Isolation and Characterization of Host mutations that Supress the Bacteriophage Lambda (λ) Rex Phenotype

by

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The Bacteriophage lambda (λ) T4r*II* exclusion (Rex) phenotype is defined as the inability of T4*rII* mutant bacteriophage to form plaques on a lawn of *E. coli* lysogenized by bacteriophage λ. More than six decades have passed following the discovery of Rex by Seymour Benzer in 1955, yet the mechanism behind this elusive exclusion system remains a mystery. The Rex system is encoded by two genes of λ (*rexA*, and *rexB*), the expression of which, is primarily regulated by the repressor gene *cI* from the P_M promoter. The onset of the Rex phenotype, somehow triggered by T4*rII* infection of a Rex^+ lysogen, results in rapid membrane depolarization and a harsh cellular environment that in many ways resembles the stationary phase in metabolism and morphology. In addition, the disruption of the RexA:RexB balance, particularly the over expression of *rexA* to *rexB*, can lead to same manifestations without infection, indicating that stoichiometry of RexA:RexB is important. Despite some cell killing, infected lysogens can to some extent recover from Rex activation. The phenotype may thus be a mutualistic protection mechanism that protects both itself and the host cell from external super-infection. In this study, I have designed a system for the rapid one-step isolation of host mutations that attenuate this phenotype, in order to identify the host genes involved in Rex and elucidate the mechanism of this enigmatic exclusion system. In so doing, I was able to isolate 13 host mutations of *E. coli* K-12 that abrogate the Rex phenotype while simultaneously identifying for the first time several outer membrane protein genes including: *ompA*, *ompF*, *ompW*, and *ompX* that are essential in imparting Rex activity. In analyzing these *omp* mutants it was noted that several of these identified *omp* candidates play a critical role in cellular osmotic balance and the onset of stationary phase. This work substantiates a model whereby the onset of Rex shunts cells into a temporary stationary phase-like state that inhibits T4*rII* phage growth.

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λ	Bacteriophage lambda
λimmF	λ immunity region forward sequence primer
λimmR	λ immunity region reverse sequence primer
λF7	Synonymous with *λDam15imm21cIts* that possesses a defective (suppressible) *D* gene and the immunity region (*imm*) of phage 21
λCI857	λCI[Ts]857; temperature sensitive as a prophage; higher temperatures are indicated by a reduction in repressor activity and expression of the downstream *rex* genes
λ*cI*[Ts]*rex*$^+$	λ*cI*857; temperature sensitive repressor
σ^E	subunit of RNA holoenzyme encoded by *rpo*E gene; activated in response to stressful environments such as hyperosmotic shock, metal ion exposure, and changes in outer membrane lipopolysaccharide structure, governs transcriptional activation of many downstream genes including the periplasmic folding proteins and degrading factors
a.a.	Amino acid
Ap^R	Ampicillin antibiotic resistance marker (β -lactamase). Cells grow in the presence of ampicillin antibiotic
Ap^S	Cells are sensitive to ampicillin antibiotic and cannot grow in the presence of ampicillin antibiotic in the growth medium
ATP	Adenosine triphosphate
bp	Base pair
cfu	Colony forming unit; number of viable cells per unit volume
CI	Lambda repressor, allows phage to reside in a lysogenic state

cI	λ *cI* repressor gene
CII	Lambda transcriptional stimulator of high-level transcription from λ P_E promoter
Cro	Lambda repressor binds to O_R and O_L operator sequences and inhibits CI stimulation of P_M transcription
clpP	Hsp100 protease that requires complexing with ClpX or ClpA in order to recognize substrates for degradation
cro	Lambda repressor binds to O_R and O_L operator sequences and inhibits CI stimulation of P_M transcription
Da	Dalton; atomic mass unit
E	T4 endolysin encoded by the *e* gene of T4; degrades the cellular proteoglycan layer and lyses the cell
EOP	Efficiency of plating; the relative viability of phage sample in comparison to a positive control
F^+ plasmids	The prototype conjugative plasmid associated with conjugation in a strain of *E. coli*. Also called fertility or F factor.
Gene l	Gene *l* of bacteriophage T5; mutant of gene *l* is sensitive to Rex-mediated exclusion
Gene 20	Mutants in gene *20*, a new T7 gene, cannot grow on *rex+* λ lysogens; Gene *20* works as *rII* genes of T4 to grow on λ lysogen
gpE	λ gene product E, a major structural protein in the λ phage capsid head

h	Hour unit
Hfq	Protein encoded by the *hfq* gene and acts as a repressor of mRNA translation
Iac	*E. coli* operon comprised of *Iac*Z-Y-A genes and required for cellular metabolism of lactose
*Iac*Z	β-galactosidase; enzyme that hydrolyses lactose
imm	Immunity region of bacteriophage P_M-*cl-rexA-rexB-timm*
IPTG	Isopropyl β-D-1-thiogalactopyranoside, a molecular biology reagent.
IS10	A component insertion sequence of transposon, Tn*10*
IU	International unit
K	Kilo unit; 1000 times
Km^R	Kanamycin antibiotic resistance marker; cells are not inhibited by the presence of kanamycin antibiotic
Km^S	Cells are sensitive to kanamycin antibiotic and cannot grow in the presence of kanamycin antibiotic in the growth medium
LIN	T4 **l**ysis **in**hibition phenotype; phage inhibition of host lysis in response to adsorption of secondary T-even phage
MCS	Multiple cloning site, a short segment of DNA within a plasmid containing numerous restriction sites that are typically used during molecular cloning or sub-cloning procedures

micA	Small RNAs, besides *rybB,* expressed under the control of σ^E-directed transcription down-regulate *ompA, ompC* and *ompW*
motA	Called *rim* gene (**R**ex **Im**munity gene); The T4 modulation of transcription gene; mutation in the *motA* gene reverses exclusion of T4 by *rex*
MOI	Multiplicity of infection; ratio of infective phage per bacterial cell
MW	Molecular weight unit; pure atomic weight
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 interon (*int*[4]) sequences
O/N	Overnight
Omp	Outer membrane protein; specific for gram negative bacteria; a complex that among other roles regulates cellular osmolarity and nutrient uptake
O_L	λ leftward promoter; regulates P_L
O_R	λ rightward operator; regulates P_R.
P_E	Establishment promoter of λ; stimulated by CII
oop	λ functional RNA that attenuates Rex exclusion phenotype when expressed from multicopy plasmid
phoA fusion	**A**lkaline **pho**sphatase is encoded by the *phoA* gene in *E. coli*. The *phoA* gene has a signal-sequence allowing export of alkaline phosphatase into the periplasm where it is

	active; due to the highly reducing environment, alkaline phosphatase is not active in the cytoplasm
pfu	Plaque forming unit; single immobilized phage that grows to form a plaque on an agar cell lawn. A means of calculating lysate titer and EOPs
P_L	λ leftward promoter; repressed by CI binding at O_L operator
P_{Lit}	λ **l**ate **i**mmunity **t**ranscription promoter; located within the C-terminal of *rexA*, transcribes *rexB*
P_M	λ low-level maintenance promoter; stimulated by CI in a repressed prophage
P_R	λ right-ward promoter; repressed by CI binding at O_R operator
R6K	Antibiotic-resistance plasmid in *E .coli*; its origin requires the π replication protein for initiation of plasmid replication
red	λ *exo bet* —general recombination genes
ren	λ Rex suppressor gene
Rex phenotype	Exclusion of T4*rII* mutant phage by λ *rex*$^+$ lysogen
rexA-rexB	λ genes that confer exclusion of T4*rII* to the λ lysogen
Rex-centric mutualism	Ability of *rex* genes to protect λ lysogens against T4*rII* infection
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-terminators.
rI	Encodes a T4 periplasmic protein that inhibits T holin activity; essential in the establishment of lysis inhibition

rII (rIIA-rIIB)	T4 genes that confer to the phage the ability to escape Rex exclusion by a λ lysogen
rIII	A T4 gene has not yet been characterized, but is hypothesized to provide a stabilizing effect during lysis inhibition that influences the interaction between *rI* and gene *T*
rIV	A T4 gene product that is hypothesized to aid T4 during host infection by repairing the broken membrane during T4 adsorption and DNA injection
r-type plaque	**R**apid lysis plaque morphology is large and sharp-edged generated by T4*r* mutants that are incapable of establishing lysis inhibition
RT	Room temperature
RPM	Revolutions per minute
rpoE	RNA polymerase, a minor sigma subunit (σ^E) *E. coli* response element, activated in response to stressful environments such as hyperosmotic shock, metal ion exposure, and changes in outer membrane lipopolysaccharide structure
rybB	Small RNAs , like *micA,* expressed under the control of σ^E-directed transcription down-regulate *ompA*, *ompC* and *ompW*
rV, *t*	T4 holin gene provides an inner membrane lesion through which the endolysin egresses
S	λ holin gene, provides a leasion for egress of phage lysozyme to the periplasm
SraD RNA	Known as MicA RNA, a small non-coding RNA that its expression is inhibited in stationary phase; it binds the Hfq protein and regulates levels of gene expression by an antisense mechanism
Tc^R	Tetracycline antibiotic resistance marker; confers growth to cells in the presence of tetracycline antibiotic

Tc^S	Cells are sensitive to tetracycline antibiotic and cannot grow in the presence of tetracycline antibiotic in the growth medium
t, rV	T4 holin gene provides an inner membrane lesion through which the endolysin egresses
T4	Bacteriophage T4
T5	Bacteriophage T5
T7	Bacteriophage T7
T-even	Family of phages with similar characteristics. Includes T4, T2, and T6
t_{imm}	λ terminator genetic element
Tn*10*	Transposon possessing IS10 insertion elements for transposition that confers tetracycline resistance to recipients
Ts	**T**emperature-**s**ensitive
tsp	**T**ail **s**pecific **p**rotease; periplasmic endoprotease that recognizes hydrophobic C-terminal residues of periplasmic proteins
unc	*E. coli* genes encoding ATPase
wt	Wild-type

CHAPTER ONE

1. Introduction

1.1. Introduction to the T4*rII* Exclusion Phenotype:

1.1.1. The Foundation of Rex Phenotype:

The Rex phenotype is defined as the ability of *rex* genes (*rexA, rexB)* of bacteriophage lambda (λ) to prevent mutant bacteriophage T4*rII* plaque formation on *E. coli* lysogenized by λ (λ lysogen). The discovery of the T4*rII* exclusion phenotype (Rex Phenotype) dates back to 1955 when Seymour Benzer found that while the wild-type T4 bacteriophage was capable of generating plaques on *E. coli* (λ) lysogens, whereas T4*rII* mutants could not (Benzer, 1955). Using a plaque-morphology-based genetic selection Benzer was able to genetically map the *rIIA* and *rIIB* genes of bacteriophage T4; this work played a very important role in our current understanding of homologous recombination and our understanding of the triplet nature of the genetic codon (Benzer, 1959; 1962). By using this unique exclusion phenotype thousands of T4*rII* mutants were isolated and confirmed in recombination experiments, examining frequency of rII^+ recombinants. Although the Rex system proved powerful enough to exclude *rII* mutants at a genetic reversion frequency of $\leq 10^{-8}$, Benzer did find that some of the isolated *rII* mutants were capable of forming tiny plaques on *E. coli* lysogens, suggesting that some residual RII function remained in these "leaky" mutants. Noting a large degree of cell death upon infection of λ lysogens by T4*rII* he concluded that Rex was a cell-killing mechanism (Benzer, 1959; 1962).

In line with Seymour Benzers' initial observations, Parma et al. (1992) presented the Rex phenotype as an altruistic cell death system, whereby Rex-mediated death would be "triggered" following infection of the host lysogen by a Rex-sensitive phage, such as T4*rII*. By this model, Rex as an a population defense mechanism directs infected lysogens to sacrifice their host, suggesting that the λ prophage would rather kill itself than to permit the propagation of a competitor phage on its infected host population (Maclean et al.,1974). This altruistic death model, put forth by Parma et al. (1992) offered the only mechanistic and phenomenological explanatory mechanism for Rex until the new millennium. Since, Slavcev et al. (2001; 2003) proposed that Rex shunts cells into stationary phase—a harsh reversible cellular environment that is associated with some cell killing, but one that also prevents propagation of the superinfecting phage.

1.1.2. The *rex* Genes and Other Immunity Regulators of Bacteriophage Lambda:

Rex Genes: The *rexA* and *rexB* genes are genes encoded within the immunity (*imm*) region of bacteriophage λ (Fig. 1). This operon consists of the *cI* repressor and *rexA*-*rexB* genes flanked by the left- and the right-ward operators (O_L, O_R) that bind the CI repressor and regulate rightward and leftward expression of all remaining λ genes by conditionally occluding the two strong left-ward (P_L) and right-ward (P_R) promoters (Heinemann and Spiegelman, 1970; Echols and Green, 1971; Meyer et al., 1980; Li and Susskind, 1994).

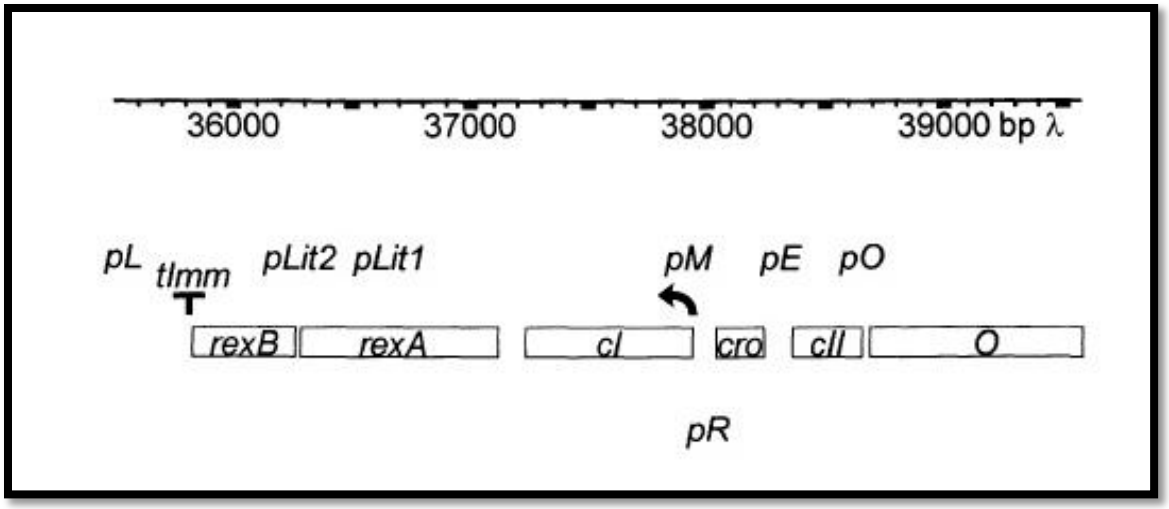

Figure 1: Immunity Region of Phage λ and Maintenance of Lysogeny. Lambda repressor CI functions by binding to the operators O_L and O_R, and overlaps promoters P_L and P_R. P_E is the promoter for repressor establishment located in *cII* gene and P_M, the promoter for repressor maintenance overlaps O_R. Binding CI to O_R serves to block transcription from P_R and stimulate transcription from P_M; rexA and *rexB* are co-transcribed with *cI*. Source: Slavcev, R. (2005)

The nucleotide sequence of the λ P_M-*cI*-*rexA*-*rexB*-t_{imm} operon is a 2,112 bp locus located from 37,940 to 35,828 bp of the 48,512 bp λ genome. Promoter P_M is positioned at λ 37,940 bp, where the transcription in a repressed λ prophage proceeds from P_M through the terminator t_{imm} at 35,804 bp (Hayes and Szybalski, 1973; Daniels et al., 1983) (Fig. 2).

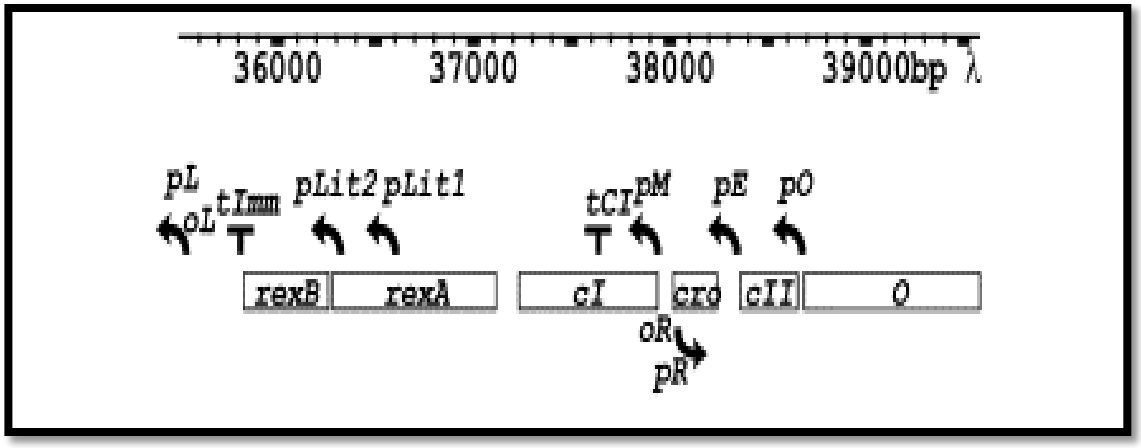

Figure 2: A Portion of Immunity Region from Phage λ: Operator sites O_L and O_R, which when bound by the CI repressor, prevent leftward and rightward transcription from promoters P_L and P_R. The terminator t_{imm} stops transcription originating from the P_{Lit} promoters for *rexB*, *cI* maintenance transcription from promoter P_M or vigorous *cI-rexA-rexB* transcription from the *cI* establishment promoter P_E. Source: Hayes et al. (2010)

Lysogeny and Rex: Bacteriophage λ can undergo both lysogenic and lytic cycles of replication upon infection, and the λ immunity region in addition to the λ stimulation of lysogeny protein regulate which cycle is established. Upon delivery of the λ phage DNA into an uninfected host, P_R expression occurs immediately conferring *cII* expression. The CII protein is an unstable, but very powerful stimulator of expression from the establishment promoter (P_E) that provides a burst of *cI-rexA-rexB* transcript (Echols and Green, 1971). This mRNA burst promotes lysogeny and is essential in the lysis-lysogeny decision of the phage upon infection (Echols and Green, 1971). Establishment of lysogeny is accompanied by prophage DNA integration into the bacterial chromosome (Echols and Green, 1971) (Fig. 3).

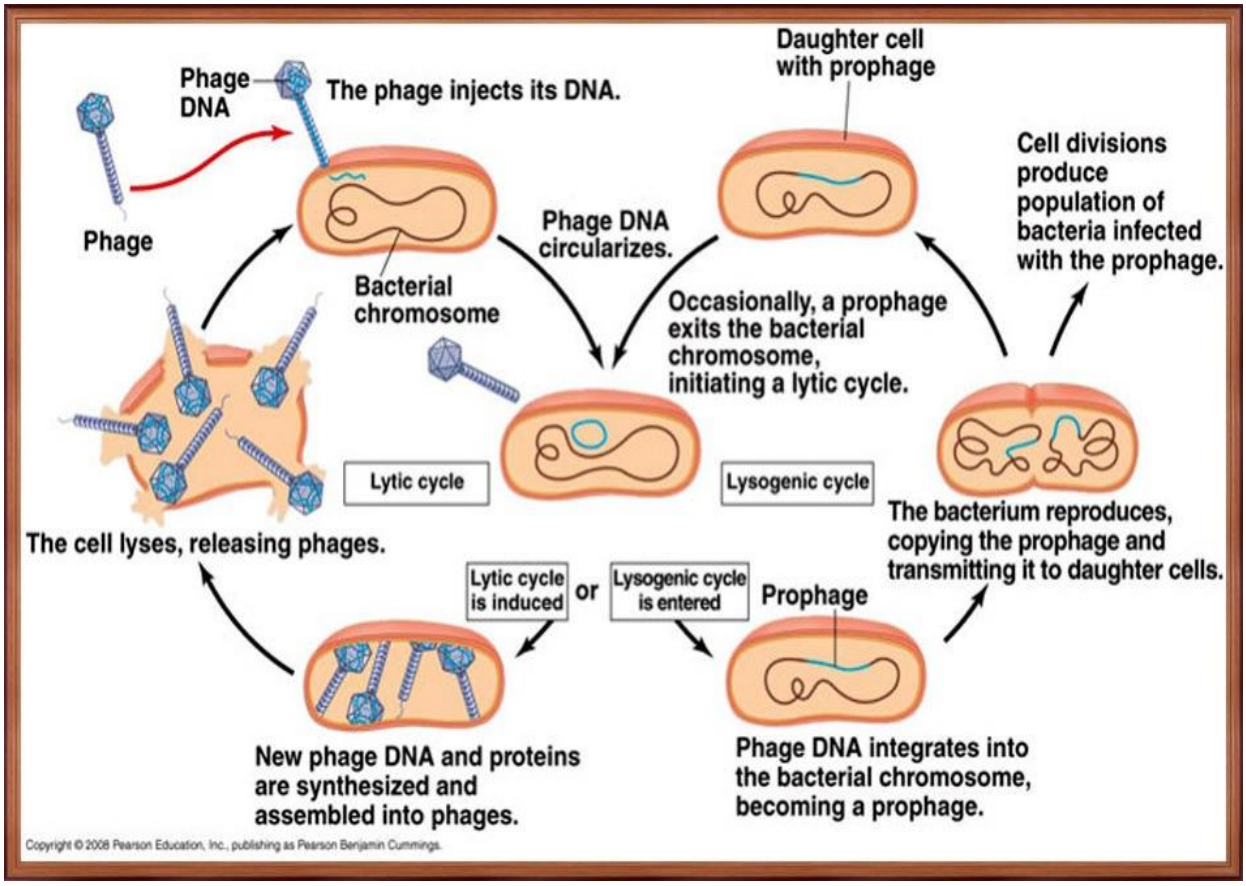

Figure 3: Lytic-Lysogenic Replication Cycles of Bacteriophage λ. Source: Reece et al., 20011, pg.386

To impart a lysogenic state, the repressor maintenance promoter (P_M) is stimulated by the binding of repressor CI to the O_R resulting in low level (lysogeny "maintenance" level) transcription through the *cI-rexA-rexB-*t_{imm} operon, conferring repressor production (Fig. 4) and a Rex^+ "ready" state (Heinemann and Spiegelman, 1970; Meyer et al., 1980).

