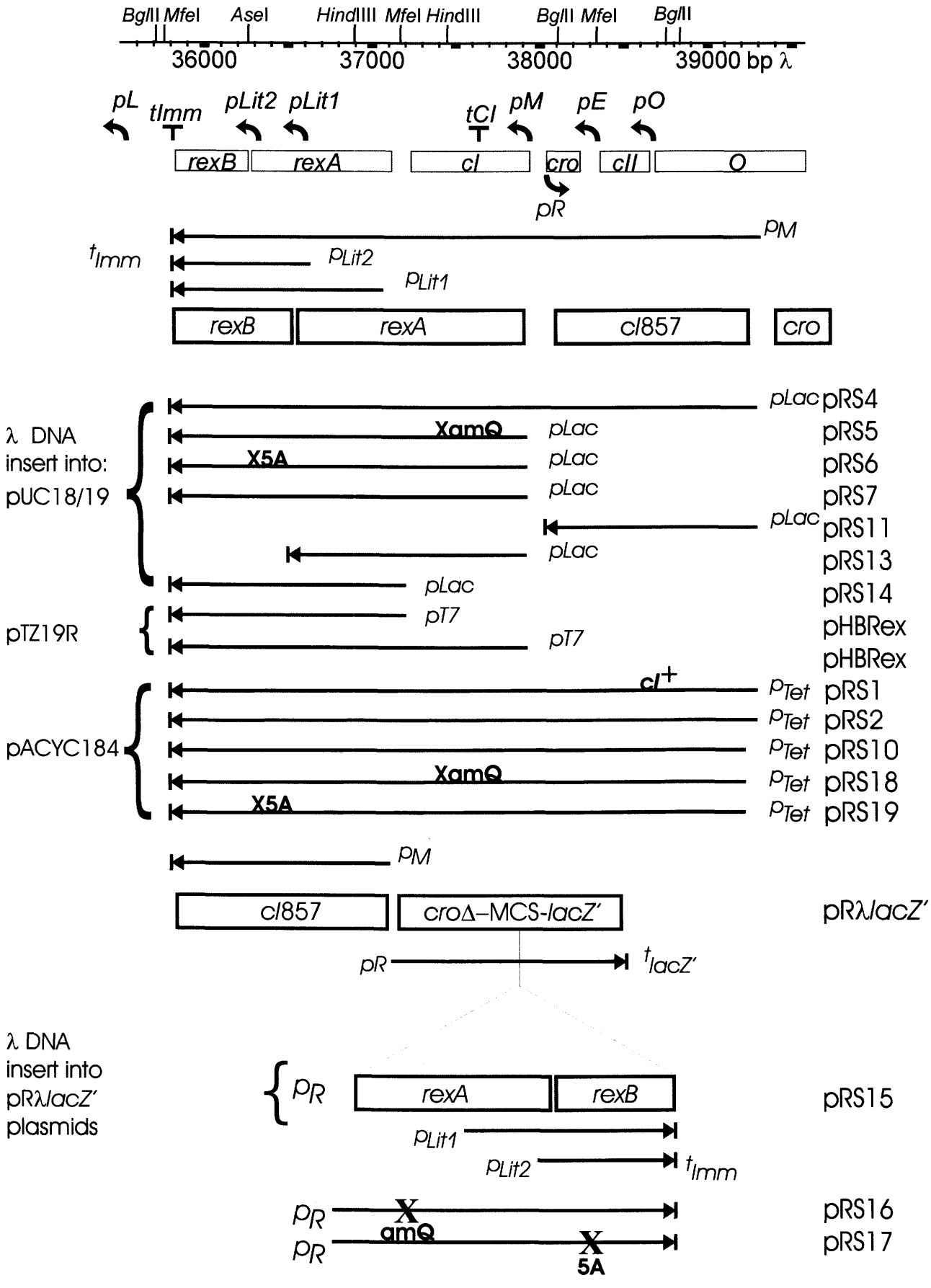
Designation	Characteristics/Genotype	Source
***E. coli* K**		
C600	*thr*-1 *leuB*6 *fhuA*21 *lacY*1 *glnV*44 *e*14$^-$ *glpR*200 *thi*-1	Bachmann, 1987, p.34
HD173	*thr*-33 *trpE*9829 *tyrA*15 *thyA*707 IN(*rrnD-rrnE*)1 *ilv*-683 *rho*702[Ts] *argH*212	*E. coli* Genetic Stock Center via M. Berlyn, p. 123
JM101	F' *traD*36 *lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/*supE thi* Δ(*lac-proAB*)	Bachmann, 1987, p.34
M72(λ*N*am7,53*cI*857ΔH1)	defective in replicative killing$^-$ (RK$^-$). ΔH1 removes prophage DNA between *cro-J*b of λ and through *chlA* of host	Greer, 1975, p. 148
MC4100	F$^-$ *araD*139 Δ(*argF-lac*)169 *flhD*5301 *fruA*25 *relA*1 *rpsL*150 *rbsR*22 *deoC*1	Bachmann, 1987, p. 34
R594	F$^-$ *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1	Bachmann, 1987, p. 34
SA500	F$^-$ *his*-87 *relA*1 *strA*181 *tsx*-83	Bachmann, 1987, p. 34
SA297	SA500(λ*cI*857Δ297) Δ297 removes *cII-J*b of λ through *chlA* of host	Dove et al., 1971, p. 147
TC600	*thr*-1 *leuB*6 *fhuA*21 *lacY*1 *glnV*44 *e*14$^-$ *glpR*200 *thi*-1 *supE*44	Hayes collection (from C600, Bachmann, 1987) strain # B8, p. 172
W3101	*galT*22 IN(*rrnD-rrnE*)1	Bachmann, 1987, p. 34

