Identification of *Escherichia coli* Host Genes That Influence the Bacteriophage Lambda (λ) T4*rII* Exclusion (Rex) Phenotype

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ABSTRACT The T4r*II* exclusion (Rex) phenotype is the inability of T4*rII* mutant bacteriophage to propagate in hosts (*Escherichia coli*) lysogenized by bacteriophage lambda (λ). The Rex phenotype, triggered by T4*rII* infection of a *rex*$^+$ λ lysogen, results in rapid membrane depolarization imposing a harsh cellular environment that resembles stationary phase. Rex "activation" has been proposed as an altruistic cell death system to protect the λ prophage and its host from T4r*II* superinfection. Although well studied for over 60 years, the mechanism behind Rex still remains unclear. We have identified key nonessential genes involved in this enigmatic exclusion system by examining T4r*II* infection across a collection of *rex*$^+$ single-gene knockouts. We further developed a system for rapid, one-step isolation of host mutations that could attenuate/abrogate the Rex phenotype. For the first time, we identified host mutations that influence Rex activity and *rex*$^+$ host sensitivity to T4*rII* infection. Among others, notable genes include *tolA*, *ompA*, *ompF*, *ompW*, *ompX*, *ompT*, *lpp*, *mglC*, and *rpoS*. They are critical players in cellular osmotic balance and are part of the stationary phase and/or membrane distress regulons. Based on these findings, we propose a new model that connects Rex to the σ^S, σ^E regulons and key membrane proteins.

KEYWORDS bacteriophage lambda; bacteriophage T4*rII*; Rex; membrane proteins; sigma factors

THE T4*rII* **ex**clusion (Rex) phenotype, first discovered in 1955 by Seymour Benzer (Benzer 1955), is defined as the ability of the *rex* genes (*rexA*, *rexB)* from bacteriophage lambda (λ) to prevent plaque formation by mutant bacteriophage T4*rII* on λ lysogenized *Escherichia coli* (Shinedling *et al.* 1987). It is thought to be a form of defense by λ to protect its host bacterium against other invading bacteriophages. Activation of the Rex phenotype imparts a severe cellular environment that results in cessation of total cellular macromolecular synthesis, depolarization of the cytoplasmic membrane, reduction of the cellular respiration, and death in the majority of cells (Garen 1961; Sekiguchi 1966). Parma *et al.* (1992) proposed that Rex exclusion occurs by way of a membrane pore (RexB), activated upon interaction with two RexA proteins, establishing a stoichiometric relationship between them (Shinedling *et al.* 1987; Parma *et al.* 1992) (Figure 1).

In addition to Rex protein stoichiometry, both extra- and intracellular ionic environments mediate Rex regulation. Rex activation is dependent upon monovalent cations such as H^+, Na^+, K^+, NH_4^+, and Cs^+ (Garen 1961; Sekiguchi 1966). In contrast, the presence of divalent cations such as Ca^{2+} and Mg^{2+}, polyamines, or sucrose can diminish exclusion activity (Garen 1961; Brock 1965; FerroLuzzi-Ames and Ames 1965). Although activation of Rex kills a majority of cells, it still protects ~1/100 of the "Rex-activated" population (Slavcev and Hayes 2002). Such cells exhibit phenotypic traits characteristic of stationary phase. This quiescent metabolic state is characterized by changes in cellular morphology: spherical appearance, flagellar production, and low cellular proton motive force (Parma *et al.* 1992). This has led to the hypothesis that Rex somehow triggers an osmotic shift that shunts cells into stationary phase—a metabolic state that is not permissive to the propagation of superinfecting phage such as T4 (Slavcev and Hayes 2003).

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Overexpression of RexA was also previously reported to result in "sticky" cells (Hayes and Slavcev 2005), suggesting that the outer membrane may play an important role in the physiological manifestations of Rex and/or its triggering mechanisms. Very similar cellular attributes have been observed in mutants of the tolerance membrane protein TolA and the ion channel outer membrane protein (Omp) OmpA; mutants of both are spherical in shape, leaky, and sensitive to external stresses, including phage infections (Wang and Lin 2001; Lazzaroni *et al.* 2002). Omps control the influx and the efflux of solutes across the membrane to adapt to external changes, while the Tol system is considered the primary system to import/export micro- and macromolecules to maintain cell structure stability and integrity (Lloubès *et al.* 2001; Lazzaroni *et al.* 2002).