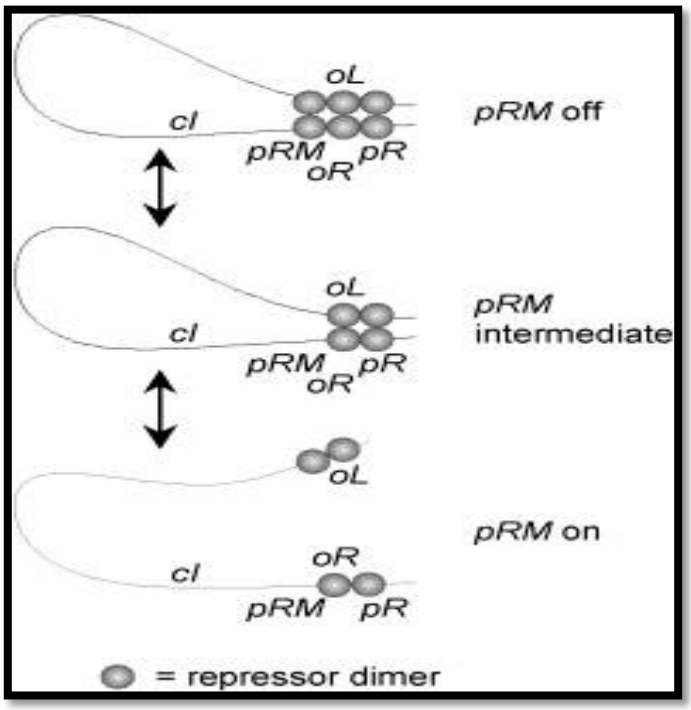

Figure 4: Model of O_L–CI–O_R Complex. The CI repressor dimer and the P_M can be open; P_M can be partially repressed when two repressor dimers bind and form an octamer, and P_M can be more strongly repressed by an additional of two repressor dimers bind to O_L and O_R to form tetramer. Source: Baek and Svenningsen (2003)

Therefore, binding of the CI repressor to O_R serves two important purposes: **i)** to repress expression of lytic genes in λ during lysogeny (Meyer et al., 1980; Ptashne, 1986, 2004) and **ii)** to promote expression of CI repressor to propagate a lysogenic state (Meyer et al., 1980; Ptashne, 1986, 2004).

Late Immunity Transcripts and Rex: In the event of a danger signal such as presence of a mutagen or any other environmental stresses, CI would be cleaved as part of the cellular SOS response and thus, no longer be capable of repressing transcription from promoters P_R and P_L (Fix, 1993). Activation the transcription from P_R would initiate the transcription of the "lysis-inducing gene" *cro* (co-repressor) and the prophage would be depressed and enter the lytic phase (Fix, 1993). Within the operon, an additional promoter is present within the C-terminal end of

rexA that governs Late Immunity Transcription (P_{Lit}; Fig. 2). P_{Lit} is activated during de-repression of the prophage and leads to high levels of *rexB* gene expression in the absence of *rexA* or *cI* genes (Hayes et al., 1997). Hayes et al. (2003) later found that the late-immunity transcript governed by two promoters (P_{lit1}, and P_{lit2}). The first is a powerful and inducible promoter, while the second is low-level and constitutive. After λ induction, P_{lit1} is activated, driving *rexB* gene transcription—a scenario that may allow λ to escape its own exclusion system due to the favoured RexB:RexA stoichiometric imbalance (discussed in detail in Section 1.1.3.1). Hayes et al. (1997) found that another λ gene, *oop,* may further protect λ from its own Rex exclusion by activating the P_{lit1} promoter, resulting in overexpression of *rexB* gene in the absence of *rexA*; a phenotype that abrogates Rex activity. The *oop* gene encodes a functional RNA (OOP RNA synthesis), which is expressed by the stimulation of *oop* promoter (*Po*) (Krinke et al., 1991).

Unlike CII, CI repressor is not involved in the lysis-lysogeny decision but is required to maintain a repressed state through its activity on the operators and to stimulate expression of itself and the *rexA-rexB* genes. Although co-expressed with *rexA* and *rexB,* the CI repressor is not required in Rex function. Thus, plasmids expressing *rexA-rexB* genes in absence of *cI* are readily used and have been shown to impart the Rex phenotype upon subsequent infection of transformed cells by T4*rII* phage (Shinedling et al., 1987; Snyder and McWilliams, 1989). In contrast, the *cI* repressor gene may modulate the severity of exclusion against lambdoid phages; a phenotype found to be suppressed by the λ *ren* gene (Toothman and Herskowitz, 1980). For instance, Toothman and Herskowitz, (1980) found that λCl[Ts]857 lysogens conferred stronger exclusion (lower plating frequencies) against sensitive lambdoid phages than λCI^{+} lysogens. Modulation of Rex is again likely dependent upon the stoichiometry of RexB to RexA that may be differentially imparted by

sensitive phage upon infection of a Rex^+ cell. However, the connection between CI-directed immunity and Rex-mediated exclusion of lambdoid phages remains nebulous.

While knowledge pertaining to the mechanism and activity of the *rex* genes is still very limited, it is known that both RexA and RexB gene products are required to confer the Rex phenotype, whereby mutation of either gene results in the loss of exclusion and restores T4*rII* plating capacity (Matz et al., 1982).

Rex Proteins Biochemistry: The RexB gene product is a 16 KDa polytopic trans-membrane protein that likely functions as an ion channel on the host cell's membrane (Landsmann et al., 1982). **A**lkaline **Pho**sphatase fusion analysis *(phoA* fusion) was used to determine RexB protein topology (Parma et al., 1992). This fusion analysis exploited the fact that alkaline phosphatase must be transferred through the periplasmic membrane to be active (Hoffman and Wright, 1985; San Millan et al., 1989), In generated RexB::PhoA fusions (spanning the *rexB* gene) the phosphatase enzyme domain would show its highest activity when the fusion is directed toward the periplasm, but if directed into the cytoplasm, the phosphatase enzyme would be inactive (Parma et al., 1992). Using this fusion strategy, RexB was observed to cross the membrane at least four times with the hydrophilic regions that carry positive charge and adhering to the "plus inside (cytoplasm) rule" (Von Heijne, 1986).

RexA, a 28.5 KDa protein, was isolated by Belfort (1978) and suggested to be a cytoplasmic protein as its primary amino acid sequence was largely hydrophilic (Parma et al., 1992). However, based on the strange "sticky cell" morphology observed in RexA over-expression studies, it was suggested (Slavcev and Hayes, 2003) that this protein may in fact localize to the outer membrane of the cell. The function of RexA is still unknown, but it is still widely believed

to function as a "sensor" that somehow activates or regulates the pore-like function of RexB, upon super-infection by a Rex-sensitive phage.

1.1.3. The Physiological Manifestations of the *rex* Genes:

1.1.3.1. *Stoichiometry of RexA to RexB*:

Activation of the Rex phenotype imparts a severe cellular environment that results in the cessation of total cellular macromolecular synthesis (Snyder and McWilliams, 1989), depolarization of the cytoplasmic membrane, reduction of the cellular respiration, and death in the majority of cells (Parma et al., 1992; Slavcev and Hayes, 2002). This rapid abortion of production similarly impacts super-infecting phage and cellular DNA, RNA, and protein synthesis. Rex can be "triggered" through either infection *via* Rex-sensitive phage, or even in the absence of super-infection, by over-expressing *rexA* relative to *rexB* (Snyder and McWilliams, 1989; Slavcev and Hayes, 2003). The stoichiometric balance of RexA to RexB has been shown to have fundamental relevance to the onset or abrogation of the Rex phenotype. The Rex phenotype is not seen and exclusion is not present in the case of over-expression of either *rexA* or *rexB* in the absence of the other, or over-expression of *rexB* to *rexA* (Snyder and McWilliams, 1989; Slavcev and Hayes, 2002; Slavcev and Hayes, 2003). In addition, increased co-expression of *rexA*-*rexB* from a multicopy plasmid not only "primes" the cell for exclusion, but also is capable of excluding T4 (RII^+) and T4*rII* alike, as well as phages T5 and T7, but not λ (Snyder and McWilliams, 1989; Slavcev and Hayes, 2003).

These differing outcomes indicate that the Rex phenotype is sensitively dependent upon the quantitative relationship between *rexA* and *rexB* "gene dosage". If *rexA* is overexpressed compared to *rexB*, Rex is activated and some cell death ensues (Slavcev and Hayes, 2003). On

the other hand, if *rexB* is overexpressed relative to *rexA*, the Rex phenotype will be suppressed, and T4*rII* phage as well as lambdoid Rex-sensitive mutants will be enabled to grow on and lyse the host cell (Parma et al., 1992; Slavcev and Hayes, 2003). Interestingly, Slavcev and Hayes (2003) found that, like after T4*rII* infection of Rex^+ cell population, over-expression of *rexA* relative to *rexB* results in a high degree of cell-killing, but at least 10% of cells survive. They proposed that Rex may not be an altruistic cell death system, but rather a mechanism by which to protect at least some λ lysogens against super-infection; a model they had formerly termed "Rex-centric mutualism" (Slavcev and Hayes, 2002).

1.1.3.1.1. Model for Rex Proteins:

Parma et al. (1992) proposed a mechanism whereby upon infection of the lysogenic host by T4*rII*, the ratio of RexA to RexB somehow increases resulting in the activation of a RexB pore protein that opens, much like a phage holin in the inner membrane of the host cell (Fig. 5).

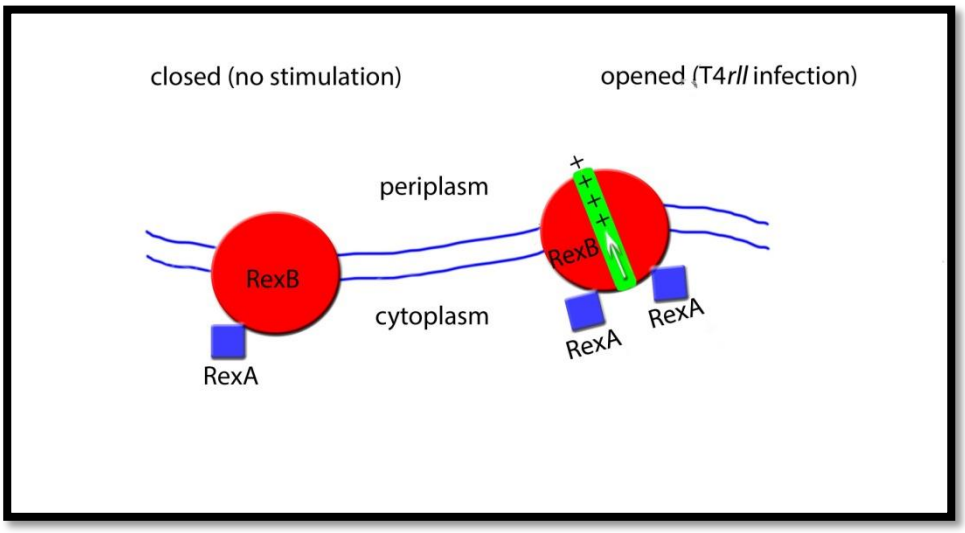

Figure 5: RexA/RexB Model. RexB acts as an ion channel and interacts with RexA at a ratio of 1:1 where no external stimulation occurs. Upon infection by T4*rII*, it was postulated that RexA accumulates relative to RexB and activates RexB pore formation, depolarizing the cell membrane and leading to loss of cell membrane potential.

This model proposes that pore formation would be "primed" and ready to "trigger" when the ratio of RexA to RexB is balanced when there is no exposure to infection; this mechanism supports the idea that RexA works as a sensor to activate the Rex system. In such a scenario RexB would interact with a single RexA protein. Upon exposure to infection, RexA increases relative to RexB and leads to two (or more) RexA proteins per RexB—a scenario that results in pore activation and Rex onset. Finally, in the case of RexB overproduction, the probability of RexB subunits to interact with multiple RexA subunits declines and results in the abrogation of the exclusion phenotype; a phenotype confirmed by multiple groups (Parma et al., 1992; Slavcev et al., 2003).

In some altered physiological states such as starvation or osmotic imbalance (Campbell and Rolfe, 1977), Rex was noted to result in only transient cessation of macromolecular synthesis (Sauerbier et al., 1969). Slavcev and Hayes (2002) noted that the Rex system, while killing a

high proportion of cells protected about a tenth of the population. Furthermore, these cells appeared in morphology similar to those seen in stationary phase (Fig. 6). This quiescent metabolic state is characterized by: **i)** changes in the cell morphology, **ii)** spherical appearance; **iii)** flagellar production, and **iv)** low cellular proton motive force. They proposed the model that Rex triggers an osmotic shift that activates stationary phase in the host—a metabolic state that is not permissive to the propagation of many super-infecting phage, including T4.

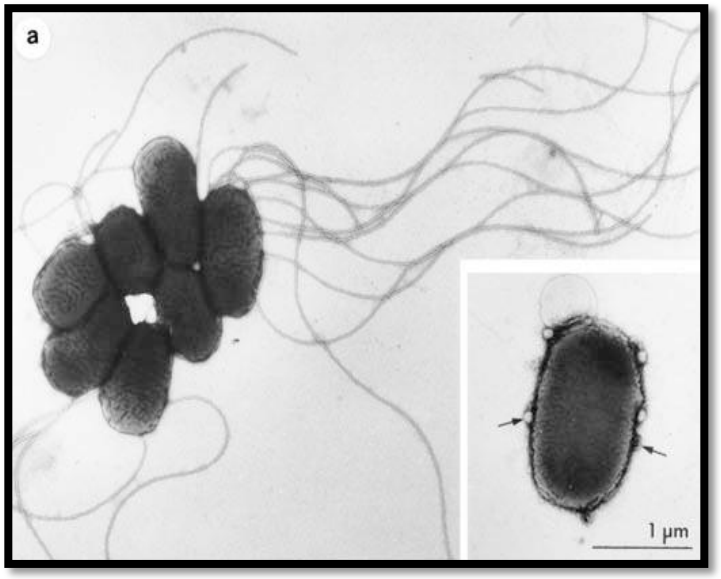

Figure 6: The morphological changes after a Rex^+ *E. coli* cell is infected by T4*rII*. Changes include: flagellar expression, and spherical appearance, similar to cellular metabolic shift to a quiescent (stationary) state. **Source:** Slavcev and Hayes (2003)

Following infection of *E. coli* (λ) lysogens by T4*rII* phage growth was noted to carry on for 10 minutes (Colowick and Colowick, 1983; Parma et al., 1992), which interestingly times to just before the RII proteins of T4 would normally be expressed that would bypass Rex (Sauerbier et al., 1969; Parma et al., 1992). It is then, that the cellular (physiological) manifestations of Rex become obvious, resulting in the shutdown of cellular metabolic systems (Sauerbier et al., 1969;

Parma et al., 1992). At ten minutes, the membrane potential and proton motive force are lost (Parma et al., 1992), which also results in a prompt drop in cellular ATP (Colowick and Colowick, 1983). However, while the preferential DNA transcription of T4*rII* and the mRNA synthesis of the host continue to the 13 minute point mark, the transcription of the host cell and the prophage has been shown to continue in surviving cells thereafter (Sauerbier et al., 1969). This finding is very much in line with the model that a sudden osmotic shift is shunting cells into a temporary quiescent state that is serves to evacuate the lysogen of the competing invading DNA. In many cases the environment is lethal to the host, and while only 10% of the super-infected population survives, this represents ~10^5-fold higher viability than that following T4*rII* infection of a Rex^- lysogen (Slavcev and Hayes, 2002).

1.1.3.2. *Rex and the Ionic Environment*:

In addition to Rex proteins stoichiometry, the ionic environment and its concentration in the surrounding medium plays an important role in regulating Rex activity, which supports the idea that RexB functions as an ion channel. The Rex system has been shown to require the presence of monovalent cations like H^+, Na^+, K^+, NH_4^+, or Cs^+ in the cellular environment to confer the exclusionary phenotype (Garen, 1961; Sekiguchi, 1966). In contrast, the presence of divalent cations such as Ca_2^+ Mg_2^+, polyamines, or sucrose in culture diminishes exclusion activity (Garen, 1961; Ames and Ames, 1965; Brock, 1965) and can prevent onset if present in culture during the first ten minutes of T4*rII* infection of the Rex^+ lysogen. Buller and Astrachan (1968) found that the reversal of the Rex phenotype is greatly diminished after the sixth minute of T4*rII* infection, which may be explained by the rapid loss of the cellular energy and membrane potential and the inability to properly uptake divalent cations introduced into the cellular environment by that point.

1.1.3.3. *The ATP Level*:

While the loss of the cellular ATP seems to be an outcome of Rex "triggering", it does not in itself lend relevance to the Rex phenotype as *unc* (ATPase) mutants, incapable of ATP hydrolysis, still interfere with T4*rII* replication when lysogenized by λ (Colowick and Colowick, 1983). The *unc* genes encode eight subunits that are responsible for the proton-translocating ATP synthase, and mutation of these genes will result in defective in H^+-ATPase synthesis and conductivity. Colowick and Colowick (1883) noted that the cellular energy of the λ *unc* mutant lysogens remained stable following infection by T4*rII* and proposed that the drop of ATP levels seen upon *T4rII* infection is not related to exclusion itself, but that ATPase activity is necessary following Rex activation in order to reinstate the proton motive force by pushing interior ATP out of the cell. Furthermore, they detected that infection of λ lysogens by wild-type T4 phages caused a rapid decline in the cellular energy, but can be recovered by aborting the source of infection. However, infection of λ lysogens by T4*rII* would cause a rapid drop of cellular energy that was unrecoverable within the time frame studied (Colowick and Colowick, 1983).

1.1.4. Additional Rex-dependent Phenotypes:

In addition to the primary enigma of T4*rII* exclusion, several other intriguing Rex-related phenotypes have been noted that are not likely directly connected to the exclusion itself, but are rather related to the physiological manifestations of the *rex* genes and Rex "triggering".

For instance, in the absence of infection and existence of amplified DNA photolyase enzyme, (gene product of the *phr* gene) amplified photolyase enzymes bind to the DNA pyrimidine dimer to remove the lesions (Li et al., 1992; Fix, 1993). In the presence of the *rexA-rexB* genes, this

protein-DNA interaction appears to activate Rex and result in subsequent cessation of the cellular metabolism (Li et al., 1992). The authors propose that the heightened interaction of photolyase with DNA simulates the "trigger" that results in the onset of Rex.

The λ *red* (*exo bet*) and *ren* loci are both involved in λ DNA recombination, and appear to also play a role also in bypassing Rex during λ infection and replication, This theory is supported by evidence suggesting that, the deletion of either *ren* or *red* genes sensitizes λ to Rex (Toothman and Herskowitz, 1980). The *red* recombination proteins may defend λ from its exclusion by either eliminating intermediates that are sensitive to Rex or by fixing the broken DNA that may be caused by Rex phenotype (Daniels et al., 1983).

While Gene *l* is important for bacteriophage T5 to transfer its DNA into the host cell as well to destroy the host DNA, Jacquemin-Sablon and Lanni (1973) found that T5*l* mutant phage were excluded on λ lysogens. Likewise, T7 *20* mutant phage was noted to be excluded only on λ *rex*$^{+}$ lysogens, and it is noted that gene *20* possesses remarkable sequence similarity to *rII* of bacteriophage T4 that are needed to suppress Rex. This Rex-dependent intervention occurs late during the lytic cycle and most likely during the DNA packaging process (Pao and Speyer, 1975).

1.2. The *rII* Genes of Bacteriophage T4:

1.2.1. Bacteriophage T4 and the *rII* Genes:

Bacteriophage T4 is one of the largest and well-studied phages in bacterial genetics to date and has been involved in many important historical genetic discoveries. T4 is a member of the T-even phages that infect *E. coli* bacteria and are distinguished by exclusively lytic replication. The "*rII* genes" of bacteriophage T4 have been extensively studied to identify their characteristics and determine the mechanisms by which they suppress Rex. However, the precise function of the *rII* locus, comprised of *rIIA* and *rIIB* genes remains obscure, despite being the most studied and mapped genes to date (Benzer, 1955). Benzer (1955) found that T4 *r* mutants possessed a "***r***apid lysis" (*r*) phenotype when plated on *E. coli* B cells.

Depending on the availability of host cells in the environment, T4 and its T-even phage relatives can control the lysis time of the host cells to maximize usage of their host and probability of propagating their DNA. Wild type T4 can rapidly lyse *E. coli* cells within 25 to 30 minutes of infection at 37^{o}C, releasing around 100 to 200 progeny per cell (Abedon et al., 2003). However, any addition of other T-even phages to the culture during the first three minutes of initial infection can delay the lytic cycle for up to several hours depending on the multiplicity of infection (MOI), during which phage continue to replicate in their host and can release up to 1000 phage per host cell (Doermann, 1948; Abedon et al., 2003).

This phenomenon of the delay in the lysis timing, which results in an increase in the amount of phage released by as much as 10-fold compared to regular lysis has been termed the **L**ysis **IN**hibition phenotype (LIN). Hershey (1946) first found that LIN can be distinguished from rapid lysis mutants on plates seeded with *E. coli* B. While T4 wild type phages make small, fuzzy-

edged plaques, T4 mutants that cannot establish LIN form large, sharp edged plaques, indicative of rapid lysis. This phenotype allowed Hershey (1946) and Benzer (1955) to map the first three rapid lysis loci *rI, rII* (*rII*A, and *rII*B), and *rIII* (described further below).

1.2.2. Types of "*r*" Mutants:

The *rI* gene encodes the T4 anti-holin, that is secreted into the periplasmic membrane and inhibits cell lysis upon super-infection by T-even phage by directly inhibiting T holin protein of bacteriophage T4 originally identified as the *rV* mutant (Paddison et al., 1998). Holins are critical proteins in dsDNA phages that accumulate in the inner membrane in a highly timed manner and control lysis of the host and release of phage progeny (Young, 1992). Late in T4 lytic cycle the T holin protein forms holes in the cytoplasmic membrane thereby permitting the gpE muralytic endolysin enzyme to translocate into the periplasm and hydrolyze peptide bonds in the peptidoglycan (cell wall) layer (Josslin, 1971; Young, 1992). The T holin is thus essential for phage release, thus the original mutants of *rV* that were isolated likely retained holin activity, but lost sensitivity to RI (Dressman and Drake, 1999). The *rIII* gene has not yet been identified but is hypothesized to provide a stabilizing effect during lysis inhibition that influences the interaction between RI and T (Ramanculov and Young, 2001). The *rIV* gene product is hypothesized to aid T4 during host infection by repairing the broken membrane during T4 adsorption and DNA injection (Abedon et al., 2003; Kai et al., 1999). The *rII* locus encoding *rII*A and *rII*B, is required for T4 to successfully establish LIN in *E. coli* B but not *E. coli* K cells, where T4*rII* and T4 plaques look identical (Doermann, 1948; Benzer, 1955). While both genes are essential for the viability of bacteriophage T4 on *E. coli* lysogenized with λ, the involvement of the r*II* genes in phage replication remains unknown. Although the localization of RII proteins is still

unknown, they are anticipated to map to the inner membrane of T4 cell (Huang, 1975; Takacs and Rosenbusch, 1975) and one of the suggested functions of RII proteins is to firmly adhere the T4 replication complex to the internal membrane (Mosig et al., 1984).