W3350A	F⁻ *lac*-3350 *galK*2 *galT*22 IN(*rrnD-rrnE*)1	Bachmann, 1987, p. 34
Y-mel	F⁺ *mel*-1 *supF*58 (*tyrT*58, su⁺)	Bachmann, 1987, p. 34
SA500(λ*bio*275*cI*857[Ts]Δ431) replicative killing defective (RK⁻) mutant:		
Y870-3	rep$^{\lambda-}$ 870 (initiation of λ replication defective)	Hayes, 1986, p. 95
100a	*P*100a::IS2	Hayes et al., 1998, p., 150
SA500(λ*bio*275*cI*857[Ts]*cro*27Δ431) RK⁻ mutants:		
101b	*P*101b	Hayes et al., 1998, p. 150
SA500(λbio10cI857[Ts]cro27Δ431) RK⁻ mutants:		
141e	*O*141e::IS2	Hayes, 1998, p. 150
145c	*P*145c::IS2	Hayes, 1998, p. 150
145d	*P*am145d	Hayes, 1998, p. 150
***E. coli* B**		
EMG31	unknown	Luria and Delbruck, 1943, p. 96
ATCC11303	unknown	American Tissue Culture Collection, p. 77
L. Gorini	harbours a P2 related cryptic prophage	*E. coli* Genetic Stock Center; source unknown, p. 77
Phages		
λpapa	wild type bacteriophage λ*rex*⁺	S. Hayes Laboratory,

		stock #271, p. 30
λ*rexB*go293	λ *rexB*$^-$-*rexA*$^+$	Matz et al., 1982, p. 23
λ*rexB*5A	λ *rexB*$^-$-*rexA*$^+$	Matz et al., 1982, p. 23
λ*rexA*30A	λ *rexB*$^+$-*rexA*$^-$	Matz et al., 1982, p. 23
λ*cI*857	λ *cI*[Ts]*rex*$^+$	S. Hayes Laboratory, stock #142, p. 202
λ*cI*857*rexA*amQ	λ *cI*[Ts]*rexB*$^+$-*rexA*[CDL]	Matz et al., 1982, p. 23
λ*cI*857*rexB*5A	λ *cI*[Ts]*rexB*$^-$-*rexA*$^+$	Matz et al., 1982, p. 23
λ*cI*857*S*am7	amber mutation of λ *S* holin	S. Hayes Laboratory, stock #759, p. 77
λ*cI*857*cro*27	λ *cI*[Ts] *cro*$^-$	Hayes, 1979, p. 149
λ*cI*72	λ *cI*$^-$	S. Hayes Laboratory, stock #510, p. 122
λ*cI*[Ts]2	λ *cI*[Ts]	S. Hayes Laboratory, stock #711, p. 202
λ*cII*68	λ*cII*$^-$	S. Hayes, Laboratory stock #152, p. 122
λvir	λ $O_R^-O_L^-$ mutations in rightward and leftward operators	S. Hayes Laboratory, stock #556, p. 123
λspi156*nin*5	λ *bio* substitution Δ(*red gam*); Δ(*ren-orf*221)	Smith, 1975, p. 211
λ*imm*434T	λ *rex*$^-$; immunity region of 434 substituted for λ	S. Hayes Laboratory, stock #539, p. 77