In *E. coli*, most membrane protein expression is under the control of sigma factors. Sigma factors (σ) are small protein subunits required for initiating transcription by binding to the core RNA polymerase and directing transcription at their specific cognate promoter. As such, gene expression may change to adapt to different environmental signals or conditions (Feklístov *et al.* 2014). The sigma E factor (σ^E) (*rpoE*) is activated in response to stress such as hyperosmotic shock, metal ion exposure, and changes in envelope structure. The sigma S factor (σ^S) (*rpoS*) maintains cell viability during stationary phase. Mutations to *rpoS* may also affect the stability of other sigma factors (Battesti *et al.* 2011). Absence of σ^S will stimulate an extracellular stress response resulting in elevation of *rpoE* expression, as well as degradation of Omps and cell lysis (σ^E-dependent cell lysis; Lima *et al.* 2013; Kosaka *et al.* 2017). In turn, small RNAs regulate sigma factors at the transcriptional, translational, and post-translational levels (Schweder *et al.* 1996; Battesti *et al.* 2015).

Given the potential role of membrane proteins in combination with the current understanding of the effect of the ionic environment on Rex activity (Garen 1961), we hypothesized that σ^S- and σ^E-dependent stress response proteins and their regulators may be involved in the mechanism of Rex. Therefore, we aimed to isolate and identify relevant *E. coli* host mutations that could influence the Rex phenotype. For the first time, we have linked the manifestation of Rex to genes underlying key host stress responses.

Materials and Methods

E. coli strains and cultures

Bacteria, phages, and plasmids used in this study are described in Table 1. Strains were grown on Luria–Bertani (LB) solid agar at 30°, supplemented with antibiotics [100 μg/ml ampicillin (Ap); kanamycin (Km) 50 μg/ml; 20 μg/ml tetracycline (Tc)]. Liquid cultures were grown in LB at 30° (with Ap for plasmid maintenance). Host cells and mutants were assessed for Rex activity by performing standard relative efficiency of plating (EOP) assays using T4 wild-type (wt) and T4r*II* stock lysates against an isogenic Rex^- parent strain.

Transformation

Electrocompetent host cells were transformed by 0.5–1.0 μg of plasmid following standard electroporation transformation using the Electroporator 2510 (Eppendorf Canada, Mississauga, CA), and plated on selective LB agar.

Plasmid construction

Colony PCR was performed on W3110 (λ) to amplify the λ*imm* (immunity) region with the λ*immF* (forward) primer: 5′GGGGGGCATTGTTTGGTAGGTGAGAGAT 3′; and the λ*immR* (reverse) primer: 5′ TTGATCGCGCTTTGATATACGCCGAGAT 3′. Amplification was completed using Phusion polymerase (Thermo Fisher Scientific): initial denaturation at 98° for 30 sec, denaturation at 98° for 10 sec, annealing at 72° for 10 sec, extension at 72° for 3 min, then final extension at 72° for 10 min; repeated 30 times. Reactions were run on 0.8% agarose gel electrophoresis (AGE). The λ*imm* region (2.4 kb) PCR fragment was extracted, purified, and digested by BgIII. The PCR insert (P_M-*cI857-rexA-rexB-*t_{imm} *rex* operon) was isolated and purified using BgIII, which cuts at λ 35,722 bp and λ 38,103 bp, a region that closely flanks the λ*imm* region and the P_M-*cI857-rexA-rexB-*t_{imm} *rex* operon on both sides. Following the digestion of pUC19 by BamHI, the λ*imm* region BgIII-O_RP_R-P_M-*cI857-rexA-rexB-*t_{imm} O_LP_L-BgIII fragment was cloned into pUC19 to form the rex^+ plasmid, pHA1 (Figure 2A and Table 1). XbaI and EcoRI were used to digest pHA1 to excise the XbaI-O_RP_R-P_M-*cI857-rexA-rexB-*t_{imm}-O_LP_L-EcoRI (2.4 kb) insert fragment. This was subcloned into the pBSL199 suicide vector to yield the suicide "pRex" plasmid, pHA2 (Figure 2B and Table 1). All plasmids were purified using the E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek). Extracted plasmid was digested to confirm presence of the *rex* operon, verifying that only a single insert fragment was subcloned per vector and to confirm the expected 5.2 (pHA1) and 8.7 (pHA2) kb vector size, respectively. pUC19 served as the rex^- plasmid control for pHA1 and pBSL199 for pHA2.