The *t* (RV) holin gene of bacteriophage T4 functions very similarly to the λ *S* holin and can even replace it during *in vivo* λ infection (Lu and Henning, 1992). Similarly, RexB over-expression can suppress both the T4 *t* and the λ *S* mutations (Slavcev and Hayes, 2005). Expression of λ *rexB*, (only in the absence of *rexA*) also suppresses LIN in *E. coli* K cells conferring the necessity of RIIA and RIIB; a scenario that is identical to that seen in *E. coli* B cells (Slavcev and Hayes, 2005). Although these findings can help to understand the role of *rII* genes in T4 replication, the mechanism of RII–mediated bypass of Rex remains unknown.

1.2.3. T4 *motA* Gene:

The T4 *motA* (**mo**dulation of **t**ranscription) gene is an additional gene that has been identified to influence Rex. Mattson et al. (1974) noted that the double T4*rII motA* mutant was insensitive to Rex, and named *motA* the ***R****ex* **Im**munity Gene (*rim*); in addition, Parma et al. (1992) found that *motA* mutants can also postpone the dropping of the membrane potential after T4*rII* infecting λ lysogen. MotA influences the transcription of early genes, including *rIIA* and *rIIB*, and is active right around the time at which Rex onset and cellular manifestations of Rex are observed (Johnson and Hall, 1974; Mattson et al., 1974).

Following the discovery of the Rex phenotype, researchers have attempted to understand the definite sequence, mechanism, and relationship of the *rexA* and *rexB* genes and their interactions with the *rIIA* and *rIIB* genes of bacteriophage T4. Rex activity is associated with some physiological manifestations that may direct cells into stationary phase to protect the host cells

along with itself from superinfection (Slavcev and Hayes, 2002). Slavcev R. (personal communication, 2003) formerly had found that mutants of *ompF,* an outer membrane protein (Omp) that functions in osmoregulation, attenuated Rex activity. The *ompF* gene encodes a porin that, like *rII* exclusion, has been shown to be inhibited by polyamines (Delcour and Vega, 1996), -where polyamines, in addition to divalent cations, have been previously shown to reduce Rex activity (Garen, 1968). Omps play an important role in controlling the passage of bacterial solutes and nutrients through the outer membrane; relevantly, Rex activity is critically affected by the ionic environment. Hence, in addition to undertaking the isolation of new mutations that attenuate Rex, we further aimed to study the Omp porins as they may be involved in the triggering of some of the physiological manifestations of Rex. In combination, we sought to be the first to identify novel host gene(s) involved in Rex in the plight to solve this longstanding scientific mystery.

1.3. Rationale, Hypothesis, and Objectives:

Rationale:

The Rex phenotype is a very complex and convoluted collection of phenotypes; the mechanism of T4*rII* exclusion, RII-mediated suppression of Rex, and other peripheral connected phenotypes after more than 60 years has yet to be resolved. It has long been believed that additional host proteins are involved in such a pleiotropic phenotype and were long sought as the "holy grail" in the elucidation process. We thus, aimed to design a system by which we could isolate and characterize *E. coli* mutants that attenuate T4*rII* exclusion as an approach to uncover important clues about the functionality of Rex and the mechanism of its activation.

In *E. coli*, the outer membrane proteins function in important cellular osmoregulation, nutrient uptake, and as a protective barrier that provides structural and functional stability to the cell. The biological composition of the outer membrane allows the ion channel Omps to control the influx and the efflux of solutes across the membrane to adapt to any external changes. In combination with previous findings that the ionic environment is critical to Rex phenotype, and deletion of *omp* genes would make cells more sensitive to external changes and less resistant to infection, we aimed to investigate the Omps and their potential involvement in Rex activity.

Hypothesis:

The Rex-mediated establishment of a harsh, but temporary cellular environment requires both phage and host proteins to enact this complicated, but reversible scenario. Rex involves the interplay of RexB and/or RexA with additional host proteins(s) in the exclusion of T4*rII*. The isolation of host mutations that abrogate Rex will identify the direct and indirect "players"

involved in “triggering” and inducing the cellular (physiological) manifestations of the Rex phenotype.

Objectives:

The primary objectives of this project are the following:

1. Design and construct a vector as part of a one-step insertional mutagenesis strategy to isolate mutants with mutations of non-essential host genes that influence *rII* exclusion.
2. Mutagenize and isolate *rex*$^+$ *E. coli* mutants that impact Rex activity.
3. Test viable candidate genes that are postulated to influence Rex activity
4. Screen isolated *rex*$^+$ *E. coli* mutants for sensitivity to T4*rII* plating and confirm they are genotypically *rex*$^+$ and phenotypically attenuated or impacted for Rex activity.

Future objective:

5. Sequence host mutations in isolated host mutants that suppress *rII* exclusion and interpret their involvement in the exclusion mechanism.

CHAPTER TWO

2. Material and Methods

2.1. Materials:

2.1.1. Strains and Plasmids:

All bacterial strains and phages strains along with plasmids that were used in this study are listed and described in Table. 1.

Table 1: Strains and plasmids used in this study

Designation	Relevant characteristics	Source
Bacterial Strains		
DH5α	F-, Δ*(argF-lac)169, ϕ80dlacZ58(*M15*), ΔphoA8, glnV44(*AS*), λ-,deoR481, rfbC1, gyrA96(NalR), recA1, endA1,thi-1,hsdR17*	*E. coli* Genetic Stock Collection (CGSC) #12384
W3110	F-, λ-, *IN*(*rrnD-rrnE*)*1, rph-1*	CGSC# 4474
BW25113 wt	F-, *567, ΔlacZ4787*(::rrnB-3), λ^{-}, *rph-1, Δ(rhaD-rhaB)568, hsdR514*	CGSC# 7636
DH5α (λ*pir*)	DH5α lysogenized by phage λ434 (heteroimmune derivative) containing the *pir* gene	Gift from Dr. Charles lab, Biology department, UW (2009)

W3110 (λ)	F^-, λ^+, *λcI578, IN(rrnD-rrnE)1, rph-1*	National BioResource Project (NBRP) # ME6104 (2009)
S17-1 (λ*pir*)	TpR SmR recA, thi, pro, hsdR-M+RP4: 2-Tc:Mu: km^R,Tn7, λpir^+	Gift from Dr. Charles lab, Biology department, UW (2013)
JW0427-1	Δ*clpP::*km^R, λ^-	CGSC# 8590
JW0554-1	BW25113 isogenic derivative: *ΔompT774::*km^R	CGSC# 8680
JW0799-1	BW25113 isogenic derivative: *ΔompX786::*km^R	CGSC# 11794
JW0912-1	BW25113 isogenic derivative: *ΔompF746::*km^R	CGSC# 8925
JW0940-6	BW25113 isogenic derivative: *ΔompA772::*km^R	CGSC# 8942
JW1248-2	BW25113 isogenic derivative: *ΔompW764::*km^R	CGSC# 9125
JW1312-1	BW25113 isogenic derivative:	CGSC# 11793

	ΔompG756::km^R	
JW1371-5	BW25113 isogenic derivative: *ΔompN740::km^R*	CGSC# 9213
JW2203-1	BW25113 isogenic derivative: *ΔompC768::km^R*	CGSC# 9781
JW3368-1	BW25113 isogenic derivative: *ΔompR739::km^R*	CGSC# 10510
JW3846-1	BW25113 isogenic derivative: *ΔompL737::km^R*	CGSC# 10779

Phage

T*4D*	Wild type T4	Obtained from Mosig G (2009), Slavcev lab Freezer Pro # 23 phage box
T4*rII*Δ1586	Δ(*rIIA*-*rIIB*)	Obtained from Mosig G. (2009), Slavcev lab

		Freezer Pro # 24 phage box
λ (*cI*-857)	*cI*[ts]*857*, (*rex*$^{+}$)	NBRP # ME6104 (2009)
λF7	*λ, Dam15*, *imm21*, *cI*[ts]*857*	Mikawa et al. (1996) Maruyama et al. (1994)

Plasmids

pUC19	High copy number plasmid; MCS-*lacZ*α, Ap^R	New England Biolabs (NEB) # N3041S, Whitby, Canada
pBSL199	*ori* R6K, *lacI*^{Q}, *Tn*10, mob (RP4), IS10, Ap^R and Tc^R	NBRP (2009), M. F. Alexeyev (1995)
pBSL180	*ori* R6K, *lacI*^{Q}, *Tn10*, mob (RP4), IS10, Ap^R and Km^R	NBRP (2009), M. F. Alexeyev (1995)
pHA1	pUC19 [*cI-rexA-rexB*]; MCS- *lacZ*α, Ap^R, *pM-cI857-rexA-rexB-*t_{imm}	This study

pHA2	pBSL199 [*cI-rexA-rexB*]; IS10-Tc^R, *pM-cI857-rexA-rexB-*t_{imm}	This study

***E. coli* Host Strain**	**Resident Plasmid**	
DH5α	pUC19	This study
DH5α [λ*pir*]	pBSL180	This study
DH5α [λ*pir*]	pBSL199	This study
DH5α	pHA1	This study
DH5α [λ*pir*]	pHA2	This study
S17- 1 [λ*pir*]	pHA2	This study
S17- 1 [λ*pir*]	pBSL199	This study
BW25113 (wt)	pUC19	This study
JW0554-1 (*ΔompT*)	pUC19	This study
JW0799-1 *(ΔompX)*	pUC19	This study
JW0912-1 (*ΔompF)*	pUC19	This study
JW0940-6 (*ΔompA)*	pUC19	This study
JW1248-2 (*ΔompW)*	pUC19	This study

JW1312-1 (*ΔompG)*	pUC19	This study
JW1371-5 (*ΔompN)*	pUC19	This study
JW2203-1 (*ΔompC)*	pUC19	This study
JW3368-1 (*ΔompR)*	pUC19	This study
JW3846-1 (*ΔompL)*	pUC19	This study
BW25113 (wt)	pHA1	This study
JW0554-1 (*ΔompT)*	pHA1	This study
JW0799-1 *(ΔompX)*	pHA1	This study
JW0912-1 (*ΔompF)*	pHA1	This study
JW0940-6 (*ΔompA)*	pHA1	This study
JW1248-2 (*ΔompW)*	pHA1	This study
JW1312-1 (*ΔompG)*	pHA1	This study
JW1371-5 (*ΔompN)*	pHA1	This study
JW2203-1 (*ΔompC)*	pHA1	This study
JW3368-1 (*ΔompR)*	pHA1	This study
JW3846-1 (*ΔompL)*	pHA1	This study

2.1.2. Solutions and Buffers:

Growth media:

Luria **B**ertani (LB) Broth (BD Difco, Mississauga, Canada)

Purpose: A Rich nutrient media for optimum growth of bacterial cultures.

Preparation: Ten g of tryptone, five g of yeast abstract, and five g of NaCl were added to one liter (L) of ddH_2O. Antibiotic was added (as needed) after LB broth was autoclaved and allowed to cool for one hour (h). Broth with antibiotic stored at 4^oC otherwise stored at room temperature (RT).

Luria **B**ertani (LB) Agar (BD Difco, Mississauga, Canada)

Purpose: A Rich semi-solid media for optimum growth of bacterial cells on semi-solid media (plates).

Preparation: Ten g of tryptone, five g of yeast abstract, five g of NaCl and 12.5 g of bacto agar were added to one L of ddH_2O and antibiotic was added (as needed) after LB agar was autoclaved and was then allowed to cool for one h. Media was poured into sterile mono-plates (Fisher Brand, Ottawa, Canada) at a volume of approximately 35 mL per plate. The media was allowed to solidify at RT for approximately two h and then stored at 4^oC.

Luria **B**ertani (LB) Top Agar (BD Difco, Mississauga, Canada)

Purpose: A rich media to suspend bacterial cultures and phages and give a good dispersion of cells as a plate overlay.

Preparation: Ten g of tryptone, five g of yeast abstract, five g of NaCl and seven g of Bacto agar were added to one L of ddH_2O. 50 mL aliquots were prepared, autoclaved, and then stored in 55^oC water bath.

Super **O**ptima Broth with **C**atabolite repression (SOC) (BD Difco, Mississauga, Canada)

Purpose: A rich media used to recover cells after electroporation.

Preparation: Ten g of tryptone, five g of yeast abstract, and five g of NaCl were added to one L of ddH_2O in addition of 10 g of tryptone, one Molar (M) $MgCl_2$ and one M glucose . After preparation, the media was autoclaved and stored at RT.

Antibiotic stocks:

Purpose: Antibiotics were added to liquid media or plates as selective markers to amplify cells carrying plasmids or to distinguish cells with antibiotic markers on their chromosome.

Ampicillin stock (Ap) (Sigma Aldrich, Oakville, Canada)

Purpose: Ampicillin was used to select for cells transformed by plasmids: pUC19, pBSL180, pBSL199, pHA1, and pHA2.

Preparation: Small aliquots of stock ampicillin were prepared at a final concentration of 50 μg/mL. Ampicillin stock powder was dissolved into sterile ddH_2O and filter sterilized. Ampicillin stock was stored at -20^oC and thawed solution was added to media to a final concentration of 100 μg/mL.

Kanamycin stock (Km) (Sigma Aldrich, Oakville, Canada)

Purpose: Kanamycin was employed as a second antibiotic marker to screen for pBS180 (*cI-rexA-rexB*) plasmid, and to distinguish Δ*clpP* and Δ*omp* mutant strains.

Preparation: Small aliquots of kanamycin stock solution were prepared at a final concentration of 25 μg/mL by dissolving kanamycin stock powder into sterile ddH_2O and filter satirizing. The stock was stored at -20^oC and thawed solution was added as necessary to media at a final concentration of 50 μg/mL.

Tetracycline stock (Tc) (Sigma Aldrich, Oakville, Canada)

Purpose: Tetracycline was employed as a second antibiotic marker to screen for pHA2 plasmid, *in tandem* with kanamycin antibiotic to screen for Rex$^-$ mutants.

Preparation: Small aliquots of tetracycline stock solution were prepared at a final concentration of 20 μg/mL by dissolving tetracycline stock powder into 1:1 50% of sterile ddH_2O and 50% of 95% ethanol. Tetracycline stock was stored in foil wrap to protect from light degradation at -20^oC, and thawed solution was added to media as necessary at a final concentration of 20 μg/mL.

Note: all unused stock, plates, or broth containing tetracycline was protected from light degradation while stored.

Buffers:

Tris **N**aCl (TN) buffer (Fisher bioreagents, Ottawa, Canada)

Purpose: Used to make serial phage dilutions and phage storage.

Preparation: 0.1 M of NaCl and 0.01 M of Tris-HCl were added to one L of ddH_2O and adjusted to PH of 7.8. Solution was autoclaved and cooled to RT for storage.

50X **T**ris-**A**cetate-**E**DTA (TAE) Buffer (Fisher Bioreagents, Ottawa, Canada)

Purpose: To separate DNA fragments (bands) based on their sizes when running samples on gel electrophoresis.

Preparation: 50X TAE buffer consists of 242 g Tris base (MW=121.1), 57.1 mL glacial acetic acid, and 100 mL of 0.5 M EDTA per one liter. 50X TAE solution was diluted in ddH_2O to a final concentration of 0.5X and stored at RT.

Screening reagents:

Iso**p**ropyl β-D-1-**t**hio**g**alactopyranoside (IPTG) solution (Fisher Bioreagents, Ottawa, Canada)

Purpose: For the induction of *lac*Z gene expression in the pUC19 vector required for white/blue colony screening, and the induction of the transposable element to be inserted into host chromosomal DNA.

Preparation: 2.38 g of IPTG powder was dissolved in 100 mL ddH_2O to a final concentration of 100 mM IPTG. Products were filter sterilized and then stored at -20^oC.

5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal) solution (Omega Bio-Tek, Norcross, USA)

Purpose: To use in combination with IPTG for white/blue colony screening denoting disruption of the *lacZα* genetic element and LacZ^- phenotype in M15 Δ*lacZ* cells (see below for genetic description). After *lac*Z induction by the gratuities inducer, IPTG, X-Gal particles are hydrolyzed by LacZ^+ cells inducing blue colour in non-recombinant colonies.

Preparation: 20 mg of X-Gal powder was dissolved in 20 mL of dimethylsulfoxide (DMSO, Fisher Bioreagents, Ottawa, Canada). Unused X-Gal plates/stock were protected from light degradation at -20^{o}C.

0.8% DNA agarose gel chromatography

Purpose: To separate DNA fragments and make them visible under UV light.

Preparation: 0.8 % agarose was dissolved in 100 mL 0.5X TAE buffer and microwaved for one minute to dissolve agarose particles.

Method: Four µL of Ethidium Bromide (EtBr) solution (Bio-Rad, Mississauga, Canada) was added to molten agarose gel just before pouring the melted agarose into the gel cast. The gel solidified at RT before use.

2.2. Methods:

2.2.1. Phage lysate preparation and purification: *E. coli* K-12 wild type host strain, W3110, was plated at 30^{o}C overnight (O/N). A culture was prepared at 30^{o}C with shaking at 225 RPM O/N. A subculture was prepared from the O/N culture at a 1:100 dilution and was grown to an optical density $A_{600} = 0.4$ (Spectrophotometer, Fisher Bio-Scientific, Ottawa, Canada). Primary lysate serial dilutions were prepared in TN Buffer (0.01 M Tris-HCl and 0.1 M NaCl, pH 7.8, (Fisher Scientific, USA) in 1:10 increments to find the best concentration prior to adding lysates to the bacterial stocks. Lysate dilutions of phage were added at a maximal volume of 300 µL to fresh cell cultures along with three mL of top agar, and the mixture was immediately poured onto LB plates and incubated O/N at 37^{o}C. The plates were then examined for consistent and

productive lysis patterns the next days and productive plates were used to prepare phage lysates, where one of the following techniques was performed:

1) *Plate lysate preparation:* Plates with productive lysis patterns incubated at 4^{o}C for 4-24 h with 10 mL of TN buffer. The solutions and top agar were transferred into pre-chilled conical tubes and 45 µL of chloroform ($CHCl_3$) was added and lysates were incubated on ice for 20 min. To remove any cellular debris, mixtures were centrifuged at 4^{o}C for 30 min and were filter sterilized using a 0.45µm polyethersulfone filter membrane (VWR, Mississauga, Canada), then stored at 4°C.

2) *Liquid lysate preparation:* A single plaque was isolated and incubated it with 300 µL of fresh O/N culture at 37^{o}C for 30 min. Next, 10 mL of pre-warmed LB was added to the culture and incubated it at 37^{o}C in shaker at 260 RPM for 4-5 h. The cultures were monitored for a pattern of turbidity followed by clearing, approximately 4-5 h, to a final A_{600}= 0.05. The lysate was incubated on ice for 10 min and 45 µL of $CHCl_3$ was added before further incubation for 10 min. Mixtures were centrifuged at 4^{o}C for 30 min at 12K RPM to remove cell debris and filter sterilized using a 0.45 µm polyethersulfone filter membrane (VWR, Mississauga, Canada) and stored at 4°C.

2.2.2. Plate overlay phage titration and efficiency of plating (EOP) assay: Serial lysate dilutions ranging from 10^{-2} to 10^{-8} phage/mL were prepared in TN buffer and added in 10 µL aliquots to 300 µL of fresh O/N W3110 culture. Three mL of top agar was added to the mixture and was immediately poured on LB plates. Ten µL of each lysate dilution was dropped on the solidified top agar then incubated O/N at 30^{o}C. Plaque counts were calculated the following day relative to the efficiency of plating compared to the 100% control strain W3110.

2.2.3. Assessing Rex and T4rII activity: Cultures of W3110 (wt) and W3110 (λ) were prepared in five mL aliquots at 1:100 dilution, cultures were then incubated shaking O/N. 300 µL of culture was added to 3 mL top agar and poured immediately onto pre-warmed LB plates, then left at RT to solidify. Serial dilutions of T4 (wt) and T4r*II* lysates were prepared in TN buffer with concentrations ranging from 10^{-2} to 10^{-8} phage/mL to quantify plating efficiency and Rex activity. Lysates were added in 10 µL drops onto W3110 (wt) and W3110 (λ) overlay plates. Plates were left for 30 min to dry, then incubated O/N at 30°C. The following day, plates were assessed and plaques (where evident) were quantified.

2.2.4. E. coli K-12 host transformation: *E. coli* strains and their resident plasmids are shown in Table 1. Five mL of O/N cultures of host cells were prepared shaking at 30°C. Subcultures 1:100 dilution from a fresh O/N parent culture were prepared the following day to an A_{600} = 0.4-0.6 or for 3-4 h. Cells were incubated on ice for 10 min, then centrifuged at 4K RPM (Avanti J-E Centrifuge, Beckman Coulter, Mississauga Canada) for 30 min at 4°C. The supernatant was discarded and the pellet was washed with 20 mL pre-chilled sterile ddH_2O to clean the cells of any charged particles that could interfere with the electroporation process. Cells were centrifuged at 4K RPM again for 10 min at 4°C, the supernatant was discarded and the pellet washed with 10 mL pre-chilled sterile ddH_2O. Cells were centrifuged at 4K RPM for 10 min at 4°C and the pellet was re-suspended to the final volume of 1.0-5.0 mL with pre-chilled sterile ddH_2O. Small aliquots (100 µL per tube) were prepared and plasmid DNA with a concentration ranging from 0.5 µg to 1.0 µg was added and mixed with electro-competent cells and incubated on ice for 10 min. The mixture was added to the pre-chilled electroporation cuvettes and pulsed for three seconds at 1000 V using the Electroporator 2510 (Eppendorf, Mississauga, Canada). The electrical pulses would help to form pores on the host cell membrane and allow the easy uptake

of plasmids. Cells were immediately re-suspended in one mL pre-warmed SOC media and incubated shaking at 50 RPM at 37ºC for one hour. 200-250 µL of cells were then plated on selection antibiotic plates and incubated at 30ºC O/N. Serial dilutions of electroporated cells were plated in order to assess the relative efficiency of transformation where necessary.