λ*imm*434*nin*5	λ immunity region of 434 Δ(*ren-orf*221)	S. Hayes Laboratory, stock #28, p.202
λ*imm*434*cII*2002*S*am7	immunity region of 434, *cII*⁻, amber mutation in *S*	S. Hayes Laboratory, stock #873, p. 77
λ*N*am7,53*imm*434	*N*[CDL] antiterminator; immunity region of 434	S. Hayes Laboratory, stock #410, p. 123
ϕ80*imm*λ*cI*857	phage ϕ80 with λ immunity region; *rexA*⁺-*rexB*⁺	R. Slavcev, this work, p. 77
ϕ80*imm*λ*cI*857*rexA*amQ	*rexB*⁺-*rexA*[CDL]	R. Slavcev, this work, p. 77
ϕ80*imm*λ*cI*857*rexB*5A	*rexB*⁻-*rexA*⁺	R. Slavcev, this work, p. 77
T4D	wild type T4	G. Mosig, p. 30
T4*rIIA*	*rIIA*⁻-*rIIB*⁺	G. Mosig, p. 30
T4*rII*Δ1589	Δ(*rIIA-rIIB*)	G. Mosig, p. 30
T4*t*A3	amber mutation of *t* holin	G. Mosig, p. 30

Appendix 2. Plasmid Maps



Appendix 3. Abbreviations

434	bacteriophage 434.
Δ*nin*5	deletion of λ DNA between *ren-orf*221.
φ80	bacteriophage φ80.
λ	bacteriophage lambda.
a.a	amino acid.
ATP	adenosine triphosphate.
Bc	*E. coli* B L. Gorini strain cured of a P2-related cryptic prophage.
bio	*E. coli* biotin operon. Illegitimate recombination during λ prophage excision can result in substitution of λ genes to the right of att^{λ} for *E. coli bio* DNA (eg. λ*bio*275 = *bio* substitution for λ *int-kil*.
cI	λ repressor.
cII	λ transcriptional stimulator of high-level transcription from λ P_E establishment promoter.
CdI-Rex	Conditional Rex exclusion Phenotype; temperature sensitive Rex exclusion phenotype—Rex^+ at 30°C and Rex^- at >39°C.
CFU	colony forming unit.
clpA	Hsp100 chaperone; interacts with ClpP to form ClpPA that

	recognizes specific C-terminal peptide tags for degradation.
clpB	Hsp100 chaperone involve in unfolding of damaged proteins; may interact with ClpP.
clpP	Hsp100 protease; requires complexing with ClpA or ClpX in order to recognize substrates for degradation.
clpX	Hsp100 chaperone; interacts with ClpP to form ClpPX that recognizes specific C-terminal peptide tags for degradation.
cro	λ repressor binds to O_R and O_L operator sequences and inhibits CI stimulation of P_M transcription.
cya	AMP cyclase; required for production of cyclic AMP.
e	T4 endolysin; degrades the cellular proteoglycan layer and lyses the cell.
Eco B	*E. coli* restriction modification system B.
Eco K	*E. coli* restriction modification system K.
Fec	ability of λ phage to plate on a *recA* mutant host due to the expression of λ *exo bet gam* genes.
fliC	*E. coli* flagellin-structural unit of flagella.
gam	confers λ protection against Exo V degradation.