Cell viability assay

Cells harboring pHA1 or pUC19 were assessed for cell viability. *E. coli* mutants from the Keio collection (Baba *et al.* 2006), each possessing a single gene deletion, were tested along with their parent strain for Rex activity upon transformation by either plasmid (Table 1). The parent strain transformed with each plasmid, BW25113[pHA1] and BW25113[pUC19], served as controls for Rex activity. The Keio Δ*ompC* mutant (Table 1) precludes T4 adsorption and was used as a negative control for T4 infection. We prepared 1:100 subcultures of cells, as previously described, in LB + Ap and incubated at 30° while shaking at 225 rpm, until A_{600} = 0.4. 200 μl aliquots of cells were mixed with T4r*II* lysate at a multiplicity of infection (MOI) of 3. Infected cells were incubated at 30° for 10–15 min, washed twice with 2 ml Tris-NaCl (TN) buffer, and resuspended to a final volume of 1 ml. The suspension was serially diluted in TN buffer. A total of 100 μl aliquots of

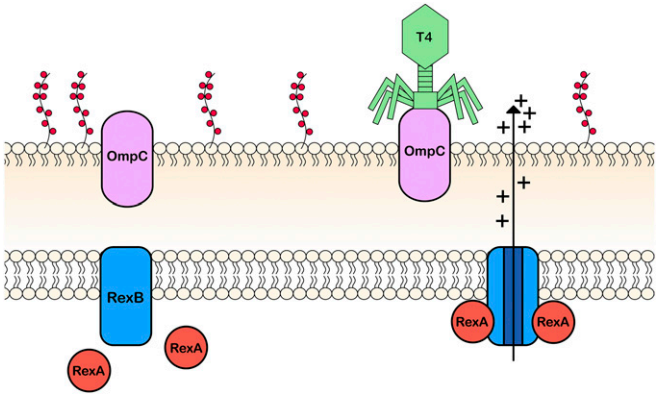

Figure 1 Upon infection by T4*rII*, RexA binds to RexB in a 2:1 ratio, activating RexB pore formation and cation efflux. This depolarizes the cell membrane.

select dilutions were spread onto LB + Ap agar and incubated at 30° for 48 hr before counting colonies.

Infective center assay

A 1:100 subculture of each Keio mutant carrying pHA1, and BW25113[pUC19] (negative Rex control), was prepared in LB + Ap as previously described, and incubated at 30° with shaking at 225 rpm, until A_{600} = 0.4. 2 ml of culture was centrifuged at 10,000 × g for 10 min and pelleted cells were resuspended in 1 ml of $CaCl_2$. T4*rII* phage were added at MOI = 3 and allowed to adsorb to cells for 15–20 min at 37°. Infected cells were washed three times in TN buffer and resuspended in 100 μl TN buffer. A total of 0.3 ml of the original subculture was added to the re-suspended cells, mixed with 3 ml top agar, poured onto prewarmed LB + Ap agar, and incubated overnight at 30°.