2.2.5. Colony polymerase chain reaction assay (PCR): A single colony of W3110 (λ) was streaked out on an LB plate and incubated O/N at 30ºC. One colony from the fresh plate was picked and diluted in 100 µL of DNase/RNase free molecular grade water (Hyclone, Ottawa, Canada), and the PCR reaction began with heating (initial heating step) the diluted colony at 94ºC for 10 min (C1000 Touch™ Thermal Cycler**,** Bio-Rad, Mississauga, Canada). The λ*imm*F (forward) primer: 5'GGGGGGCATTGTTTGGTAGGTGAGAGAT 3', and the λ*imm*R (reverse) primer: 5' TTGATCGCGCTTTGATATACGCCGAGAT 3' (Sigma Aldrich, Oakville, Canada) were diluted to small aliquots (one mL/ tube) to a final concentration of one IU/mL. The PCR reaction was set up as follows:

Five µL of diluted pre-heated template was mixed with 10 µL of each primer, 10 µL DNA polymerase enzyme (Phusion Flash high-Fidelity PCR Master Mix, Fisher Scientific, Ottawa, Canada) to a final volume of 25 µL per reaction with DNase/RNase free molecular grade water (Hyclone, Ottawa, Canada). A negative control without template was also prepared. The cycling thermal reaction included initial denaturation at 98ºC for 30 sec, then denaturation at 98ºC for 10 sec, then annealing at 72ºC for 10 sec, then extension at 72ºC for three min, then finally, extension at 72ºC for 10 min. Cycles of denaturation, annealing and extension were repeated 30X. After passing through the final extension cycle, the thermal cycling reaction cooled to 4ºC where the PCR reaction was completed. Reactions were then collected and run on 0.8 % agarose gel electrophoresis, and identified based on the fragment size. The λ immunity region of 2.4 kb

PCR fragment was gel extracted using gel extraction kit (Omega Bio-Tek, Norcross, USA), purified using PCR cycle-pure kit (Omega-Bio-Tek, Norcross, USA), and then digested by *BgI*II (NEB, New England Biolabs, Whitby, Canada) restriction enzyme and purified again using mini-prep kit (Plasmid Mini Kit I, Omega Bio-Tek, Norcross, USA). Finally, the DNA concentration was determined using the Nanodrop 2000 spectrophotometer at $A_{260/280}$ (Thermo Fisher Scientific, Ottawa, Canada).

2.2.6. Plasmid extraction and purification: A single colony isolated from a successful transformation was cultured O/N at 37^{o}C shaker in LB + antibiotic to a total volume of 10 mL and resident plasmid was extracted and purified using mini-prep kit (Plasmid Mini Kit I, Omega Bio-Tek, Norcross, USA). Plasmids were then eluted in 50-100 µL of DNase/RNase free molecular grade water (Hyclone, Ottawa, Canada), and stored at 4^{o}C for frequent use. DNA concentration was then determined using the Nanodrop 2000 spectrophotometer at $A_{260/280}$ (Thermo Fisher Scientific, Ottawa, Canada).

2.2.7. Plasmid digestion and DNA purification: Plasmids were digested using specific restriction enzymes (New England Biolabs, Whitby, Canada). In a 600 µL sterile micro-centrifuge tube (Eppendorf, VWR, Mississauga, Canada), one µg of plasmid, 10X CutSmart NEB buffer, one IU restriction enzyme were mixed and DNase/RNase free molecular grade water (Hyclone, Ottawa, Canada) was added to complete a volume of 50 µL. *Bam*HI-HF restriction enzyme was used to cut pUC19 at a specific site in the MCS, double digests using *X*baI and *Eco*RI were performed to cut pHA1 and plasmid pBSL199. *Hind*III-HF was used to cut the construct pHA1 at three different sites. The PCR insert product (the P_M-*cI857-rexA-rexB-*t_{imm} *rex* operon) was isolated and purified using *BgI*II at λ 35722 bp and λ 38103 bp that closely

flanks the operon on both sides (Fig. 7). The parent plasmid, pUC19, served as the plasmid control for pHA1, and pBSL199 served as the control for pHA2.

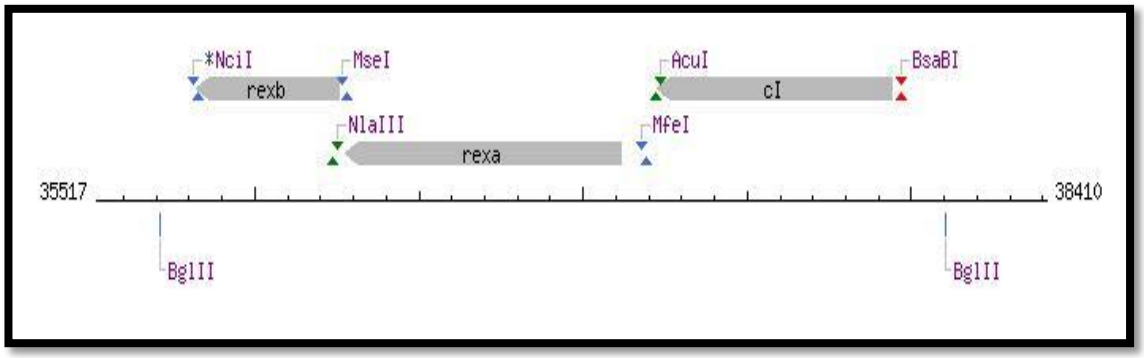

Figure 7: Linear sequence of P_M-*cI*-*rexA*-*rexB*-t_{imm} PCR fragment showing the two sites of *Bgl*II restriction enzyme.

The digestion reaction was then mixed gently and incubated in 37^{o}C water-bath for 1-2 h. Where required, the reaction was inactivated *via* heat shock at 64^{o}C for 10 min. The concentration of the DNA fragments was measured and assessed using a Nanodrop 2000 spectrophotometer at $A_{260/280}$ (Thermo Fisher Scientific, Ottawa, Canada).

2.2.8. DNA extraction and purification: The digested insert (*X*baI-P_M -*cI*-*rexA*-*rexB*-t_{imm}-*Eco*RI) and vector (*X*baI -pBSL199- *Eco*RI) fragments were separately mixed with 6X loading dye (New England Biolabs, Whitby, Canada) and electrophoresed against one kb DNA ladder (New England Biolabs, Whitby, Canada) on a 0.8% agarose gel with four µL EtBr at 100 V for one h or at 50 V for three h. The DNA bands were then visualized under Spectroline UV Trans-illuminator (Thermo Fisher Scientific, Ottawa, Canada) and the vector and insert bands were recognized based on their sizes and compared to the one kb ladder sizes control. The correct bands were then cut, collected and purified using a gel extraction kit (Omega-Bio-Tek, Norcross,

USA) and the vector/insert concentrations were measured using a Nanodrop 2000 spectrophotometer at $A_{260/280}$ (Thermo Fisher Scientific, Ottawa, Canada). Gel extraction was repeated as necessary in order to collect a concentrated volume of DNA required for subsequent manipulation.

2.2.9. DNA ligation: A 1:3 or 1:5 ratio of vector: insert fragment was ligated using one IU T4 DNA ligase enzyme (New England Biolabs, Whitby, Canada), 10X ligase buffer and DNase/RNase free molecular grade water was added to a final volume of 25 µL. Negative control of insert only, vector only, and insert + vector without ligase enzyme were set up. Ligation reactions were mixed gently and incubated O/N at RT. The reactions were inactivated by heat shock at 64^{o}C for 10 min the next day and then kept on ice to proceed with transformation preparation as formerly described.

2.2.10. Cell viability assay: Cultured cells of outer membrane protein genes (*omp*) mutants containing [pUC19-*rex*$^{+}$] plasmid (pHA1) or pUC19 were assessed for cell viability. The parent strain, BW25113 wt [pHA1], was used as a positive control and BW25113 [pUC19] as a negative control for Rex. The Δ*ompC* mutant that inhibits T4 attachment was used as the negative control for infection. Cells were grown in LB+ Ap and incubated with shaking at 225 RPM O/N at 30^{o}C. A 1:100 subculture dilutions of each was then prepared from the O/N cultures in LB+ Ap and incubated with shaking at 225 RPM at 30^{o}C until A_{600}= 0.8. The 1:100 subcultures preparations were then prepared in TN buffer, and 200 µL aliquots were mixed with phage T4*rII* lysate at a multiplicity of infection (MOI) of 3 and mixed gently. The infected cells were incubated at 30^{o}C for10-15 min, then washed twice with two mL TN buffer and re-suspended to a final volume of one mL. The suspended cells were diluted again in 1:1000 TN

buffer and 100 µL aliquots were prepared to be mixed with 3 mL top agar before being poured onto pre-warmed LB+ Ap plates. The preparations were incubated at 30^{o}C for 48 h.

2.2.11. Infective center assay: Overnight cultures of *omp* [pHA1] cells as experimental strains, the parent strain BW25113 wt [pHA1] as the positive control, and BW25113 [pUC19] and Δ*ompC* [pHA1] as the negative controls, were grown in LB+ Ap and incubated with shaking at 225 RPM at 30^{o}C. The 1:100 subculture were then prepared in LB+ Ap and incubated with shaking at 225 RPM at 30^{o}C until an A_{600} = 0.8. Approximately two mL of culture was then centrifuged at 10K RPM for 10 min and cells were re-suspended in one mL of $CaCl_2$. T4*rII* lysate was then added to MOI of 3, and allowed to be adsorb for 15-20 min. Infected cells were washed 3X in TN buffer and re-suspended in 100 µL TN buffer. 200 µL of the original O/N cultures were then added to the re-suspended cells, mixed with three mL top agar, and then poured onto pre-warmed LB+ Ap plates and incubated O/N at 30^{o}C.

2.2.12. E. coli outer membrane protein (omp) mutant conjugation with the rex^{+} suicide plasmid: Overnight cultures of Δ*omp* mutants (recipients) and S17-1(λ*pir*) [pHA2] (donor) were prepared at 30^{o}C, O/N with shaking at 225 RPM. The Δ*clpP* strain was used as a Rex^{-} control recipient as there is no evidence that ClpP is involved in Rex phenotypic activity. We have further verified Δ*clpP::km^{R}* as a suitable negative recipient control via executed experiments described here in (please see Results). The parent strain BW25113 (Km^{S}) was used as a negative control recipient and the S17-1λ*pir* pBSL199 strain was used as a plasmid control. One mL of each culture was centrifuged at 13K RPM for 10 min at RT and pellets were washed 2X in 0.5 mM NaCl (Fisher bioreagents, Ottawa, Canada). A ratio of 1:2 of donor: recipient was used. Mixtures were then centrifuged again for another 10 min at 13K RPM at RT and re-suspended in 80 µL 0.5 mM NaCl before being mixed with 100 µL pre-warmed fresh LB. During the first one

to two hours of incubation time and before plating, 0.1 mM IPTG was added and the mixture was incubated for further 1-2 h at 37ºC. Samples of mixtures without IPTG (donor cells only) and recipient cells were directly plated and incubated O/N at 37ºC. Mixtures with IPTG were spot plated (made one large spot of growing cells) on LB plates and incubated O/N at 37ºC.

2.2.13. Insertional mutagenesis of rex genes and isolation of Rex⁻ mutants: Candidate of trans-conjugants were diluted in one mL LB and 200 µL aliquots were prepared for plating. Fresh pre-warmed Tc + Km plates were prepared and seeded with 10^{-5} T4r*II* fresh lysate (prepared in TN buffer). A volume of 200 µL of cells were plated and incubated O/N at 30ºC. The next day, the trans-conjugation frequencies for Rex⁻ mutants were calculated and the Rex⁻ bitten colonies (colonies with irregular morphology which indicates the sensitivity of these cells to T4*rII* is increased after mutation) and frequencies were determined.

2.2.14. Assessing the effect of omp mutations on Rex activity: Following the spot test of T4 and T4*rII* plating assay, we were able to assess the Rex activity of Δ*omp* mutants. Cultures of Rex⁻ isolated mutants of Δ*omp* were prepared in 5 mL aliquots at 1:100 dilution and incubated in shaking O/N. 300 µL of culture was added to 3 mL top agar and poured immediately onto pre-warmed LB plates, then left on bench to solidify. Serial dilutions of T4 (wt) and T4r*II* lysates were prepared in TN buffer with concentrations ranging from 10^{-2} to 10^{-8} phage/mL to quantify plating efficiency and Rex activity. Lysates were added in 10 µL drops onto isolated Rex⁻ mutants overlay plates. Plates were left for 30 min to dry, then incubated O/N at 30ºC. The following day, plates were assessed and plaques (where evident) were quantified.

2.2.15. Phage λ (rex⁺) lysogenization of Rex⁻ isolated mutants: Fresh O/N cultures were prepared from a single colony of each *omp* and isolated Rex⁻ mutants, in LB at 30ºC. Overlay

plates were prepared by mixing 300 µL of the cell cultures with three mL of top agar and plated on LB plates. Plates were left on bench for 20 min until the top agar solidified and then 10 µL of 10^{-4} of fresh λ*cI*-857 and λ wt lysates in TN buffer and were then spotted onto the experimental plates. Phage spots were allowed to dry and the plates were incubated O/N at 30°C for λ*cI*857 lysogens and at 42°C for λ wt lysogens. The next day, large turbid plaques were visualized and isolated using sterile toothpicks to confirm λ lysogeny by immunity assay. The λ*cI*857 lysogens were grown at 30°C and then tested through an incubation at 42°C to inactivate the CI repressor where any lysogens would be induced for phage growth and lyse their resident cells, thereby killing the host. For λ wt lysogens, cells that were able to grow at both 30°C and 42°C were tested for confirmation of the presence of λ wt lysogens by stabbing cells in top agar of λF7 overlay plating assay to test for presence of λ immunity to confirm lysogenization.

2.2.16. Lambda immunity assay: Lambda (wt) lysogens that grew at both temperatures (30°C and 42°C) were streaked out to test a single colony per lysogen. An O/N culture of heteroimmune lysogen W3899 (λF7) was prepared and 300 µL of this fresh culture was mixed with 10 µL of 10^{-4} λF7 lysate diluted in TN buffer and added to three mL of top agar before pouring onto an LB plate. The experimental colonies were stabbed in the overlay top agar using sterile toothpicks, and incubated O/N at 30°C.

CHAPTER THREE

3. Results

3.1. Construction of a one-step transposable *rex*$^{+}$ insertional mutagenesis vector:

The objective behind the construction of a transposable *rex*$^{+}$ suicide vector was to mutagenize and provide a naturally expressed *rex*$^{+}$ genotype through a single transposition event into recipient cells. Through the use of this vector and the collection of thousands of mutants, it was expected that we would be able to isolate and identify host gene(s) that were involved in the *rII* exclusion phenotype (Rex phenotype) by using transposon-mediated mutagenesis, followed by subsequent characterization.

3.1.1. Assessing T4/T4rII (Rex) activity on E. coli (λcI857): We employed *E. coli* W3110 (λ*cI857*), a wild type *E. coli* K-12 strain lysogenized by Rex^{+} λ prophage as the source for the P_M-*cI-rexA-rexB-*t_{imm} operon. To ensure that the strain was Rex^{+}, the lysogen was first tested for exclusion of T4*rII* to ensure Rex activity and for RII-suppression (wild type RII^{+} T4*D* (RII^{+}) at various dilutions (Fig. 8).

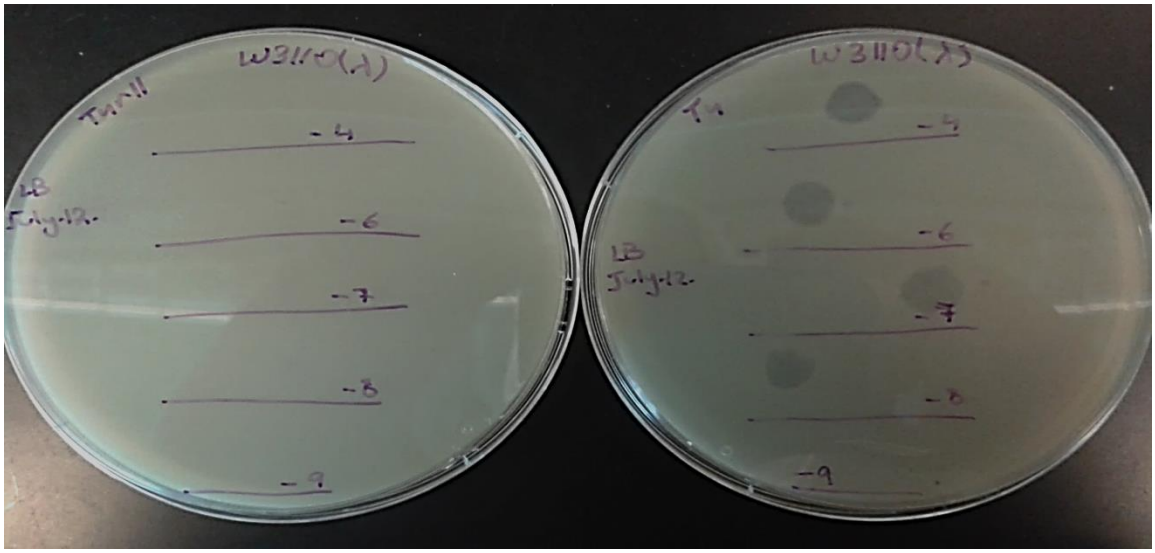

Figure 8: T4*rII* exclusion phenotype. Infection *E. coli* W3110 (λ*cI857*) by T4*rII*Δ1589 (left) and T4*D* (right) at increasing dilutions. 10 µl of each T4 and T4*rII* in serial dilutions from a stock concentration of about 6.9 x 10^9 pfu/mL were spotted onto a W3110 (λ) host cell overlay at phage dilutions of: 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Phage T4 (*rII*$^+$) shows plaques at all dilutions10^{-4}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. In contrast, T4*rII* replication was aborted for infection (*rII* exclusion) on W3110 (λ*cI857*), indicative of the Rex phenotype.

Serial dilution spots of T4*D* (T4 wild type) and T4*rII*Δ1589 phage (RII$^-$ phage deletion that spans *rIIA* into *rIIB*) were placed as labeled on *E. coli* W3110 (λ*cI857*) overlays, and incubated upright O/N at 30ºC. Phage T4 plaque formation on W3110 (λ*cI857*) was observed at all dilutions ranging from 10^{-2} to 10^{-7}. T4 plating compared to a W3110 100% control was equivalent (1.0) or higher, indicating perfect infectivity on this strain. In contrast, T4*rII* was incapable of forming plaques even at highest concentration/lowest lysate dilutions and exhibited Rex-mediated aborted infection even at a concentration of 6.9 x 10^7 pfu/mL (10^{-2} dilution) T4*rII*, although evidence of cell-killing (feint cell background) was evident at this concentration. Based on these results and confirmation of Rex activity, W3110 (λ*cI857*) was deemed a reliable source from which to amplify the λ immunity region for subsequent *rex*$^+$ vector construction.

3.1.2. Lambda (λ) imm operon (rex$^+$) fragment synthesis: Once the Rex$^+$ phenotype of λ*cI857* was confirmed, the λ immunity region (λ*imm*) from the W3110(λ*cI857*) lysogen served as a template for PCR amplification, using designed ImmF (forward) and ImmR (reverse) primers (Fig. 9A)

Imm L forward: 5' GGGGGGCATTGTTTGGTAGGTGAGAGAT 3'

Imm L reverse: 5' TTGATCGCGCTTTGATATACGCCGAGAT 3'

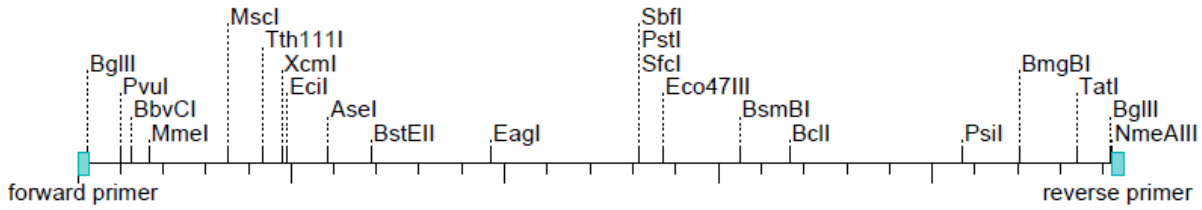

Figure 9A: Lambda *imm* operon (*rex*$^+$) shows sites of λ*imm*F (forward) and λ*imm*R (reverse) primers resultant fragment *Bgl*II cut sites at 35,711 bp and 38,103 bp of λ *imm* region.

The λ*imm* region was amplified with the 28 bp sequence of forward primer, ImmF encoding the sequence of λ 35,739 bp to 35,711bp, and with the 28 bp sequence of reverse primer ImmR encoding the sequence of λ 38,103 bp to 38,131 bp. The amplified PCR fragment was predicted at 2.42 kb and was confirmed by 0.8 % agarose gel electrophoresis. One µg of PCR DNA product per reaction was then digested at two sites flanking the P_M-*cI*-*rexA*-*rexB*-t_{imm} operon for cloning (Fig. 9B, C) and the final *Bgl*II-*cI*-*rexA*-*rexB*-*Bgl*II size fragment was purified at the expected DNA band size of 2.39 kb (Fig. 9D).

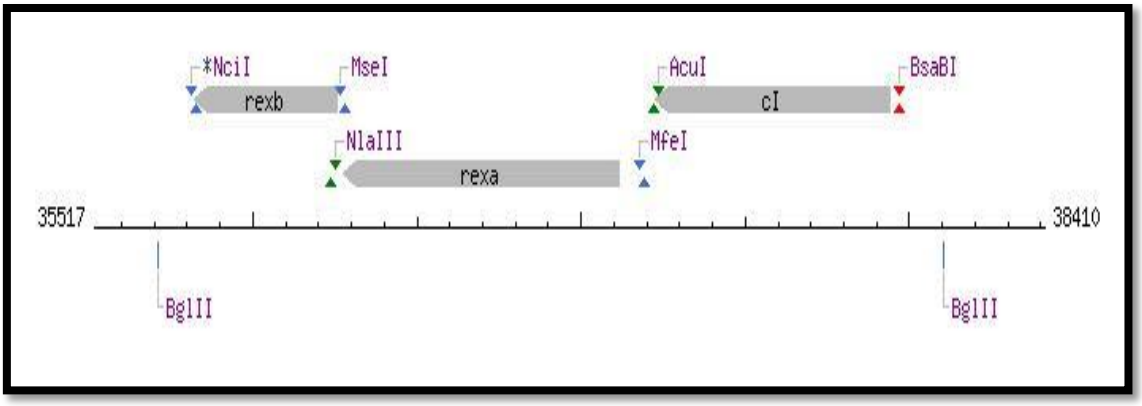

Figure 9B: Linear sequence of *Bgl*II-P_M- *cI-rexA-rexB*-t_{imm}- *Bgl*II PCR fragment showing the two sites of *Bgl*II restriction enzyme.