hflA	Hsp70 (*hflC-K*) modulate activity of essential protease *hflB.* Modulates degradation of λ CII protein
himA	host integration factor A subunit; involved in stationary phase gene regulation and integration of λ into the host chromosome.
ilr	initiation of λ replication; requires intact λ *O P* genes and itn^4 (iteron) sequences.
IS*2*	insertion sequence *2.*
lac	*E. coli* operon comprised of *lacZ-Y-A* genes and required for cellular metabolism of lactose.
lacZ	β-galactosidase; enzyme that hydrolyses lactose.
LIN	T4 lysis inhibition phenotype; phage inhibition of host lysis in response to adsorption of secondary T-even phage.
lrp	leucine-responsive regulatory protein; global regulatory DNA-binding protein.
mazEF	*E. coli* apoptotic module in response to amino acid starvation; addiction module where MazE antidote is unstable and MazF toxin is stable.
MOI	multiplicity of infection; phage:cell ratio.
N	λ antiterminator; binds to *nutR* and *nutL* sites on λ mRNA. Required for bypass of t_R and t_L transcriptional terminators.

O	λ initiation of replication; O binds iteron (*itn*^{4}) sequences.
oop	λ functional RNA that attenuates Rex exclusion phenotype when expressed from multicopy plasmid.
P	λ initiation of replication; P binds O prebound to iteron (*itn*^{4}) sequences.
P1	bacteriophage P1; used for transduction of markers from donor to recipient *E. coli* strains.
P_E	λ high-level establishment promoter; stimulated by CII.
PFU	plaque forming unit.
P_L	λ leftward promoter; repressed by CI binding at O_L operator.
P_{Lit}	λ late immunity transcription promoter; located within the C-terminal of *rexA*, transcribes *rexB*.
P_M	λ low-level maintenance promoter; stimulated by CI by a repressed prophage.
PMF	proton motive force across the inner membrane of the cell.
P_R	λ rightward promoter; repressed by CI repressor binding at O_R operator.
PTA	phosphotungstic acid; negative stain employed for visualization of

	cells by electron microscopy.
rI	T4 probable periplasmic protein that may negatively regulate holin activity; essential in the establishment of lysis inhibition.
rIIA-rIIB	T4 genes that confer to the phage the ability to escape Rex exclusion by a λ lysogen.
rV,t	T4 holin gene provides an inner membrane lesion through which the endolysin egresses.
red	λ *exo bet* general recombination genes
Rex-Centric Mutualism	ability of *rex* genes to protect λ lysogens against T4*rII* infection.
Rex exclusion	exclusion of T4*rII* mutant phage by λ lysogen.
relA	ribosome dependent (p)ppGpp synthetase I (or stringent factor).
rexA-rexB	λ genes that confer exclusion of T4*rII* to the lysogen.
ren	λ gene allows the phage to escape its own exclusion system.
rho	*E.coli* mRNA hexameric helicase that dislodges RNA polymerase from the DNA and mRNA from the RNA polymerase. Required for efficient termination at Rho-dependent terminators.
RK	replicative killing phenotype; cryptic λ prophage trapped in chromosome initiate replication upon prophage induction. The

	event is lethal to the host.
rpoS	σ^S stationary phase sigma factor.
r-type	rapid lysis plaque morphology generated by T4 mutants that are incapable of establishing lysis inhibition.
S	λ holin gene; provides a lesion for egress of phage lysozyme to the periplasm.
Spi	sensitivity of λ phage to plating on a P2 lysogen due to the expression of λ *exo bet gam* genes.
spoT	ribosome-independent (p)ppGpp 3'-pyrophosphohydrolase-(p)ppGpp synthetase II.
ssrA	10Sa RNA; tags peptides stalled in translation with the 11 a.a. degradation signal AANDENYALAA.
t, rV	T4 holin gene provides an inner membrane lesion through which the endolysin egresses.
T4	bacteriophage T4.
Tn*10*	transposon that confers Tetracycline resistance.
Ts	conditional mutation rendering the gene product temperature sensitive.
tsp	tail specific protease; peripalsmic endoprotease that recognizes hydrophobic C-terminal residues of periplasmic proteins.

unc *E. coli* genes encoding ATPase.