Conjugation assay

Overnight cultures of JW0427-1 (Δ*clpP*, Km^R) (recipient) and S17-1(λ*pir*)[pHA2] (*rex*$^+$ suicide plasmid and donor) were prepared at 30° with shaking at 225 rpm. JW0427-1 (Δ*clpP*, Km^R) was used as a recipient as the *rex*$^+$ derivative. We previously found that Δ*clpP* mutants retained full Rex activity, so the gene, to our knowledge, is not involved in Rex (Hayes and Slavcev 2005). Cells were pelleted from 1 ml of each culture, washed twice in 0.5 mM NaCl, and mixed in a 1:2 donor:recipient ratio. Mixtures were pelleted and re-suspended in 80 μl of 0.5 mM NaCl, then added to 100 μl prewarmed LB. After 1–2 hr of incubation and before plating, 0.1 mM IPTG was added and the mixture was incubated for an additional 1–2 hr at 37°. Samples without IPTG (donor cells only) and recipient cells were directly plated and incubated overnight at 30°. Mixtures with IPTG were spot-plated on LB agar and incubated overnight at 30°.

Insertional mutagenesis by transposable rex$^+$ cassette and screening for Rex$^-$ mutants

Cells from conjugation assays were diluted in 1 ml LB. A total of 200 μl aliquots were prepared for plating. Prewarmed LB + Tc + Km agar plates were seeded with 10^5 PFU of T4*rII* phage diluted in TN buffer to screen for a T4*rII*-sensitive phenotype (T4*rII* "biting" of growing colonies). Then, 200 μl of cells were plated and incubated overnight at 30°. The *trans*-conjugation frequency for Rex$^-$ mutants and the frequency of Rex$^-$ bitten colonies were determined (data not published).

Phage λ (rex$^+$) lysogenization of Rex$^-$ mutants and immunity assay

Overlay plates of isolated Rex$^-$ mutants were prepared as follows: 10 μl of 10^{-4} dilutions of fresh wt λ or *cI*857 λ in TN buffer were spotted onto LB top agar. After drying, plates were incubated overnight at 30° to generate λ lysogens. Cells within large turbid plaques were isolated to confirm for λ lysogeny. The *cI*857 λ lysogens were grown at 42° to inactivate the *cI* repressor, where any lysogens would be induced for phage amplification and lysis of their resident cells. Lysogenized cells able to grow at both 30° and 42° were confirmed for the presence of wt λ lysogens by an immunity assay. Cells were stabbed into a top agar overlay on LB agar containing ~10^8 PFU of phage (λ*imm*21) as well as 3 × 10^8 CFU W3899(λ*imm*21) lysogens, to test for presence of λ immunity to confirm lysogenization. Colonies were stabbed in the overlay top agar and incubated overnight at 30°. Large lysis spots arising from λ*imm* recombinants were visualized the next day.

PCR mapping and sequencing of mini Tn10 insertions in the chromosome

Inverse colony PCR was performed using *Taq* DNA polymerase. A single fresh colony of each isolated mutant was diluted into 50 μl of ddH_2O, where 1 μl was used as a template for the PCR reaction. Four primers were used during two rounds of inverse PCR as described by Nichols *et al.* (1998). Primers: first round PCR: primer #1 (JEP83) 5′ TTGCTGCTTATAA CAGGCACTGAG 3′ and primer #2 (JEP5) 5′ GGCCACG CGTCGACTAGTACNNNNNNNNNNGCTGG 3′; second round PCR: primer #3 (JEP84) 5′ CTTTGGTCACCAACGCTTTTCCCG 3′ and primer #4 (JEP6) 5′ GGCCACGCGTCGACTAGTAC 3′ (Peters and Craig 2000; Shi *et al.* 2008). The cycling thermal reaction for the first round (primers 1 and 2) proceeds as follows: 95° for 5 min, 95° for 30 sec, 30° for 1 min, 72° for 1 min, repeated 10 times. Next, samples were heated to 95° for 30 sec, 42° for 1 min, and 72° for 1 min, repeated 30 times. A 1:10 dilution of this first PCR reaction was used as a template for a second round (primers 3 and 4), which proceeds as follows: 95° for 5 min, 95° for 30 sec, 50° for 45 sec, and 72° for 1 min, repeated 30 times. Reactions were separated by AGE. The presenting band (~800 bp) was extracted using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek). PCR fragments amplified from primers 3 and 4 were commercially sequenced (Bio-Basic Inc., Markham, Canada).