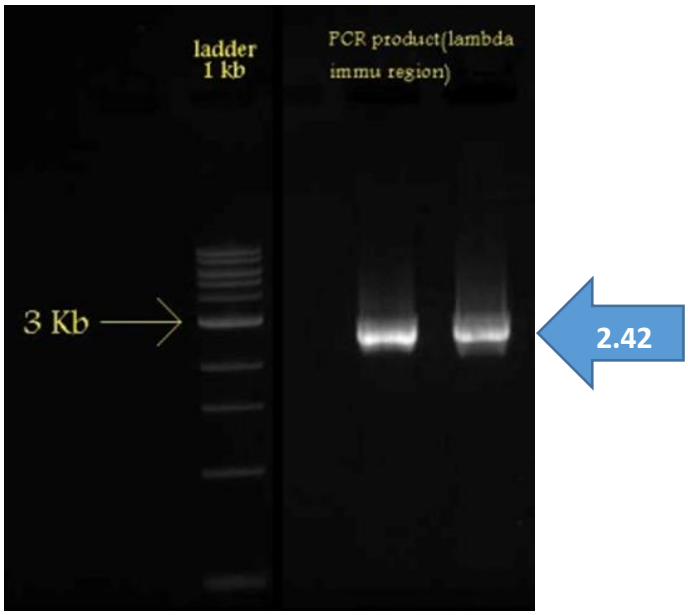

Figure 9C: The uncut PCR operon *cI-rexA-rexB* shown as expected 2.4 kb band

Lane 1: 1 kb ladder; Lane 2, 3: uncut PCR fragment

Description: BglII cuts at 35711, and 38103 sites

Cut Action: Cut with BglII
Molecule left end is blunt; right end is blunt

Enz L	Start	End	Size	Enz R
Left end	1	671	671	BglII
BglII	672	3063	2392	BglII
BglII	3064	3120	57	Right end

Figure 9D: The final PCR fragment *Bgl*II-P_M- *cI-rexA-rexB-*t_{imm}- *Bgl*II size and cut positions. Final size 2.39 kb

3.1.3. Construction of the high copy (pHA1) rex*$^+$ *plasmid: The commercial high copy plasmid, pUC19 was employed to clone the P_M- *cI-rexA-rexB-*t_{imm} operon/fragment employing a compatible cut site in the multiple cloning site (MCS) of pUC19 (Fig. 10A-C)

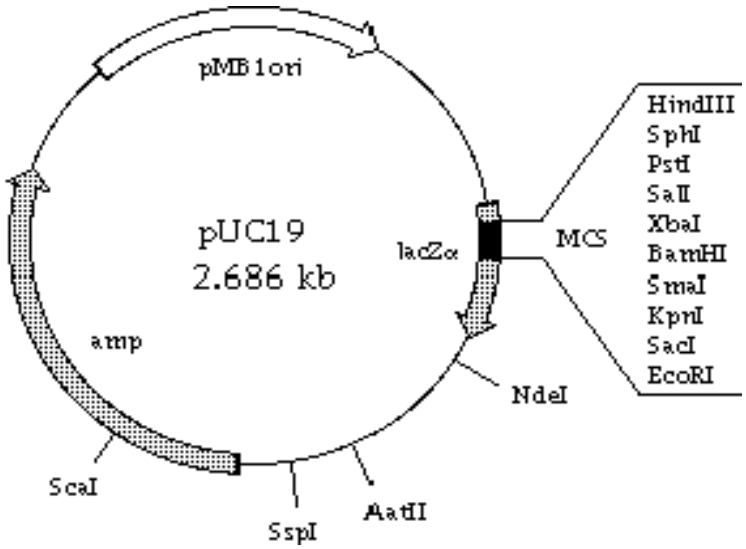

Figure 10A: Map of plasmid pUC19 shows *Bam*HI endonuclease target site in the MCS.

Following the digestion of pUC19 with *Bam*HI, the λ*imm* region *Bgl*II-*cI-rexA-rexB-Bgl*II fragment was sub-cloned into the linear plasmid pUC19 (Fig. 10B, 10C) to form the high copy *rex*$^{+}$ plasmid, pHA1. Successful candidate clones were isolated using X-Gal and IPTG by blue/white screening as described in Methods. The new pHA1 construct was confirmed at the expected ~5 kb size.

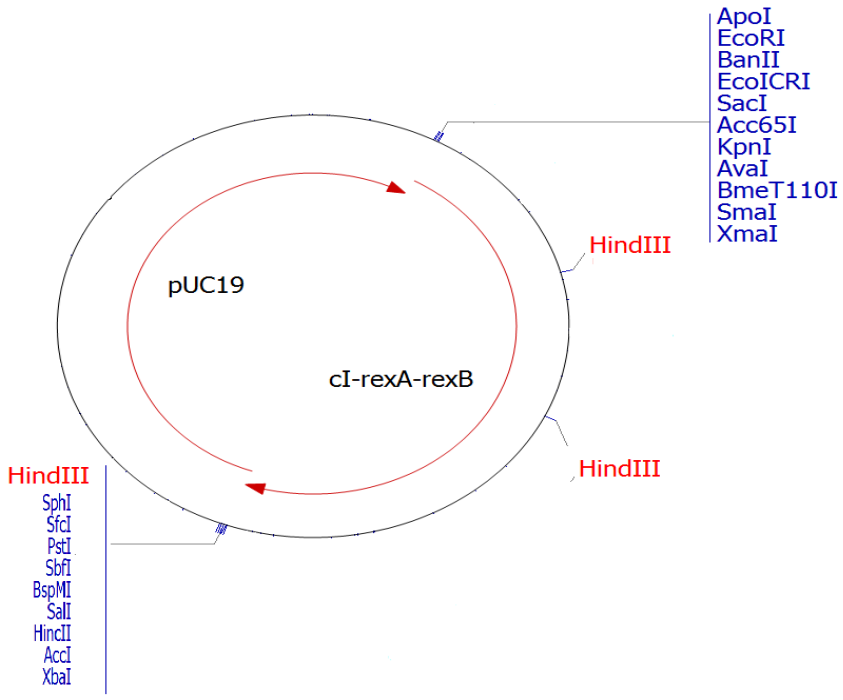

Figure 10B: The new construct pHA1 showing the insert orientation and direction within the MCS. *Hind*III cuts at three different sites.

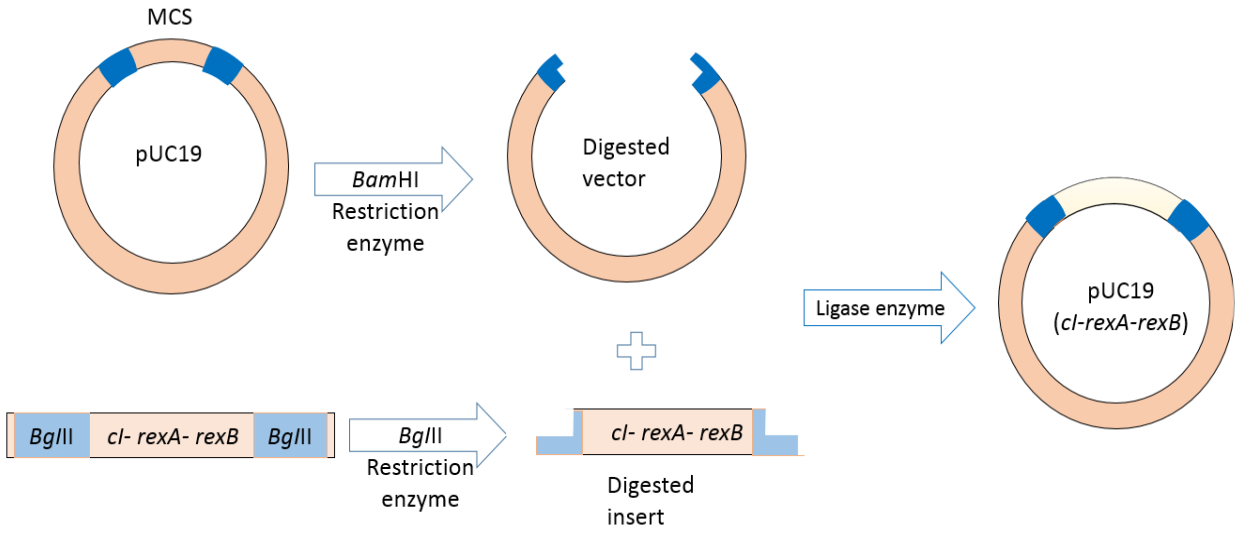

Figure 10C: pHA1 construct cloning: 1) vector and insert preparation. 2) vector digested by *Bam*HI and insert digested by *Bgl*II restriction enzyme to yield compatible ends 3) digested vector and insert were ligated using T4 DNA ligase enzyme.

IPTG was used as a gratuitous inducer of the *lac* operon, binding to the LacI repressor, thereby inactivating it and inducing the *lac* operon in harbouring cells. Cloning within the MCS inactivates *lacZα*, and in the presence of X-Gal, recombinant colonies appear white; while non-recombinants retained functional *lacZα* and colonies exhibited X-Gal hydrolysis and blue colour. As the cloning was not directional, the insert sequence orientation was confirmed by restriction fragment analysis (Fig. 10D-E). The desired insert fragment was confirmed for proper insertion and orientation in vector pHA1 based on the fragment analysis.

Figure 10D: *Hind*III cuts at three different sites of the pHA1; one site within *cI857*, one site within *rexA*, and a third site within the MCS of the pUC19 backbone. Lane 1: 1 kb ladder; Lane 2, 3: Digested pHA1 plasmid by *Hind*III

Cut Action: Cut with HindIII

Enz L	Start	End	Size	Enz R
HindIII	1062	1625	564	HindIII
HindIII	1626	2839	1214	HindIII
HindIII	2840	1061	3300	HindIII

Figure 10E: Band sizes of pHA1 fragments after digestion by *Hind*III that cuts once in *cI* and once in *rexA*.

3.1.4. Assessing Rex activity and RII-resistance of the pHA1 high copy Rex plasmid: Cells were transformed by the pHA1 plasmid and subjected to EOP assays for T4 and T4*rII*Δ1589, using W3110 (λ*cI587*) and W3110 (Rex$^-$) as positive and negative controls, respectively. As expected, strains harbouring the pUC19 (*rex*$^-$) plasmid strains that controlled for plasmid backbone and cell background were both sensitive to T4 and T4*rII* plating at near complete efficiency, whereas, the Rex$^+$ strains, W3110(λ*cI857*) and DH5α [pHA1] *(rex*$^+$*)* strains displayed expected sensitivity to the RII$^+$ phenotype of T4 plating at an EOP approaching 1.0, but exhibited no T4*rII* plating (EOP <10^{-6})—indicating a difference in EOP of more than 10^6-fold imparted by the *rII*$^+$ genotype of T4, and confirming a powerful Rex phenotype as imparted by the pHA1 plasmid, specifically by the λ*imm* operon (*cI-rexA-rexB*) cloned into this plasmid.

Table 2: Efficiency of plating of T4*D* and T4*rII*Δ1589 phage on Dh5α [pHA1]:

Strain	**Plasmid**	**Relative Efficiency of Plating (E.O.P)[1]**	
		T4	**T4*rII***
W3110 (wt)	None; *rex*−	~ 1.0	~ 1.0
W3110 (λ*cI857*)	None; λ lysogen; one chromosomal copy *rex*$^+$	~ 1.0	$< 10^{-6}$
DH5α	None: *rex*−	~ 0.96	0.98
DH5α	pUC19; *rex*$^-$plasmid control for Rex activity	~ 0.98	0.98
DH5α	pHA1; *rex*$^+$; high copy *rex* expression plasmid	~ 0.97	$< 10^{-6}$

[1] W3110 (λ*cI857*) lysogen was used as the positive control for Rex activity and W3110 was used as 100% control for phages plating. All EOPs are resultant average of EOPs from three independent trials.

3.1.5. Preparation of the rex$^+$ suicide plasmid: The strain DH5α (λ*pir*) that encodes π, the initiation of replication protein for the R6K origin of replication (R6K *ori*), was used as a host cell to construct and amplify the intended *rex*$^+$ suicide plasmid, pHA2. The pHA2 plasmid is comprised of the *λimm* (Rex$^+$) operon inserted into the BSL199 suicide plasmid (Fig. 11A). The suicide plasmid possesses two antibiotic resistance markers: ampicillin (Ap^R) found on the plasmid backbone and tetracycline (Tc^R) within the transposable Tn*10* element itself. The plasmid was ideal to serve as a suicide vector as replication of the plasmid requires the π initiation protein, which is not present in wild type cells and thus the plasmid is incapable of replication and cannot confer antibiotic resistance.

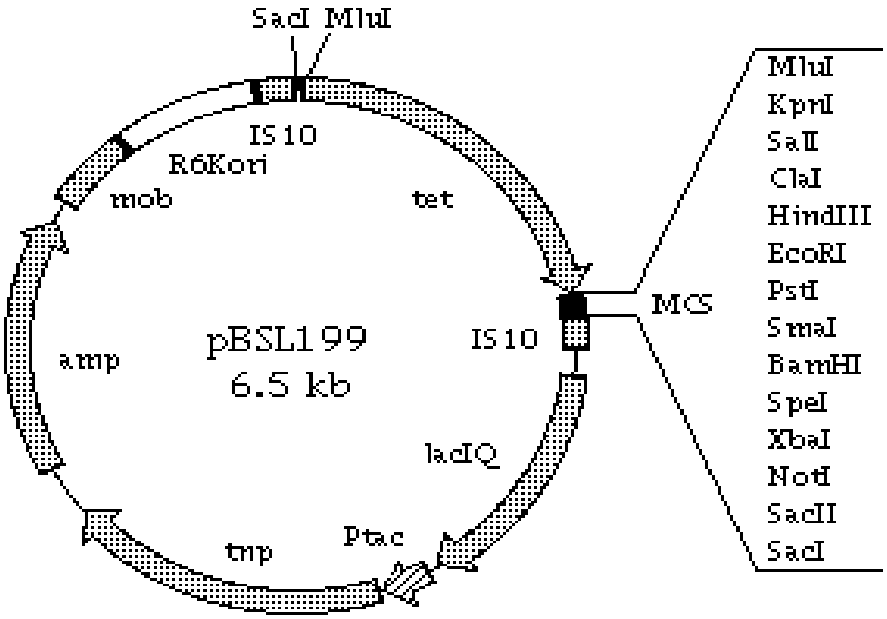

Figure 11A: Map of suicide plasmid, pBSL199, showing the antibiotic marker sites on the plasmid backbone (Ap^R) and within the transposable element (Tc^R), and the multi-cloning site (MCS). Source: Alexeyev MF (1995).

Following transformation, plasmid extraction and purification, plasmids were digested to generate a suitable backbone for the formation of pHA2 (Fig. 11B) and confirmed at the expected size (6.4 kb).

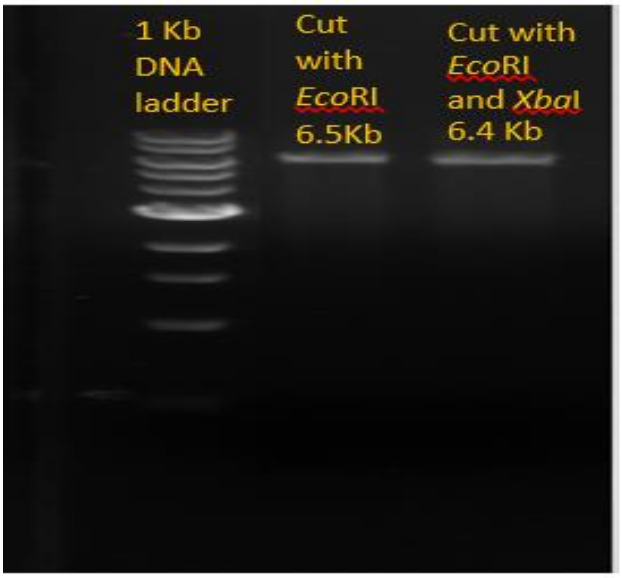

Figure 11B: Gel image shows band sizes of pBSL199 cut once with *Eco*RI (6.5 kb), and once with *Eco*RI and *X*baI (desired 6.4 kb fragment. Lane 1: 1 kb ladder; Lane 2: pBSL199 cut with *Eco*RI; Lane 3: pBSL199 cut with *Eco*RI and *X*baI.

The *cI-rexA-rexB* fragment from pHA1 was confirmed (2.4 kb) in preparation for cloning into pBSL199 and construction of the suicide *rex*$^{+}$ plasmid, pHA2 (Fig. 11C-E).

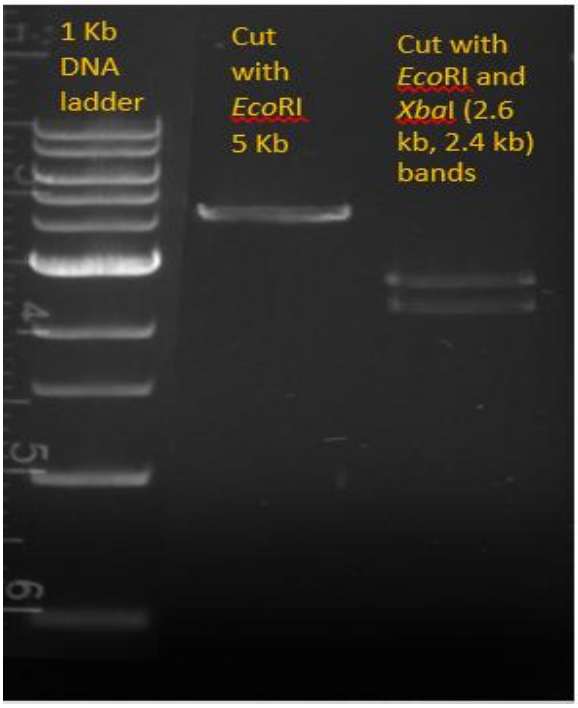

Figure 11C: Gel image shows pHA1 fragments after cutting with *Eco*RI alone (band size 5 kb) and with *X*baI+ *Eco*RI (two bands: pUC19 backbone 2.6 kb-upper band, and *cI-rexA-rexB* 2.4 kb- lower band). Lane 1: 1 kb ladder; Lane 2: pHA1 cut with *Eco*RI; Lane 3: pHA1 cut with *Eco*RI and *X*baI

Cut Action: Cut with EcoRI + XbaI

Enz L	Start	End	Size	Enz R
XbaI	258	2676	2419	EcoRI
EcoRI	2677	257	2659	XbaI

Figure 11D: The pHA1 fragment sizes after cut with *X*baI+ *Eco*RI restriction enzymes

The desired 2.4 kb DNA insert encoding (*Xba*I-*cI-rexA-rexB-Eco*RI) was purified in preparation for construction of the final pHA2 plasmid (Fig. 11C-E).

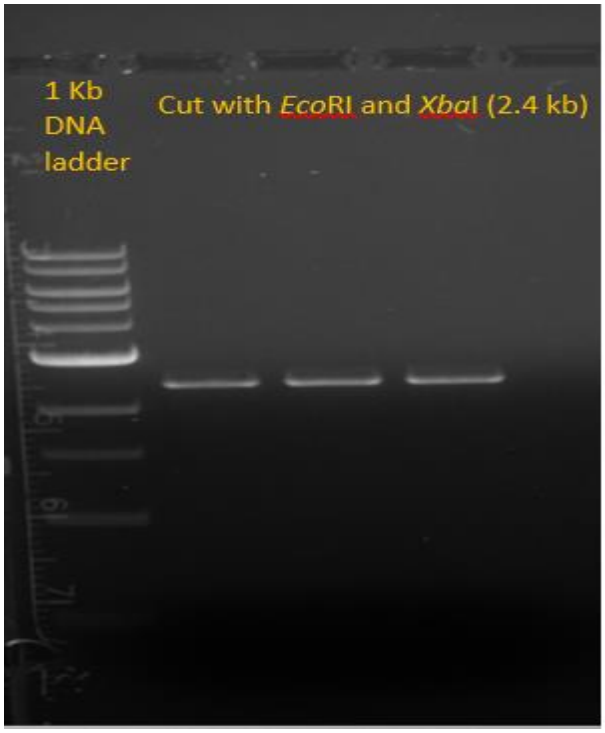

Figure 11E: Gel image shows the pure 2.4 kb (*X*baI-*cI-rexA-rexB-Eco*RI) insert band. Lane 1: 1 kb ladder; Lane 2, 3, 4: purified insert fragment

3.1.6 Constructing and assessing the final rex$^+$ suicide plasmid [pHA2]: The *cI-rexA-rexB,* (2.4 kb) insert was confirmed and ligated into the suicide plasmid (6.4 kb) to form the final *rex*$^+$ suicide vector (Fig. 12A). Extracted plasmid was then digested to confirm the presence of the *rex* operon, to confirm that only a single insert fragment was sub-cloned per a linear pBSL199 vector and to confirm the 8.7 kb size (Fig. 12B).

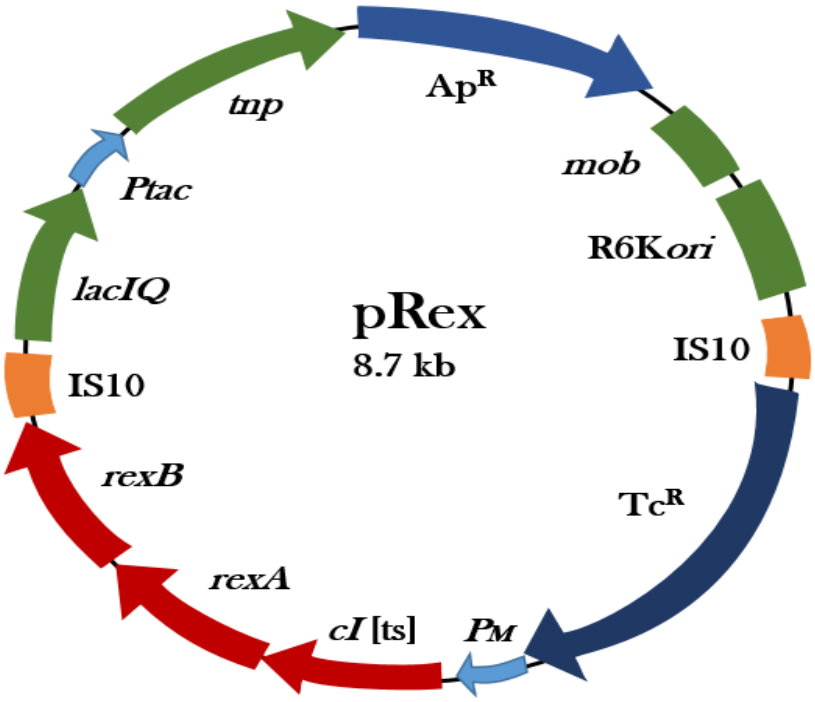

Figure 12A: The pHA2 pRex suicide construct: The R6K *ori*-based suicide vector, pBSL199, carries the thermo-regulated P_M-*cI857*-*rexA*-*rexB*-t_{imm} cassette flanked by two IS10 sequences. This plasmid is incapable of replication in wild type *E. coli* (*pir*$^-$) cells, but conditional expression of the *Tn*10 transposase allows the mini *Tn*10 cassette between IS10 elements (encoding the *rex* operon) to jump into the host chromosome resulting in the generation of a library of host gene insertional inactivations that are simultaneously genotypically *rex*$^+$, ensuring that any isolated Rex$^-$ cells are generated only by transpositional *E. coli* insertional inactivation mutations. This construct encodes also: *lacI*^{Q}: mutant lac repressor that modulates LacI expression; P_{tac}: promoter regulates expression of all genes downstream in the presence of inducer IPTG; *tnp*: transposase enzyme stimulate transposition of transposable element; *mob*: mobility or transfer gene for conjugative transfer of plasmids between donor and recipient cells; Ap^R: ampicillin resistance marker on plasmid backbone; Tc^R: tetracycline resistance marker on transposable element.