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Table 1 Bacterial strains, phages, and plasmids used in this study

Designation	Relevant characteristics	Source
Bacterial strains		
DH5α	F$^-$, Δ*(argF-lac)169*, *φ80dlacZ58(*M15*)*, Δ*phoA8*, *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*, Δ*lacZ4787*(::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 T. Charles. Kvitko *et al.* (2012)
W3110 (λ)	F$^-$, λ$^+$, λ*cI578*, *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, λ*pir*$^+$	Gift from T. Charles. Matsumoto-Mashimo *et al.* (2004)
JW0427-1	Δ*clpP*::*km*^{R}, λ$^-$	CGSC #8590 Baba *et al.* (2006)
JW0554-1	BW25113 isogenic derivative: Δ*ompT774*::*km*^{R}	CGSC #8680 Baba *et al.* (2006)
JW0799-1	BW25113 isogenic derivative: Δ*ompX786*::*km*^{R}	CGSC #11794 Baba *et al.* (2006)
JW0912-1	BW25113 isogenic derivative: Δ*ompF746*::*km*^{R}	CGSC #8925 Baba *et al.* (2006)
JW0940-6	BW25113 isogenic derivative: Δ*ompA772*::*km*^{R}	CGSC #8942 Baba *et al.* (2006)
JW1248-2	BW25113 isogenic derivative: Δ*ompW764*::*km*^{R}	CGSC #9125 Baba *et al.* (2006)
JW1312-1	BW25113 isogenic derivative: Δ*ompG756*::*km*^{R}	CGSC #11793 Baba *et al.* (2006)
JW1371-5	BW25113 isogenic derivative: Δ*ompN740*::*km*^{R}	CGSC #9213 Baba *et al.* (2006)
JW2203-1	BW25113 isogenic derivative: Δ*ompC768*::*km*^{R}	CGSC #9781 Baba *et al.* (2006)
JW3368-1	BW25113 isogenic derivative: Δ*ompR739*::*km*^{R}	CGSC #10510 Baba *et al.* (2006)
JW3846-1	BW25113 isogenic derivative: Δ*ompL737*::*km*^{R}	CGSC #10779 Baba *et al.* (2006)
JW5437-1	BW25113 isogenic derivative: Δ*rpoS746*::*km*^{R}	CGSC #11387 Baba *et al.* (2006)
JW0727-1	BW25113 isogenic derivative: Δ*tolQ786*:: *km*^{R}	CGSC #8793 Baba *et al.* (2006)
JW0728-1	BW25113 isogenic derivative: Δ*tolR787*:: *km*^{R}	CGSC #8794 Baba *et al.* (2006)
JW0729-3	BW25113 isogenic derivative: Δ*tolA788*:: *km*^{R}	CGSC #8795 Baba *et al.* (2006)
JW5100-1	BW25113 isogenic derivative: Δ*tolB789*:: *km*^{R}	CGSC #11174 Baba *et al.* (2006)
JW5437-1	BW25113 isogenic derivative: Δ*rpoS746*:: *km*^{R}	CGSC #11387 Baba *et al.* (2006)
JW5503-1	BW25113 isogenic derivative: Δ*tolC732*:: *km*^{R}	CGSC #11430 Baba *et al.* (2006)
JW0739-1	BW25113 isogenic derivative: Δ*galM728*:: *km*^{R}	CGSC #8802 Baba *et al.* (2006)
JW0740-3	BW25113 isogenic derivative: Δ*galK729*:: *km*^{R}	CGSC #8803 Baba *et al.* (2006)
JW0741-1	BW25113 isogenic derivative: Δ*galT730*:: *km*^{R}	CGSC #8804 Baba *et al.* (2006)
JW0742-1	BW25113 isogenic derivative: Δ*galE740*:: *km*^{R}	CGSC #8297 Baba *et al.* (2006)
JW1224-1	BW25113 isogenic derivative: Δ*galU745*:: *km*^{R}	CGSC #9110 Baba *et al.* (2006)
JW2027-2	BW25113 isogenic derivative: Δ*galF731*:: *km*^{R}	CGSC #9664 Baba *et al.* (2006)
JW2135-1	BW25113 isogenic derivative: Δ*mglC775*:: *km*^{R}	CGSC #9730 Baba *et al.* (2006)
JW2136-1	BW25113 isogenic derivative: Δ*mglA776*:: *km*^{R}	CGSC #9731 Baba *et al.* (2006)
JW2137-1	BW25113 isogenic derivative: Δ*mglB777*:: *km*^{R}	CGSC #9732 Baba *et al.* (2006)
JW2138-1	BW25113 isogenic derivative: Δ*galS778*:: *km*^{R}	CGSC #9733 Baba *et al.* (2006)
JW2805-1	BW25113 isogenic derivative: Δ*galR762*:: *km*^{R}	CGSC #10192 Baba *et al.* (2006)
JW2910-2	BW25113 isogenic derivative: Δ*galP789*:: *km*^{R}	CGSC #10251 Baba *et al.* (2006)
JW3996-1	BW25113 isogenic derivative: Δ*lamB732*::*km*^{R}	CGSC #10877 Baba *et al.* (2006)
JW1667-5	BW25113 isogenic derivative: Δ*lpp-752*::*km*^{R}	CGSC #9417 Baba *et al.* (2006)
JW0731-1	BW25113 isogenic derivative: Δ*pal-790*::*km*^{R}	CGSC #8796 Baba *et al.* (2006)
Phages		
T*4D*	Wild-type T4	Gift from G. Mosig (2009)
T4*rII*Δ1586	Δ(*rIIA-rIIB*)	Gift from G. Mosig (2009)
λ (*cI*-857)	*cI*[ts]*857*, (*rex*$^+$)	NBRP #ME6104 (2009)
λF7	λ, *Dam15*, *imm21*, *cI*[ts]*857*	(Gi Mikawa *et al.* 1996)
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), Alexeyev and Shokolenko (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

Results and Discussion

Despite playing a rich and historic role in evolving our understanding of modern molecular genetics, the mechanism governing the λ T4*rII* exclusion phenotype has remained a mystery for >60 years. Identification of host genes influencing Rex activity has long been sought to better understand the exclusionary mechanism. Through our work here, we have identified several key groups of host genes and linked them together in a new proposed model.

Assessing Rex activity of host genes suspected to be involved in T4rII exclusion

Based on the current understanding of the interplay of the ionic environment with Rex, we evaluated genes related to transmembrane ion transport, specifically Omps and their regulators. We observed the direct effect of each gene's deletion on Rex activity in single-gene knockouts. Selected knockout mutants (Baba *et al.* 2006) (Table 1) were transformed by a high-copy plasmid encoding *rexAB* (pHA1; Figure 2A), and were assayed for Rex activity (Tables 2 and 3). Overexpression of *rexAB* has been previously demonstrated without change in the resulting exclusion phenotype so long as the stoichiometric balance between *rexA* and *rexB* is maintained (Slavcev and Hayes 2003). By vastly increasing the dosage of *rex*, we expected only deletions of critical host players to demonstrate significant modulation of Rex activity. We then confirmed these results by examining key deletions with the natural dosage of Rex (Table 4). The wild-type parent strain (BW25113) carrying pHA1 acted as a positive control for Rex, demonstrating complete resistance to T4*rII* plating and near complete sensitivity to RII suppression of Rex activity by wild-type T4 (Tables 2 and 3). The $>10^6$-fold difference in plating between T4 (rII^+) and T4*rII* demonstrates the powerful Rex phenotype imparted by the *rex* locus encoded on pHA1 and the ability of the *rIIA* and *rIIB* genes of T4 to suppress this phenotype.