The pHA2 vector permits the movement of the transposable fragment including the *rex*$^+$ insert of interest from the originating plasmid indiscriminately to other sequences by a transposase-mediated cut and paste mechanism in a controllable manner. The plasmid was constructed to serve as a *rex*$^+$ mutagenic tool to systematically generate single mutations in host cells due to the transpositional capabilities of the resident *Tn*10 insertion sequences in a controllable manner,

while simultaneously conferring a *rex*$^{+}$ phenotype to mutant integrants. To ensure that the resultant pHA2 suicide vector was in fact *rex*$^{+}$, replication- permissive DH5α (λ*pir*$^{+}$) cells were transformed by pHA2 and transformants were assessed for Rex activity and RII-suppression (Table. 3). Compared to the cell and pBSL199 (*rex*$^{-}$) control, pHA2 conferred a 10^{7}-fold lower EOP of T4*rII*, while T4 plated with near perfect efficiency, despite that the plasmid is mid-copy number, offering multiple copies of the *rex* locus. These results confirmed the previous assumption that the new pHA2 construct could impart a powerful Rex phenotype and was sufficient to employ toward subsequent mutagenesis assays.

Table 3: Transformation efficiency of pHA2:

Strain	**Plasmid**	**Relative E.O.P** [2]	
		T4	**T4*rII***
DH5α [1]	None ; (Rex^{-})	1.0	1.0
DH5α (λ*pir*)	None ; (Rex^{-})	~ 1.0	~ 1.0
DH5α (λ*pir*)	pBSL199 ; (Rex^{-})	~ 1.0	0.97
DH5α (λ*pir*)	pHA2[3] ; (Rex^{+})	0.93	< 10^{-7}

[1] *E. coli* strain DH5α (*pir*$^{-}$) was used as 100% control with relative T4 and T4*rII* EOP of 1.0

[2]The relative EPOs are the average of the minimum of three trials.

[3] pHA2 is pBSL199- *cI-rexA-rexB*

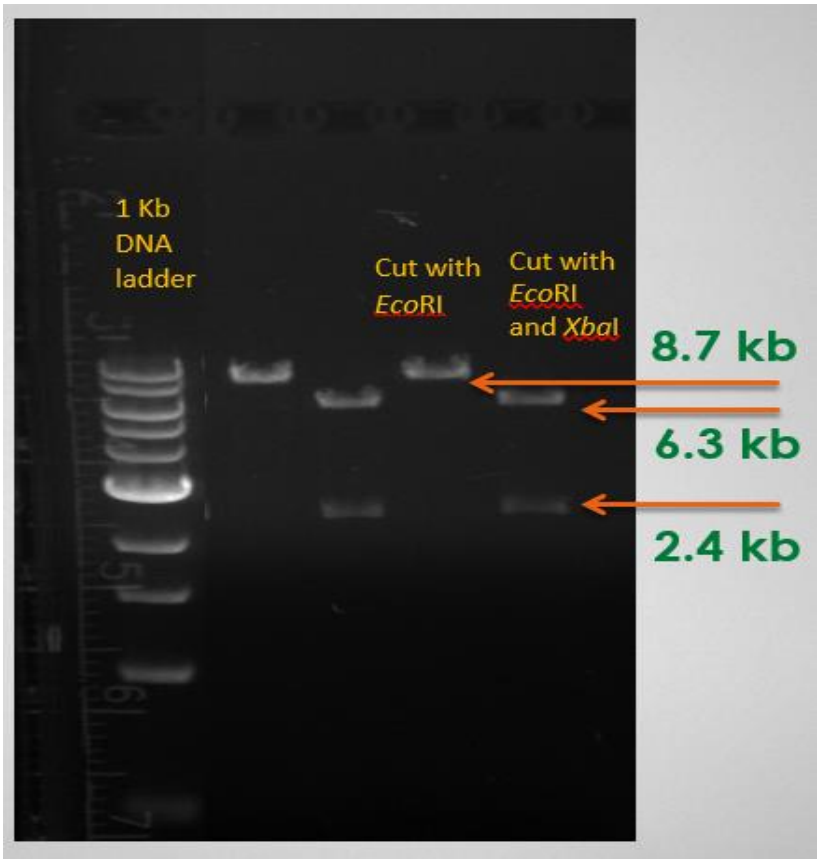

Figure 12B: Fragment analysis after pHA2 digestion by *Eco*RI alone (linear plasmid 8.7 kb) and by *X*baI+ *Eco*RI together (two fragments (plasmid pBSL199 backbone 6.3 kb and insert cassette 2.4 kb). Lane 1: 1 kb ladder; Lane 2, 4: plasmid pBSL199 backbone 6.3 kb; Lane 3, 5: insert cassette 2.4 kb

3.2.Mutagenesis and isolation of *rex*$^+$ second site *E. coli* mutant(s) that abrogate the Rex phenotype:

Using the *rex*$^+$ suicide pHA2 plasmid system, a one-step mutagenesis system was employed to systematically knock out non-essential host genes that are involved in Rex activity, while ensuring the wild type expression of the *rex* genes. Hence, candidates would be genotypically *rex*$^+$, but phenotypically influenced for Rex activity.

3.2.1. Transformation-mediated transposition of rex$^+$ element from pHA2: Cells were first transformed by the pHA2 construct under transposase-inducing conditions to stimulate transposition from the plasmid to random sites in the chromosome. Plates were also seeded with T4*rII* lysate in the attempt to isolate Rex$^-$ mutants as "bitten" colonies (colonies with irregular morphology which indicates the sensitivity of these cells to T4*rII* is increased after mutation). This approach proved unsuccessful as no bitten colonies of Rex$^-$ integrants were detected, this was likely due to the observed very low efficiency of transformation. As such, we next sought to mutate cells by conjugation-mediated transposition of the *rex*$^+$ element from pHA2 conjugative plasmid.

3.2.2. Conjugative transposition rex$^+$ element from pHA2: The pBSL series of plasmids are also RP4 conjugative plasmids that encode a *mob* gene (mobility/transfer gene) that can be horizontally transferred into host cells via conjugation between donor and recipient cells. *E. coli* strain S17-1 (λ*pir*) encodes RP4 helper-transfer functions, which can confer the transfer of the pHA2 plasmid into the intended recipient. The S17-1(λ*pir*) was employed in bi-parental mating (horizontal mating) to deliver pHA2 to our intended *pir*$^-$ recipient.

Transposition of the Tc^R marker into recipient cells (Km^R) indicated successful transposition and integration of the Tn*10* carrying the *cI857-rexA-rexB* operon. The recipient JW0427-1 (Δ*clpP*, Km^R) *E. coli* derivative cells of the parent Keio series strain BW25113 were horizontally conjugated with [pHA2] donor cells as described. Following the one-step mutation isolation of Rex$^-$, we were able to insert the *rex* genes at random location to confer the rex$^+$ genotype and at the same time cause a mutation of one gene on the host strain (Fig. 13A).

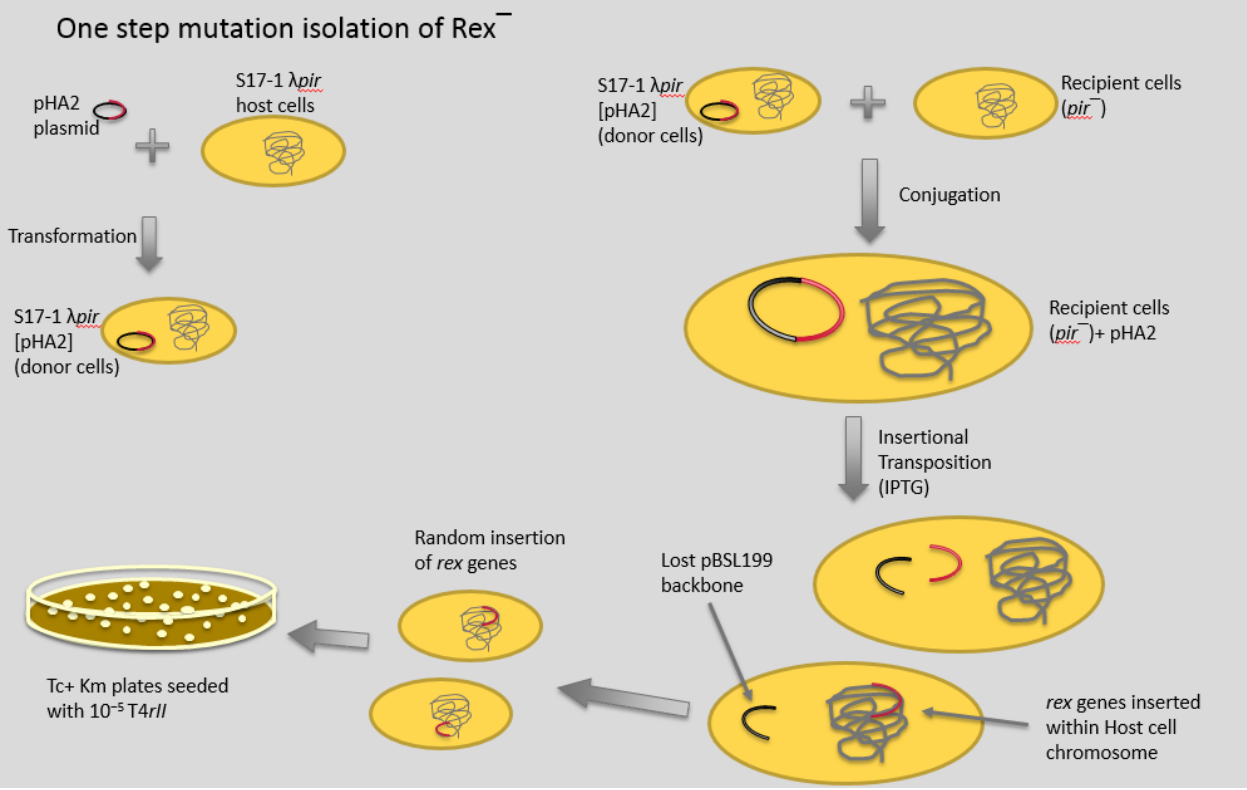

Figure 13A: One-Step mutation isolation of Rex⁻ mutants: 1) transformation of S17-1 *λpir* cells by pHA2 plasmids and use them as donor cells; 2) conjugation between donor cells (*pir*$^{+}$) and recipients cells (*pir*$^{-}$) to transfer pHA2 suicide plasmid; 3) add IPTG to induce transposition of *Tn*10-*rex*$^{+}$ fragment directly from its originality plasmid into host cell chromosome at random locations to make library of gene insertional inactivations and confer *rex*$^{+}$ genotype on host strains. 4) plating of cells on plates seeded with 10^{-5} T4*rII* and screen for bitten colonies (Rex^{-}).

ClpP has previously been shown (Slavcev and Hayes, 2005) not to influence Rex activity, and as such this strain was employed as a suitable mutant strain carrying a *km*^{R} contra-selection marker for conjugation. Several selected isogenic outer membrane protein mutants (Δ*omp*) were also conjugated to generate a single chromosomal copy of *rex*$^{+}$ and to later analyse for Rex activity, following integration of the *rex*$^{+}$ element. Successful transposition of the *rex*$^{+}$ element under inducing conditions generally ranged from 10^{-6} to 10^{-7} between strains, and the lowest frequency was from the *ompA* mutant that is considered to play a role in conjugation (Table 4). The non-

induced control exhibited no integrants, as expected, while we were pleased to find that the employed wild-type (*clpP::km*) offered the greatest frequency of transposition under induced conditions.

Table 4: Transposition frequencies via Rex conjugative suicide plasmid:

***E. coli* Recipient Strains**	**Δ*omp* (km^R) Location**	**Frequency of *rex*$^+$ integrants[1]**
JW0427-1 (Δ*clpP::km*)	-	4.1×10^{-6}
Un-induced control	-	$< 1.0 \times 10^{-9}$
BW25113 (wt)	-	-
JW0554-1	Δ*ompT*	1.5×10^{-6}
JW0799-1	Δ*ompX*	7.0×10^{-7}
JW0912-1	Δ*ompF*	9.0×10^{-7}
JW0940-6	Δ*ompA*	4.0×10^{-7}
JW1248-2	Δ*ompW*	8.7×10^{-7}
JW1312-1	Δ*ompG*	3.3×10^{-7}
JW1371-5	Δ*ompN*	5.0×10^{-7}
JW2203-1	Δ*ompC*	1.0×10^{-6}
JW3368-1	Δ*ompR*	1.2×10^{-6}
JW3846-1	Δ*ompL*	1.8×10^{-6}

[1]The frequency of transposition was given per donor cell and was scored as number of Tc^R-Km^R CFU/total number of Km^R donor CFU. The frequencies of transposition were averaged based on a minimum of three trials for each strain. 0.1 mM IPTG was added two hours after conjugation reaction started.

3.2.3. Screening of mutant integrants, confirmation of rex$^+$ genotype and assessment of Rex activity: We next sought to generate a Rex$^-$ mutant library of integrants using conjugative transposition of the pHA2 plasmid on *clp::km* cells by plating resulting integrants directly on plates seeded with T4*rII* as part of a one-step screen. Mutants that are Rex$^+$ are able to exclude T4*rII* and are thus, unaffected by the presence of phage seeded on plate and go on to form regular, smooth and circular colonies. In contrast, Rex$^-$ mutants that cannot abort T4*rII* infection are characterized by irregular, "bitten" colonies (Fig. 13B, C). Integrants were incubated for 24 h without showing any sign of growing, so they were incubated for another 16-24 h where colonies were finally visible. Several hundreds of integrants were isolated and screened. The number of bitten colonies varied per plate with the ratio of bitten to regular colonies averaging approximately 1:1500 in JW0427-1 (Δ*clpP*) recipient cells (antibiotic marker control), which was within the expected frequency of 0.1- 0.5% compared to the total number of *E. coli* genes. We noted that the number of persistent Rex$^-$ mutant candidates dramatically decreased upon confirmatory screening, and that the Rex activity of the 13 confirmed mutants exhibited a decrease in Rex activity of between 10 to 10^4-fold compared to the Rex$^+$ lysogen control. Although these mutants are gentoypically *rex*$^+$, the noted reduction in T4*rII* plating strongly suggests the successful mutation of a host gene that is either directly or indirectly involved in Rex phenotypic activity.

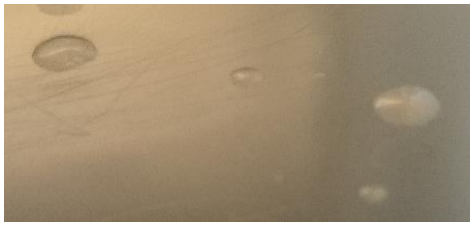

Figure 13B: Normal Rex$^+$ colonies

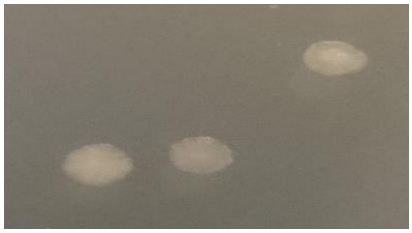

Figure 13C: Bitten Rex$^-$ colonies

Table 5: Confirming isolated *rex*$^{+}$ *E. coli* mutant(s) for sensitivity to T4*rII* plating:

***E.coli* JW0427-1** **# of Mutant**	**Mutations** **(Δ*clpP*) (*rex*$^{+}$)**	**Relative Efficiency of Plating [1]** **(T4*rII*)**	**Rex Activity**
JW0427-1	*rex*$^{-}$	1.0	< 1 x 10^{7}
JW0427-1 (λ)	λ^{+}*(rex*$^{+}$*)*	< 1 x 10^{-7}	1.0
JW0427-1 [2]	*rex*$^{+}$	< 1.0 x 10^{-7}	1.0
# JW-HA 1	*rex*$^{+}$	2.3 x 10^{-5}	< 100 fold
# JW-HA 2	*rex*$^{+}$	7.0 x 10^{-6}	< ~100 fold
# JW-HA 3	*rex*$^{+}$	1.5 x 10^{-5}	< 100 fold
# JW-HA 4	*rex*$^{+}$	1.0 x 10^{-3}	< 10K fold
# JW-HA 5	*rex*$^{+}$	1.7 x 10^{-5}	< 100 fold
# JW-HA 6	*rex*$^{+}$	1.4 x 10^{-4}	< 1K fold
# JW-HA 9	*rex*$^{+}$	5.0 x 10^{-6}	< 10 fold
# JW-HA 11	*rex*$^{+}$	1.6 x 10^{-5}	< 100 fold
# JW-HA 12	*rex*$^{+}$	1.3 x 10^{-5}	< 100 fold
# JW-HA 19	*rex*$^{+}$	2.0 x 10^{-4}	< 1K fold
# JW-HA 20	*rex*$^{+}$	8.0 x 10^{-6}	< ~100 fold

# JW-HA 21	*rex*$^+$	1.6 x 10^{-5}	< 100 fold
# JW-HA 25	*rex*$^+$	1.8 x 10^{-5}	< 100 fold

[1] *E. coli* strain JW0427-1 (Δ*clpP*) (*rex*$^-$) used as 100% control with relative T4*rII* EOP of 1.0. All values were from three replicates in three independent experiments.

[2] JW0427-1(Δ*clpP*) (*rex*$^+$) was an experimental mutant that appeared to be Rex$^+$ based on the colony morphology (smooth circular with entire edges shape).

To confirm that these mutants were in fact *rex*$^+$ and the modulated Rex activity was not due to a faulty *rex* cassette, the Rex-attenuated integrants were then lysogenized by λ (*rex*$^+$) to deliver yet another copy of the *rex* genes. These lysogens were then plated with T4*rII* phage and tested again for Rex activity (Table 6). Again, the parent Δ*clpP::km* (λ) lysogen served as a working positive control for Rex activity exhibiting no T4*rII* plating activity as compared to the non-lysogenized Δ*clpP* (Rex$^-$) control. As previously found with the pHA1 plasmid, mutants JW-HA6/9/19 each exhibited similar attenuation of Rex activity, and in a relatively gene dosage-independent manner.

Table 6: Rex activity of isolated *rex*$^+$ integrant λ lysogens:

***E.coli* JW0427-1 (# of mutant)**	**Mutations (Δ*clpP*) (*rex*$^+$)(λ$^+$(wt))**	**Relative Efficiency of Plating[1] (T4*rII*)**	**Rex Activity**
JW0427-1 (Rex$^-$)	*rex*$^-$	≈ 1.0	< 1 x 10^7
JW0427-1 (Rex$^+$)	λ$^+$(wt)	< 1 x 10^{-7}	1.0

# JW-HA 1	*rex*$^+$, λ$^+$(wt)	7.5 x 10^{-6}	~1.0
# JW-HA 2	*rex*$^+$, λ$^+$(wt)	5.2 x 10^{-6}	< 10 fold
# JW-HA 3	*rex*$^+$, λ$^+$(wt)	1.7 x 10^{-5}	< 100 fold
# JW-HA 4	*rex*$^+$, λ$^+$(wt)	5.0 x 10^{-4}	< ~1K fold
# JW-HA 5	*rex*$^+$, λ$^+$(wt)	5.0 x 10^{-6}	< 10 fold
# JW-HA 6	*rex*$^+$, λ$^+$(wt)	1.0 x 10^{-4}	< 1K fold
# JW-HA 9	*rex*$^+$, λ$^+$(wt)	4.0 x 10^{-6}	< 10 fold
# JW-HA 11	*rex*$^+$, λ$^+$(wt)	1.3 x 10^{-4}	< 1K fold
# JW-HA 12	*rex*$^+$, λ$^+$(wt)	16 x 10^{-4}	< 1K fold
# JW-HA 19	*rex*$^+$, λ$^+$(wt)	1.5 x 10^{-4}	< 1K fold
# JW-HA 20	*rex*$^+$, λ$^+$(wt)	6.8 x 10^{-6}	< 10 fold
# JW-HA 21	*rex*$^+$, λ$^+$(wt)	1.7 x 10^{-5}	< 100 fold
# JW-HA 25	*rex*$^+$, λ$^+$(wt)	4.0 x 10^{-6}	< 10 fold

[1] *E. coli* strain JW0427-1 (*ΔclpP*) (*rex*$^-$) used as 100% control; *E. coli* strain JW0427-1(λ) used as 100% control of Rex phenotype with relative T4*rII* EOP of < 1 x 10^{-7}. All values were from three replicates in independent experiments.

3.3. Assessing Rex activity of host genes suspected to be involved in T4*rII* exclusion:

3.3.1. T4/T4rII infective center assay: Initial attempts to employ a standard T4/T4*rII* overlay were unsuccessful due to the very small plaque size formed by the phage on *omp* host mutants that attenuated Rex activity. As such, the infective center assay was performed, whereby cells are infected and then cells are diluted and plated on host cells as infected centres, to generate larger, more observable phage bursts. The Δ*omp* mutants and isogenic parent strains were transformed by the pHA1 construct and T4*rII*-infected centers were tested for plating efficiency at an MOI of 3 that by Poisson distribution expects an actual infection of each cell by a single phage. The relative efficiencies of T4 and T4*rII* are shown Table 7. The results of this Rex activity assay on *rex*$^{-}$ Δ*omp* derivatives (no plasmid) are shown in Table 8.