Omps:

The Omps all play roles in solute transmembrane transport. Based on this, we expected their absence to impede establishment of the harsh ionic environment that is a hallmark of the Rex phenotype (Figure 1). T4*rII* infectivity for all Omp deletion (Δ*omp*) mutants carrying the high-copy rex^+ pHA1 was observed to be similarly exclusionary with important exceptions (Table 2). To examine the fate of rex^+ Δ*omp* mutants following infection by T4*rII* (a state that normally would confer the onset of Rex cellular phenotypes), we also employed a cell viability assay to determine whether specific Δ*omp* mutants carrying pHA1 (rex^+) were as sensitive to T4*rII*-mediated cell killing following T4*rII*-infection as wild-type (rex^-) (Table 5). All rex^+ cells challenged with T4*rII* generally grew more slowly and colonies were only visible after 48 hr of incubation at 30° compared to the uninfected control strains that were visible within 24 hr, as previously described (Slavcev and Hayes 2002, 2003). The viability of most Δ*omp* mutants harboring the rex^+ plasmid was reduced by $\sim 10^2$-fold compared to that of the rex^+ control, indicating that these *omp* genes exert weak influence over Rex-mediated cellular fate (Table 5).

All Δ*omp* mutants, including the parent strain, were sensitive to T4 (rII^+) plating with the exception of the Δ*ompC* derivative that is deficient in the adsorption protein for T4 and is, hence, a host-range mutant (Table 2). In contrast, all nontransformed (rex^-) mutants exhibited near complete T4*rII* plating efficiency, except the *ompT* outer membrane protease mutant and the *ompL* porin mutant (Table 2). Below, we discuss key Omps and their roles in Rex.

OmpA: The hyper-Rex phenotype that similarly inhibited T4 and T4*rII* plating (Table 2) speaks to the significant role *ompA* expression plays in Rex (Figure 3); *ΔompA* also reduced cell viability upon T4*rII* infection in a rex^+ context (Table 5). Interestingly, we found that the *ompA* mutant carrying the multicopy rex^+ pHA1 not only reduced plating of T4*rII*, but also reduced plating of T4 (rII^+), suggesting that OmpA may play a role in RII's escape of Rex. Upon infection of *E. coli* cells, T4 normally causes rapid degradation of the messenger RNA (mRNA) of two main membrane proteins, OmpA and Lpp, within ~1.5 and 2.4 min respectively (Qi *et al.* 2015); this further supports some interplay between OmpA and RII to avoid Rex.

Exposure to stress in Δ*ompA* mutants has been shown to induce phenotypic changes in *E. coli*, whereby cells become spherical in shape, similar to stationary-phase cells (Wang 2002), highlighting the role of OmpA in stimulating the σ^E stress response. The heightened Rex activity seen in the *ompA* mutant (Figure 3A) may thus arise due to the predisposition of these host cells to be shunted into osmotic irregularity and a stationary-like phase. Because of the additional osmotic and structural instability caused by the *ompA* mutation, recovery of the cell's osmotic rebalance and membrane potential may be irreversible in a rex^+ context, which may account for the observed low viability of rex^+ *ompA* mutants (Table 5). Conversely, the inability of *ompA* mutants to effectively passage and evacuate invading DNA out of the cell (Wang and Lin 2001) during Rex onset could similarly doom the cell to the lethality of the superinfecting DNA. In either case, the irreversible stationary-like state effected by Rex activation in the *ompA* mutant would also account for the powerful Rex-dependent exclusion of T4*rII* and T4 (rII^+) alike (Snyder and McWilliams 1989; Slavcev and Hayes 2003), as stationary phase prohibits the propagation of T-even species (Bryan *et al.* 2016). 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 mean that the survivors are able to eventually evacuate invading T4*rII* DNA—a role that may be mediated by OmpA in concert with Rex proteins.