Table 7: T4/T4*rII* plating efficiency on Δ*omp* (*rex*$^{+}$) infective centers:

Strain	Δ*omp* location	Plasmid	Efficiency of Plating [1]		
			T4(wt)[2]	T4*rII*[2]	Rex Activity [1]
BW25113(wt)	_	pHA1	0.2	$< 3.0 \times 10^{-7}$	1.0
JW0554-1	Δ*ompT*	pHA1	0.16	$< 2.0 \times 10^{-7}$	1.0
JW0799-1	**Δ*ompX***	pHA1	0.2	3.0×10^{-4}	$< 10^3$
JW0912-1	**Δ*ompF***	pHA1	0.15	3.0×10^{-5}	$< 10^2$
JW0940-6	Δ*ompA*	pHA1	3.0×10^{-4}	$< 4.0 \times 10^{-7}$	1.0
JW1248-2	**Δ*ompW***	pHA1	0.22	1.0×10^{-4}	$< 10^3$
JW1312-1	Δ*ompG*	pHA1	0.12	$< 2.0 \times 10^{-7}$	1.0

JW1371-5	Δ*ompN*	pHA1	0.3	$< 4.0 \times 10^{-7}$	1.0
JW2203-1	Δ*ompC*	pHA1	$<1 \times 10^{-7}$	$< 1.0 \times 10^{-7}$	_ [3]
JW3368-1	Δ*ompR*	pHA1	3.0×10^{-5}	$<4.0 \times 10^{-7}$	1.0
JW3846-1	**Δ*ompL***	pHA1	0.13	1.7×10^{-5}	$< 10^2$

[1] The EOPs of T4/T4*rII* were determined compared to BW25113 (wt) carrying pHA1 100% control of Rex phenotype. The visible plaques were tiny in size and the infectious center assay was conducted a total of three times and averaged for all strains

[2] Cells were infected by phage at MOI = 3 and phage were adsorbed to cells for 15-20 min before cells were plated

[3] Δ*ompC* mutant was not able to form plaques as OmpC is the absorption site for T4 phage, so neither phage can infect this strain.

Table 8: T4*rII* plating efficiency on Δ*omp* (*rex*−) infective centers:

Strain	**Δ*omp* location**	**T4*rII* E.O.P. [1]**
BW25113 wt (Rex^+)[2]	_	$< 3.0 \times 10^{-6}$
JW0554-1	Δ*ompT*	1.0×10^{-4}
JW0799-1	Δ*ompX*	0.2
JW0912-1	Δ*ompF*	0.9
JW0940-6	Δ*ompA*	0.6
JW1248-2	Δ*ompW*	0.2

JW1312-1	Δ*ompG*	0.4
JW1371-5	Δ*ompN*	0.3
JW2203-1	Δ*ompC*[3]	$< 1.0 \times 10^{-6}$
JW3368-1	Δ*ompR*	0.2
JW3846-1	Δ*ompL*	1.0×10^{-2}

[1] The EOPs of T4*rII* were determined compared to BW25113 wt (Rex^-) 100% control. Cells were infected by phage at MOI = 3 and plated on sensitive cells.

[2] BW25113 wt (Rex^+) is BW25113 [pHA1].

[3] Δ*ompC* mutant was not able to form plaques as OmpC is the absorption site for T4 phage, so neither phage can infect this strain

The wild type parent strain carrying the Rex^+ pHA1 plasmid was employed as positive control of Rex activity and as expected, demonstrated complete resistance to T4*rII* plating ($<10^{-7}$ EOP), but exhibited near complete sensitivity to T4 plating, indicating infective capacity and expected *rII*-deficient exclusion. T4*rII* infectivity for all outer membrane protein deletion (Δ*omp*) mutants carrying the *rex*$^+$ plasmid were observed to be similarly exclusionary with important exception of the following mutants: 1) The Δ*ompX* and Δ*ompW* mutants exhibited a reduction of Rex activity more than 10^3-fold compared to the control; 2) The Δ*ompF* and Δ*ompL* mutants reduced Rex activity more than 100-fold compared to the positive Rex control. This finding was found to be Rex-specific and not due to host range mutation as these mutants including the parent strain were found to be fully sensitive to T4 plating with the exception of the Δ*ompC* control that encodes the adsorption protein for T4 and hence is not capable of being infected by a T4

derivative. Also of notable relevance, the Δ*ompR* and Δ*ompA* mutants were unexpectedly found to be less sensitive to T4 plating under *rex*$^{+}$ conditions, a phenotype which was not observed by *rex*$^{-}$ derivatives, suggesting that this phenotype is specific to Rex. In contrast to these results, all *omp* mutants exhibited near wt T4*rII* plating efficiency except for the *ompT* mutant. Interestingly, this mutant plated T4 efficiently in a *rex*$^{+}$ context, but demonstrated a 10^4 fold reduction in plating in a *rex*$^{-}$ context. This outcome is being confirmed again, but if valid, may suggest a relationship between RII around OmpT.

As these results were found using the high copy Rex^{+} plasmid, pHA1, we next sought to confirm these results using wild type *rex* expression levels. As such, each Δ*omp* mutant was lysogenized by λ*cI*857(*rex*$^{+}$) phage and assayed for Rex activity (Table 9). As expected, with lower *rex* gene dosage, attenuation of Rex activity by specific mutants was generally exacerbated. Notable reduction of Rex activity was again observed for λ lysogens of Δ*ompX*, Δ*ompF*, Δ*ompW* and Δ*ompL* mutants with > 100-fold reduction in Rex activity observed for Δ*ompF*(λ), > 10-fold Δ*ompL*, and > 10^3-fold reduction in Rex activity exhibited by Δ*ompX*(λ), and most notably, > 3X10^3-fold reduction for Δ*ompW*(λ) lysogens, compared to the parent strain BW25113(λ) Rex^{+} control. We also observed a new finding from Δ*ompA*(λ) where it showed no T4*rII* activity in the presence of the pHA1 plasmid, but it demonstrated more than 1K-fold greater T4*rII* infectivity in the presence of one copy of *rex* genes from λ*cI*857 lysogen, suggesting a gene dosage-dependent interplay between Rex and OmpA.

Table 9: T4/T4*rII* plating efficiency on Δ*omp* λ*cI-857* lysogens following the infective center assay:

Strain	**Δ*omp* (del) Location**	**Relative Efficiency of Plating [1] T4*rII***	**Rex Activity [1]**
BW25113(wt)	_	$< 3.0 \times 10^{-7}$	1.0
JW0554-1	Δ*ompT*	$< 2.0 \times 10^{-7}$	1.0
JW0799-1	**Δ*ompX***	4.5×10^{-4}	$< 10^3$
JW0912-1	**Δ*ompF***	3.3×10^{-5}	$< 10^2$
JW0940-6	**Δ*ompA***	5.0×10^{-4}	$< 10^3$
JW1248-2	**Δ*ompW***	1.0×10^{-3}	$< 10^4$
JW1312-1	Δ*ompG*	$< 2.0 \times 10^{-7}$	1.0
JW1371-5	Δ*ompN*	$< 4.0 \times 10^{-7}$	1.0
JW2203-1	Δ*ompC*	$< 1.0 \times 10^{-7}$	1.0
JW3368-1	Δ*ompR*	$< 4.0 \times 10^{-7}$	1.0
JW3846-1	**Δ*ompL***	3.0×10^{-6}	< 10

[1] The EOPs of T4*rII* were determined compared to BW25113 (wt) λ*cI*857 lysogen 100% control of Rex phenotype. Cells were infected by phage at MOI of 3 using infective centres technique.

3.3.2. Cell viability of Δomp mutants following infection by T4rII: The Rex mutualism model suggests that Rex activity, despite some cell killing, may in fact protect cells against T4*rII* infection by shunting cells into stationary phase. To further investigate this model in the context of osmotic induction, we employed a cell viability assay to determine whether specific Δ*omp* mutants carrying pHA1 following T4*rII*-infection were as subject to T4*rII*-mediated cell killing as Rex^- control cells in a rex^+ and rex^-context (Table 10). Cells challenged with T4*rII* started to grow and be visible after 48 h of incubation at 30^{o}C compared to the control strains where they were able to grow within 24 h. In agreement with the infective center assay findings, Δ*ompX*, Δ*ompF*, Δ*ompA*, and Δ*ompW* once again exhibited >100-fold lower viability compared to other *omp* mutants and the Rex^+ control following T4*rII* infection. This finding again suggests that these genes may be involved in Rex activity. The viability of other Δ*omp* mutants harboring the Rex^+ pHA1 plasmid was similar to that of the wild type strain (pHA1) indicating that these *omp* genes are not likely directly involved in Rex activity. Once again, the exception was the Δ*ompC* mutant, as expected, that demonstrated complete resistance to the T4 and T4*rII* infection, serving as the negative control for T4 infection.

Table 10: Cell viability of T4*rII*-infected Δ*omp* (rex^+) [pHA1] hosts:

Strain	**Δ*omp* Mutation Location**	**Plasmid**	**Relative Efficiency of Plating (T4*rII*)[1]**	**Reduction in Cell Viability[1]**
BW25113WT	-	pHA1 (rex^+)	8×10^{-4}	1.0
BW25113WT	-	No plasmid (rex^-)	$< 2 \times 10^{-7}$	$< 10^3$
JW0554-1	Δ*ompT*	(rex^+)	4×10^{-4}	1.0

JW0799-1	**Δ*ompX***	(*rex*$^+$)	1.2 x10^{-6}	< 10^2
JW0912-1	**Δ*ompF***	(*rex*$^+$)	1 x10^{-6}	< 10^2
JW0940-6	**Δ*ompA***	(*rex*$^+$)	5 x10^{-6}	< 10^2
JW1248-2	**Δ*ompW***	(*rex*$^+$)	7 x10^{-6}	< 10^2
JW1312-1	Δ*ompG*	(*rex*$^+$)	9 x10^{-3}	~1.0
JW1371-5	Δ*ompN*	(*rex*$^+$)	5 x10^{-4}	1.0
JW2203-1	Δ*ompC*	(*rex*$^+$)	≈1	~ 0
JW3368-1	Δ*ompR*	(*rex*$^+$)	7 x10^{-3}	~1.0
JW3846-1	Δ*ompL*	(*rex*$^+$)	9 x10^{-4}	~1.0

[1] Efficiency of plating showing CFU EOP arising at 30°C after infection by T4*rII* at MOI of 3. Average of three trials. 100% control of T4*rII* infectivity was BW25113 (Rex$^-$); BW25113 (wt) carrying [pHA1] plasmid was employed as the 100% positive control of Rex activity.

CHAPTER FOUR

4. Discussion

4.1. One step-mutagenesis and screening of host mutations attenuating the *rII* exclusion phenotype:

The Rex phenotype is quite simply defined as the inability of the mutant bacteriophage T4*rII* to form plaques on a lawn of *E. coli* λ lysogenic cells (Benzer, 1957) and yet it is associated with and hence, convoluted by, the consideration of so many additional and diverse related findings. This enigmatic phenotype dates back to Seymour Benzer's discovery of the phenotype in 1955, and despite that it has played a crucial role in our understanding of modern molecular genetics, still no solution to the mechanism of Rex exists. In this work, we sought to further understand the mechanism of Rex by identifying host genes involved in central exclusionary and survival phenotypes associated with the onset of the phenotype. Such mutants have long been sought and believed to hold the secret to Rex, but until now, attempts to isolate host mutations outside of the *rexA*-*rexB* genes by other researchers have proven unsuccessful or reasons of limited molecular biological tools and confounding complexity.

We report here, for the first time, the isolation of 13 confirmed mutants that dramatically attenuate Rex activity even in the presence of a rex^+ high-copy genotype. We have constructed a single-step approach to mutagenize and screen for *E. coli* mutants that are genotypically rex^+ and yet phenotypically compromised for Rex activity. The final constructed Rex suicide plasmid, pHA2, was employed to identify *E. coli* host genes that were either directly or indirectly involved in T4*rII* (Rex) exclusion activity, while also preventing isolation of undesirable mutations in the *rexA* or *rexB* genes that would abrogate Rex activity. We employed a suicide

plasmid-delivered transposon delivery system that *via* the insertional mutagenic capabilities of the *Tn*10 transposable element can impart relatively random illegitimate recombination events into chromosomal DNA (Alexeyev and Shokolenko, 1995; Kleckner et al., 1991). Depending on the site of insertional mutagenesis, if the *rex* genes were inserted into the middle of a non-essential gene not involved in Rex activity, cells would be phenotypically Rex^+ and grow normally on plates seeded with T4*rII* phage. As expected this was the most commonly occurring scenario as relevant isolates occurred of less than 0.2% of integrants, which was reasonable considering the roughly 2000 protein-encoding genes encode by *E. coli* most of which would be expected to be unrelated in Rex. Applying this one-step genetic selection/screening system we successfully isolated selected 13 bitten colonies and they were confirmed to be genotypically rex^+, but strongly attenuated for Rex activity.

Interestingly, we did not observe any colonies at all during the first 24 h following plating on T4*rII*-seeded plates, where colonies became visible only after a minimum of 36 h incubation. This observation is in agreement with previous observations by Slavcev et al. (2003) and is aligned with their proposed model that suggests "Rex-triggered" cells undergo an arrested growth phase to protect host cells despite some cell killing. While these mutations have yet to be mapped using an *E. coli* library, we noted that the attenuation of Rex activity between several of the isolates varies by up to 100-fold, which suggests that more than one gene has been isolated here to be either directly or indirectly involved in Rex. Furthermore, the isolated mutants continued to attenuate Rex activity even after being lysogenized by λ phage, which further increased and replenished *rex* genes dosage. This finding also suggests that the loss of Rex activity in these mutants is likely due to a relevant generated host mutation rather than a cellular metabolic state that might down regulate the expression of *rex* genes. While these mutations may

be involved in the exclusion phenotype, and given some that offer only mild attenuation of Rex, the involvement of these genes may be revealed to be peripheral or indirect, or perhaps a complex genetic circuit or signalling pathway. We also noted that for some of the isolates, the degree of attenuation closely resembled the Rex attenuation noted for some of the *omp* mutants found to diminish Rex activity. For instance, isolated mutants JW-HA-4, 6, 11, and 19 exhibited between a 1000 and 5000-fold reduction in Rex activity, which was similar to that noted for *rex*$^+$ *ompW* or *ompX* mutants, while others resembled levels of attenuation more similar to the 100+-fold reductions seen for *rex*$^+$ *ompL* and *ompF* mutants; the Omp proteins and potential involvement in Rex activity are discussed below.

4.2. Outer Membrane Proteins (Omp) and Rex activity:

Slavcev R. noted (unpublished, personal communication) that *ompF* mutants may attenuate Rex activity in preliminary 2003 studies. The *ompF* gene encodes a porin that like *rII* exclusion, has been shown to be inhibited by polyamines (Delcour and Vega, 1996), where polyamines, in addition to divalent cations, have been previously shown to dramatically compromise Rex activity (Garen, 1968). Furthermore, the over-expression of RexA was also noted to result in sticky cells (Slavcev, 2005), potentiating that the outer membrane may in fact be involved in the triggering of some of the physiological manifestations of Rex. In combination, with the current understanding of the effect of the ionic environment and how Rex activity requires Na^+, but can be attenuated by divalent cations and polyamines (Garen, 1968), it reasonable to anticipate that valuable information could be gleaned from assessing the impact of mutation of various outer membrane protein (*omp*) genes on Rex activity.

The outer membrane (OM) of Gram-negative bacteria is comprised of lipopolysaccharide (LPS) decorated lipid membrane littered with an array of proteins (DiRienzo et al., 1978; Koebnik et

al., 2000; Wexler, 2002; Ruiz et al., 2015). The outer membrane confers an effective barrier located outside of the cytoplasmic membrane and is considered to be the most important structural component for bacterial survival, acclimation and protection in harsh external environments. The OM carries out a number of critical cellular activities, including cellular nutrient and solute uptake, achieved by way of translocation through pore-like channels called porins, generally formed by Omp homotrimers (Koebnik et al., 2000). Omp porins constitute more than 50% of the outer membrane density and as yet few highly expressed Omps have been structurally and functionally characterized (Koebnik et al. 2000) (Fig. 14). Omp porins control the influx and efflux of solutes across the membrane and are crucial for bacterial cell stability and cell function (DiRienzo et al., 1978; Wexler, 2002). The surface-exposed position of some of these proteins on the outer membrane also define bacteriophage host range by serving as attachment receptors for phage adsorption, or as superinfecting signal receptors against super-infecting phage (Koebnik et al. 2000; Wang and Lin, 2001).

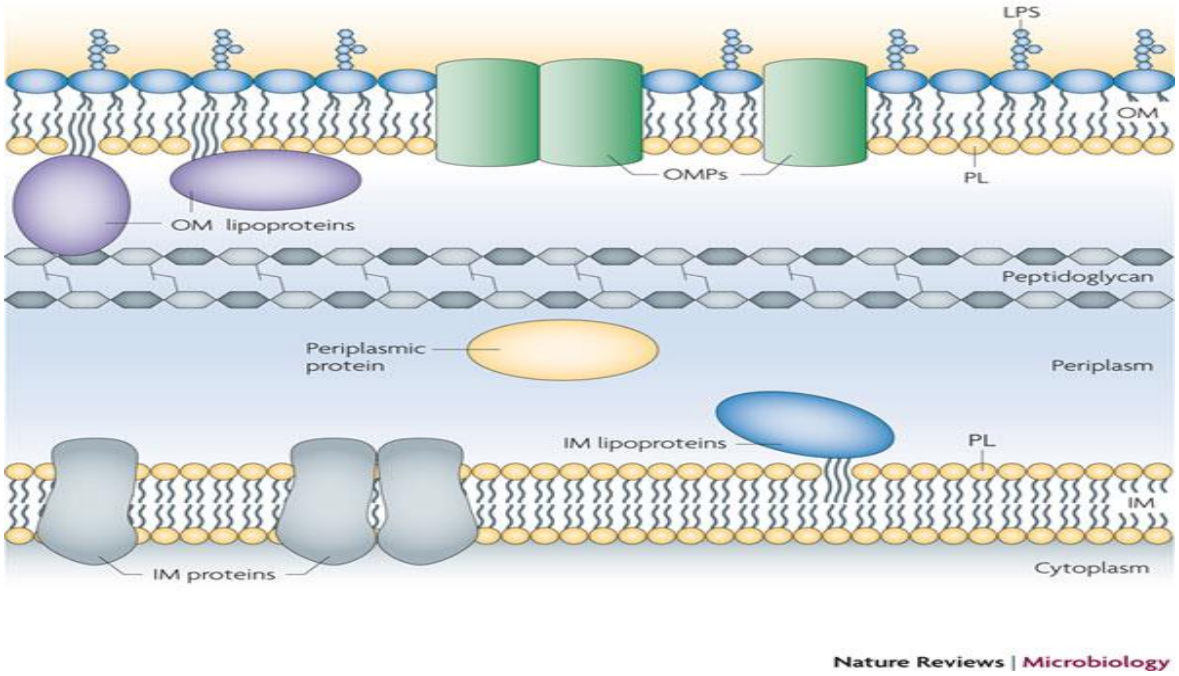

Figure 14: Gram-negative envelope components: The outer membrane consists of lipopolysaccharide (LPS) in the outer leaflet and phospholipid in the inner leaflet, as well as porin diffusion channels. The periplasmic space consists of periplasm and peptidoglycan. The inner cell membrane or plasma membrane maintains proton motive force and membrane potential.
Source: Natividad Ruiz, Daniel Kahne & Thomas J. Silhavy, Nature Reviews Microbiology 7, 677-683 (2009)

Omp A has been well-studied and plays an important role in the maintenance of the outer membrane structure and stability of *E. coli* and has been shown to be involved with one of the bacteriophage receptors and in the bacterial conjugation process required for gene transfer of F^+ plasmids and in DNA transformation (Ried and Henning, 1987). Although RP4 plasmids differ in conjugative apparatus from F, the finding that the *ompA* mutant permitted the lowest frequency of conjugative plasmid-mediated transposition aligns with OmpA involvement in cellular DNA transfer (Ried and Henning, 1987). OmpA also functions as nonspecific diffusion channel for small solutes (Sugawara and Nikaido, 1992) that can control its pore formation protecting the bacterial cells against osmotic stress (Hong et al., 2006). OmpA has been shown to serve as the

first line of cell defense against environmental stressors, where any mutations to the protein induces the cell to become more sensitive and less resistant to infection (Wang and Lin, 2001). Changes to the cell membrane structure have also shown cells to be more exposed and sensitive to superinfecting phage like T4 (Wang and Lin, 2001), which made our finding that the *ompA* mutant carrying the high copy pHA1 plasmid not only to show full Rex activity, but was also able to reduce T4 plating by more than 10^3-fold, very interesting. Interestingly, stress exposure in Δ*ompA* cells have also been found to induce phenotypic changes in *E. coli*, whereby cells become spherical in shape similar to stationary-phase *E. coli* (Wang, 2002). This may suggest that these mutants are actually more susceptible to enter stationary phase. The stability of the *ompA* mRNA transcript correlates inversely with the bacterial growth rate and is growth stage-dependent exhibiting more than 15 minute turnover in log-phase cells, but only ~4 min in stationary phase quiescent cells (Rasmussen et al., 2005). Regulation of the transcript is determined by the 5'-un-translated region, which possesses cleavage sites for RNAse E. Decay of the *ompA* mRNA is dependent upon another factor, Hfq, which binds to this region in addition to the short SraD RNA (highly conserved among Enterobacteriaceae that destabilizes the *ompA* transcript when rapidly grown cells enter the stationary phase of growth (Rasmussen et al., 2005). Interestingly, stationary phase cells promote also promote the increased expression of the small RNA, MicA that in association with Hfq binds to the *ompA* transcript and promotes degradation by RNAse E post-translationally, thus further reducing the presence of OmpA in stationary phase cells (Rasmussen et al., 2005).

The observed heightened Rex activity seen in the *ompA* mutant may thus arise due to the predisposition of these host cells to being shunted into osmotic irregularity and a stationary (-like) phase. Due to additional osmotic and structural instability caused by the *ompA* mutation,

recovery of the cell's osmotic rebalance and membrane potential may in the Rex-activated context, be irreversible, which would account for the abrogation of the Rex-centric mutualism phenotype and much lower viability of *rex*$^{+}$ *ompA* mutants. Although, conversely, the inability of the *ompA* mutant to effectively passage and evacuate invading DNA out of the cell during Rex onset would similarly subject the cell to the lethality of the superinfecting DNA. In either case, the irreversible metabolic state ensued by Rex activation within the *ompA* mutant would also account for the powerful Rex-dependent exclusion of T4*rII* and T4 (RII^{+}) alike, as stationary phase prohibits the propagation of all T-even species. The powerful inhibition of plating seen for the *ompA rex*$^{+}$ mutant was Rex-dependent as it was not seen in the *rex*$^{-}$ mutant (in its ability to reduce T4 plating efficiency), and also could not completely abrogate T4 plating efficiency. These findings may suggest that OmpA is either a target for inactivation by Rex protein(s) and this activity may be competitively inhibited by RII proteins, or rather, RII requires interaction with OmpA to suppress Rex. Either way, the incomplete abortion of T4 plating, along with the effect of other mutations in the attenuation of Rex activity, suggests that *ompA* is not the only host gene involved.

OmpX, like OmpA has been implicated in the cell's defense against virulence (Vogt and Schulz, 1999), and has been shown to play an important role in *E. coli* cell adhesion and biofilm formation, although the precise mode of action remains unknown (Vogt and Schulz, 1999; Otto and Hermansson, 2003). Deletion mutants for *ompX* are also similarly more exposed and hence, sensitive to stressful environments, imparting easier cell infection and killing by cells (Otto and Hermansson, 2003). Otto and Hermansson (2003) found that deletion of *ompX* causes a significant alteration in cell surface hydrophobicity and increases the negative charge of the cell surface that may also increase bacterial cell-target surface interaction. The *ompX* gene is over-

expressed in λ lysogens and was thus, suggested to function as a host defense-response neutralizer upon exposure to any stress (Heffernan et al., 1994; Koebnik et al., 2000). Overproduction of OmpX is also linked to the overexpression of Sigma E factor (σ^E) and a corresponding increase in σ^E activity (Ades et al., 2003; Mecsas et al., 1993). The σ^E, subunit of RNA holoenzyme encoded by *rpo*E gene, is a minor sigma subunit *E. coli* response element, activated in response to stressful environments such as hyperosmotic shock, metal ion exposure, and changes in outer membrane lipopolysaccharide structure (Ades et al., 2003; Valentin-Hansen et al., 2007). The degree of σ^E expression depends on the degree of exposed stress from the extra-cytoplasmic compartment of *E. coli*, and the expression level will last until the cell once again reaches homeostasis (Ades et al., 2003). The sigma E factor (σ^E) governs transcriptional activation of many downstream genes including the periplasmic folding proteins and degrading factors and has been implicated in a novel pathway directing cell lysis that proceeds in a cascade that entails σ^E-governed expression of *micA* and *rybB* small RNAs that modulate a reduction in Omp proteins and the eventual disintegration of the outer membrane (Murata et al., 2012). A strikingly similar scenario has been noted when *E. coli* cells enter stationary phase, where elevation of active σ^E leads to the decrease in the transcription and formation of OmpA, OmpC, and OmpW (Kabir et al., 2005). The *micA* and *rybB* transcripts, expressed under the control of σ^E-directed transcription further down-regulate *ompA* and *ompC* translation (Valentin-Hansen et al., 2007). The consequent, disruption of outer membrane proteins formation enhances cell lysis and was noted by the high resultant protein density in the culture medium (Kabir et al., 2005).

The *ompF* and *ompC* genes are differentially expressed based on changes in medium osmolarity, as sensed by the membrane-bound EnvZ sensor that through kinase activity upon the cytoplasmic OmpR osmoregulation transcriptional regulator, leads to the inverse stimulation of

expression of *ompF* and *ompC* under varying conditions of medium osmolarity (Srividhya and Krishnaswamy, 2004). OmpF forms approximately a 10-fold wider channel than OmpC and is thus more general of a passaging route and preferentially expressed under hypoosmotic medium conditions as would be expected in stationary phase (Benz, 1988). In contrast OmpC proteins preferentially decorate the outer membrane during hyperosmotic conditions when lower level of solute passaging are required (Benz, 1988). Both OmpF and OmpC function as cation-charge selective diffusion channels for the passive membrane diffusion and pore formation for small molecules (Cowan et al., 1992; Apirakaramwong et al., 1998). OmpC also functions as the primary adsorption receptor for T4, where as we similarly confirmed, deletion of this gene results in the loss of the T4 attachment and consequently, loss of T4 ability to infect (Yu and Mizushima, 1982).

Interestingly, overproduction of either OmpC, or OmpF increase the σ^E activity and activate stress response causing cell lysis, and decreased production of these proteins reduce σ^E activity (Mecsas et al., 1993). Due to the prohibitive state of *ompC* mutants toward T4 plating, we cannot definitively conclude any involvement of OmpC in Rex activity, although based on a culmination of current and previous observations and understanding of regulation, potential scenarios can be envisioned for to OmpC. For instance: 1) As the adsorption receptor for T4, OmpC would make a very logical Rex activation target or sensor, triggered by subsequent T4 super-infection; 2) the *ompF* mutation was noted to reduce Rex activity dramatically by 100+-fold, but could not abrogate the phenotype, suggesting that while it likely plays a role in Rex, its role may be more indirect, imparted perhaps through osmotic dysregulation and stimulation of a stationary-phase like state; 3) OmpR regulates both *ompF* and *ompC* transcription levels, and as the *ompR* mutant was observed to dramatically reduce T4 plating efficiency in a Rex-dependent

context, poor T4 infectivity here could be partially attributable to reduced expression of *ompC*, that again, is essential for T4 adsorption; 4) OmpR regulates transcription of the MicC small RNA that stimulates degradation of the *ompC* mRNA transcripts, thus further reducing expression of OmpC in an OmpR-deprived environment (corroborating data seen in *ompR* mutants) (Guillier et al., 2006); 5) *ompC* expression can be stimulated independently of OmpR by sucrose, previously shown to powerfully suppress Rex activity (Ozawa and Mizushima, 1983); 6) OmpC and OmpF are expressed under inverse osmolarity conditions by OmpR. As such, an *ompF* mutant, while unaffected by OmpR stimulation, would continue to produce dephosphorylated product that would stimulate *ompC* transcription (Srividhya and Krishnaswamy, 2004); 7) Expression of *ompC* is down-regulated during stationary phase, aligning with the similar cellular state observed during the onset of Rex.

The OmpW protein forms porins in the outer membrane that generate long narrow hydrophobic channels that serve as an ion channels in the transport of small hydrophobic molecules across the membrane (Hong et al., 2005). OmpW is also one of the bacterial virulence proteins in conjugation with OmpA and OmpF, which have also been documented to protect *E. coli* against any environmental stressors, in particular, versus super-infections (Wu. et al., 2013), once again suggesting relevance as a potential triggers of Rex. OmpW was also found to confer a powerful reduction in Rex activity that was also Rex specific. The summary of Omp protein function and physiological manifestations of their mutation are shown in Table 11.

Table 11: Outer membrane proteins (Omps), primary function and physiological manifestations of mutation:

Outer membrane protein (Omp)	Primary Function(s)	Mutation
OmpA	A virulence-related membrane protein, cell wall integrity and stability, bacteriophages receptor, adhesion and invasion and biofilms formation, F^+ conjugation, and diffusion channel for small solutes	Cell morphology similar to stationary phase *E. coli* cells; heightened sensitivity to superinfection phage
OmpX	A virulence-related membrane protein, bacterial adhesion and bio-films formation, naturalizing host defense response	Alteration in cell surface hydrophobicity and increases the cell surface negative charge, increase cell-surface interaction and sensitivity to external stress
OmpF	Cation-charge specific transport channel for passive diffusion, and maintains cell osmolarity with OmpC under the control of the regulator OmpR	Change cell osmolarity, overproduction causes increase the σ^E activity and activate stress response

OmpW	A virulence-related membrane protein, and an ion channel to transport small hydrophobic molecules across the membrane	Loss bacterial cell defenses and increase in the infectivity by phage
OmpC	Cation-charge transport channel for passive diffusion, maintains cell osmolarity with OmpF under the control of the regulator OmpR, and as T4 attachment site	Change cell osmolarity, overproduction causes increase the σ^E activity and activate stress response, and prevent T4 infection
OmpR	Transcriptional activator of both *ompF* and *ompC* porins as a part of osmoregulation system, and cell adhesion and biofilms formation control	Increase curli production and loss adhesive properties in some strains
OmpT	Omptin protease that cleavages foreign peptide elements	Deletion has no effect on bacterial cell growth rate or inducing cell-lysis
OmpL	A small molecules diffusion porin	Disable the cell to efflux the low molecular weight solutes into the medium, but it does not affect bacterial growth

OmpG	A non-selective large molecules channel transport	Unknown
OmpN	A low-expression non-specific pore forming porin	Unknown

The *ompC, ompF*, and *ompW* porins regulate function as virulence factors and further regulate various solutes across the OM (Koebnik et al., 2000). As such, they are potential targets in directing osmolar-stress conditions that can shunt cells into a varied osmolar states. OmpF and OmpW reduce Rex activity and as ion channels agree with former findings that fatty acids, polyamines and divalent cations can attenuate Rex activity. The *ompF* and genes are regulated by OmpR, which together with EnvZ plays a role in activation of σ^E in inducing the stress regulon that responds to osmotic stress (Darcan et al., 2009). To study the possibility that Omps may be involved in Rex activity we sought to test *E. coli* strains Omp mutants and see if they can attenuate the T4*rII* exclusion phenotype. We ordered ten *E. coli* mutants each with one *omp* deletion and also ordered their parent strain BW25113 (wt) as a positive control of Rex phenotype employing the Keio Collection of single gene Knock-outs that permit the testing of non-essential *E. coli* mutations that may abrogate Rex activity. The Keio collection is a new analysis approach that allows for the experimentation on cells in the absence of a single known gene (Baba et al., 2006).

In this study, we found as we expected, poor T4*rII* activity was observed on the Δ*ompC* confirming the need of T4*rII* to OmpC protein as an adsorption site for infection and the absence of the *ompC* causing poor infection (Yu and Mizushima, 1982). Interestingly, we found some

Δ*omp* mutants were able to form plaques, indicating that they were able to attenuate Rex activity. Δ*ompX,* Δ*ompW,* Δ*ompF,* and Δ*ompL* mutations were able to attenuate T4*rII* exclusion from > 150- to > 3K-fold) compared to controls even in the presence of hundreds of *rex* genes in the infected cells indicating that the deletion of these *omp* genes stunted the formation of these proteins and allowed T4*rII* to infect *rex*$^+$ host cells. In contrast, interestingly, the Δ*ompA* mutant exhibited a hyper-Rex phenotype that similarly inhibited T4 and reduced cell viability upon T4rII infection in a *rex*$^+$ context.

The colonies were arising after 48 hours of incubation, which again supporting the model that the Rex system encodes a protection mechanism rather than an altruistic death system by entering a long arrested growth phase upon infection (Slavcev and Hayes, 2002). This reduction in the viability of some of *ompX, ompW, ompF*, mutants indicates that these *rex*$^+$ cells were attenuated for Rex activity and sensitive to T4*rII* growth and as such, were more readily lysed upon infection. OmpA mutant reduction in cell viability was in contrast accompanied by a perceived heightened Rex activity and as such, increased killing is likely not due to phage-mediated lysis (model offered below).

4.3. Proposed model for Rex mechanism:

While *ompA* mutants have been found to impart heightened sensitivity to T4 infection in the absence of *rex* genes, we noted that T4 plating on *ompA* mutants carrying the high copy [pHA1] plasmid exhibited such a strong Rex phenotype that they were able to exclude T4*rII* and T4 alike. In addition, these cells were 100-fold more sensitive to T4*rII* killing upon infection, again suggesting that Rex activity is heightened in these cells and is likely irreversible, resulting in cell death upon the induction of Rex. OmpA inhibition may thus be involved in "priming" *E. coli*

cells for exclusion and may be targeted/inhibited by RexA and/or RexB under natural exclusionary conditions. The super-Rex phenotype noted upon excessive expression of *rexA-rexB* genes (Shinedling et al, 1989) may be thus, due to the more effective inhibition of the OmpA, which itself is highly expressed and represented in the outer membrane (Wang, 2002). Its use as a target by Rex may further improve sensitivity to T-even phage super-infection through inhibition.

Given the observed modulation of Rex activity by both OmpA and OmpW and their relationship to σ^E, it is quite enticing to postulate that σ^E, *via micA* and/or *rybB* expression, is directing the harsh physiological cellular conditions associated with Rex due to the known osmotic imbalance associated with membrane destabilization induced by Rex triggering (Parma et al., 1992) that may involve the reduction of OmpA in the outer membrane. The cell-killing effect associated with Rex may thus be directly related to the σ^E –mediated OM destabilization from the down-regulated expression of *ompA* in the outer membrane. Underproduction of OmpX has also been noted to reduce σ^E activity in *E. coli* as a "strain-dependent" phenotype (Mecsas et al., 1993), which may further explain the reduction in Rex activity associated with this mutation in gentoypically *rex*$^+$ cells. While each noted *omp* mutant appears to partially reduce Rex activity, the combinatorial effect of double/multiple mutants of *ompW*, *ompX*, and *ompF* would be very interesting to pursue and could lead to important hierarchical understanding of the role of these Omps in Rex mechanism.

Using our knowledge about Omps and their functions and our findings in this study, we suggest a model of the involvement of Omps in Rex phenotype (Fig. 15).

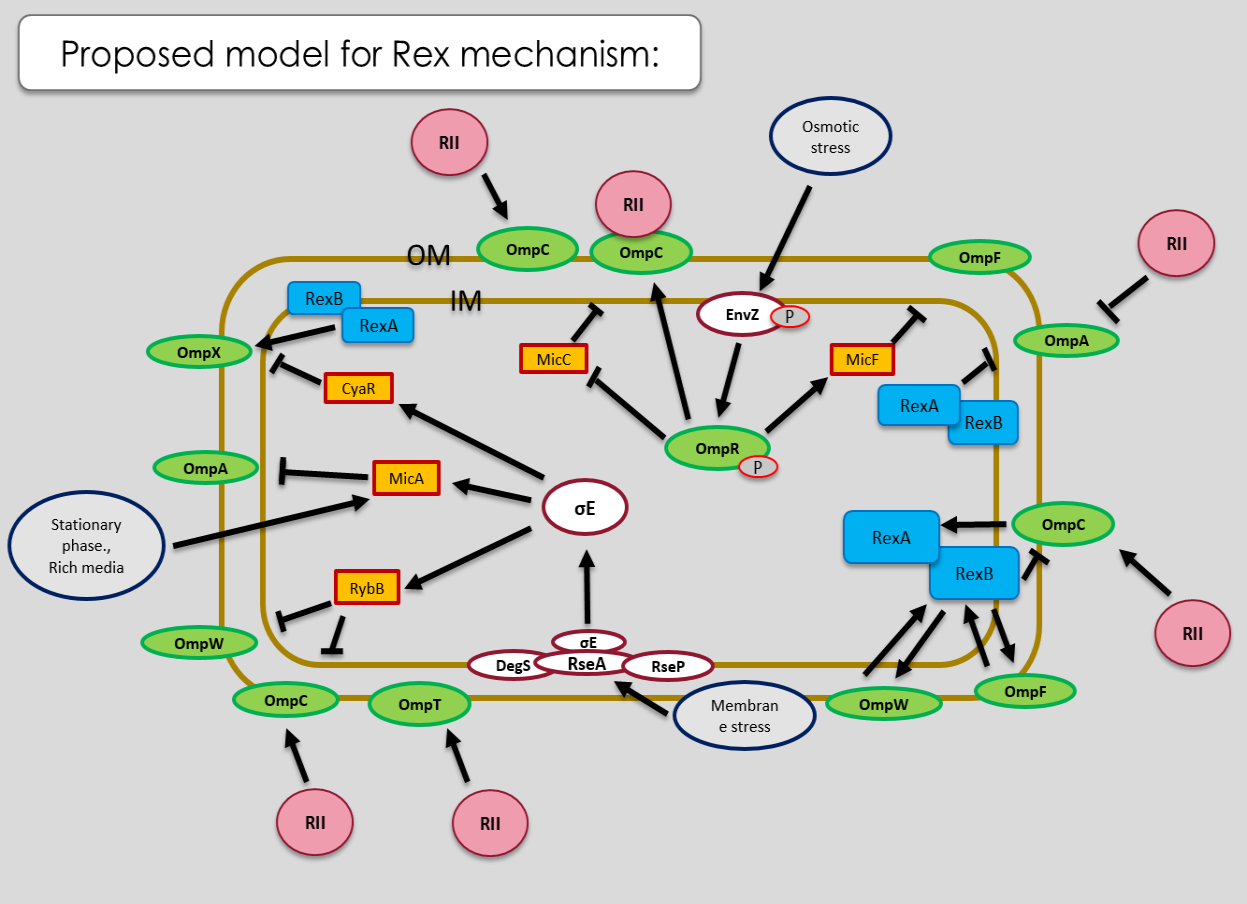

Figure 15: The Proposed model for Rex mechanism: upon infection, outer membrane proteins (Omp) production would be stimulated resulting in the elevation of σ^E expression and a state similar to stationary phase. Rex system activation may either be trigged by some Omp activity or the activity of specific Omps, resulting in a physiological cellular shunt into stationary phase. Figure keys: Green ovals are Outer membrane proteins; Pink circles are RII protein of T4 phage; Blue boxes are Rex proteins of λ; yellow boxes are small RNAs that regulate the transcription of *omp* genes and the expression of Omp proteins; blunted arrows indicate inhibition/downregulation/ blocking expression; regular arrows indicate stimulation/upregulation/ initiation expression; OM: outer membrane; IM: inner membrane.

Omp proteins protect cells against harsh environment, at the same time, they have other functions to sustain cell integrity and stability. *E. coli* cells enter stationary phase upon exposure to any external stresses and causes a σ^E activation where all the genes that are expressed during growth phase are switched off, and the stress response genes turned on (Chen et al., 2004).

Although the Omps are the first to sense the external stress which triggers the activation of σ^E, this activation causes degradation of Omp proteins and changing cell structure and cell osmolarity to enter stationary phase (Chen et al., 2004). Given the hyper-Rex phenotype induced by the *ompA* mutation, OmpA may serve as the target for Rex, inhibited directly by the Rex proteins, thereby changing morphology and further resembling a stationary phase state of arrest. We suggest that either OmpX, or OmpF in correlation with OmpC are the viable candidates that are involved in Rex activity based on their functions and the information we know about Rex proteins. The ionic environment is crucial for the onset or abrogation of Rex phenotype, where monovalent cations are essential for the onset and divalent cations can attenuate the phenotype (Ames and Ames, 1965; Brock, 1965; Garen, 1961; Sekiguchi, 1966). Upon infection with T4*rII*, OmpX as a virulence factor can protect *E. coli* cells by maintaining the surface hydrophobicity and the negative charge on the cell surface (Otto and Hermansson, 2003). Puncturing the cell surface by phage would disturb the surface stability, as a result, Rex would be activated and OmpX would re-stabilize the surface hydrophobicity and send a signal to σ^E to start the stress response. That would affect the cell adhesion and infectivity in addition to the cell stability. In the absence of OmpX, σ^E activation would not ensue.

In contrast, OmpW, OmpF and OmpC may be involved indirectly in the triggering of Rex via the modulation of osmolar stress initiated by activated RexB. Deletion of one of these genes would reduce the rate, ability of cells to shift the osmolar environment and to enter stationary phase before T4*rII* is able to replicate, increasing plating efficiency of this phage in these *rex*$^+$ mutants. T4*rII* will bind OmpC as it is a recognized adsorption site for T4 phage (Yu and Mizushima, 1982). OmpC and OmpF function as cation-charge channels that make them important for the Rex phenotype and more reasonable to be the target candidates. OmpF and OmpC control the

cell osmolarity in response to changes of the growth medium osmolarity. When cells grow under high osmotic medium, the *ompC* would be preferred to be expressed; on the other hand, in low osmotic medium, *ompF* would be expressed to stabilize the cellular osmosis. Penetration of T4 phage's tail will lyse the peptidoglycan of the outer membrane and the inner membrane and cause a disturbance in periplasmic and cytoplasm osmolarity (Arisaka, 2005); as a result, an activation of the OmpF where cells are now more osmotic than extracellular. The cytoplasm is now more acidic than it should be, causing stimulation of phosphatase enzyme and *ompF* expression to restore that cell osmolarity (K^+ efflux). This action results in: 1) increase diffusion of cation charge through OmpF ion channel outside cells, 2) the presence of monovalent cation charges in the medium activates Rex, 3) σ^E activation and stationary phase starts. Deletion of OmpF can allow T4*rII* plating where the normal osmotic state inside the cells cannot be restored and Rex cannot be activated by the absence of monovalent cation in the growth medium.

Finally, OmpA has also recently been implicated in DNA transformation in *E. coli* and DNA transfer (Sun et al., 2013). The ability of some cells to recover from Rex activity would require that surviving cells are able to eventually evacuate invading T4*rII* DNA from the cell—a role that may be mediated by OmpA in concert with Rex proteins.

This work has uncovered host mutations of *E. coli* that modulate Rex activity. These findings have substantiated a model whereby Rex shunts cells into stationary. While these findings have made inroads into the understanding of the Rex phenotype mystery, new questions now arise in the continued elucidation of this legendary mystery.

4.4. Future research objectives:

1) Identification of the interrupted genes that caused the Rex⁻ phenotype:

a) By preparing an *E. coli* library of non-essential known genes and transform them into the isolated mutants that showed some Rex attenuation. Then, Test transformed cells for their T4*rII* sensitivity, the cell that restores the Rex activity indicates that the gene on the plasmid is the gene that is involved in Rex activity. The plasmid will be extracted and sequenced to identify the encoded gene.

b) By using the *Tn*10 sequence and design one primer that against one of the IS10 elements and another random primer to PCR amplify the knocked out gene. The amplified fragment will be purified and sent for sequencing, then the sequence will be blasted against *E. coli* genes to identify the involved gene in Rex attenuation.

2) Adding to our understanding of the relevant *omp* genes functions and involvement in Rex activity which would, in turn, help to understand the mechanism of *rex* genes in excluding T4*rII*.

3) Identification the location of Rex proteins and interactions with Omp candidates such as OmpA.

4) Understanding the role of RII proteins in suppressing Rex activity and the exclusion mechanism.

Conclusion

This project aims to understand the mechanism behind the λ Rex phenotype through identifying and understanding the functions of non-essential host proteins that are involved in Rex activity. Parma et al. (1992) suggested that the *rII* exclusion phenotype (Rex phenotype) is regulated by the stoichiometric relationship of RexA to RexB proteins, and the resulting exclusion of T4*rII* is due to the sudden physiological changes in the host cells that program them for apoptosis. In contrast, Slavcev and Hayes (2003) suggest that despite some cell killing, the actual purpose of Rex is to artificially and temporarily shunt cells into a stationary-like phase to protect both λ and its host from infection. The Rex mystery has overwhelmed researchers for 60 years with little progress. In this study, we attempted to make important progress in trying to tie the accumulated Rex-mediated phenomenological observations together by understanding the mechanism and the functions of *omp* genes that are involved in the suppression of Rex activity. We have shown for the first time that OmpA, OmpW, ompX and OmpF proteins are involved in Rex activity, supporting the model, whereby Rex activation shunts cells into a stationary phase-like state to prevent T4*rII* growth. For that, we generated and isolated 13 Rex$^-$ mutants that are carrying *rex* genes but on plates they are allowing the T4*rII* infection to replicate and form plaques (genotypically *rex*$^+$ and phenotypically Rex$^-$). Identifying what genes that were knocked-out and attenuated Rex$^-$ phenotype, we definitely can get noteworthy clues about *rex* genes functions and their mechanism of exclusion. Understanding Rex can provide important clues and strategies for use toward eukaryotic exclusion systems and control mechanisms.

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