OmpX: We found that there was a $>10^3$-fold increase in T4*rII* plating in *ompX* rex^+ mutants, compared to the rex^+

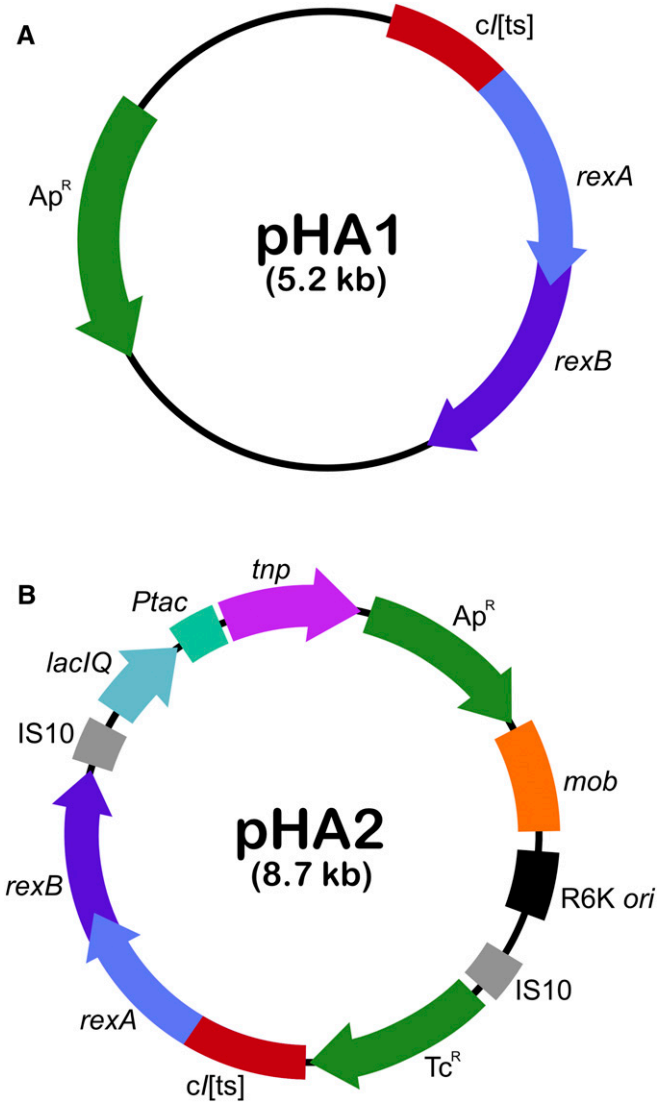

Figure 2 (A) Plasmid pHA1 carries *rex*A and *rex*B on a high-copy backbone derived from pUC19. (B) pHA2 carries *rex*A and *rex*B with a transposable element Tn*10* to randomly transpose the cassette into the host genome.

Table 2 Membrane proteins that attenuate the Rex phenotype

		Relative efficiency of plating[b]	
Mutation	Rex plasmid[a]	T4 (*rII*$^+$)	T4*rII* (*rII*$^-$)
–	+	1.0	$<3.0 \times 10^{-7}$
	–	1.0	1.0
Δ*ompA*	+	N/A[c]	N/A[c]
	–	1.4	0.6
Δ*ompC*[d]	+	$<3.0 \times 10^{-7}$	$<3.0 \times 10^{-7}$
	–	$<3.0 \times 10^{-7}$	$<3.0 \times 10^{-7}$
Δ*ompF*	+	0.2	$<3.0 \times 10^{-5}$
	–	1.0	0.9
Δ*ompG*	+	0.1	$<3.0 \times 10^{-7}$
	–	1.0	0.4
Δ*ompL*	+	0.1	2.0×10^{-5}
	–	1.0	1.0×10^{-2}
Δ*ompN*	+	0.3	$<3.0 \times 10^{-7}$
	–	1.0	0.3
Δ*ompR*	+	3.0×10^{-5}	$<3.0 \times 10^{-7}$
	–	1.0×10^{-2}	0.2
Δ*ompT*	+	0.2	$<3.0 \times 10^{-7}$
	–	1.0	1.0×10^{-4}
Δ*ompW*	+	0.2	7.0×10^{-4}
	–	1.0	0.2
Δ*ompX*	+	0.2	3.0×10^{-4}
	–	1.0	0.2
Δ*lamB*	+	1.0×10^{-6}	$<3.0 \times 10^{-7}$
	–	0.7	0.8
Δ*lpp*	+	4.0×10^{-4}	3.0×10^{-3}
	–	0.8	1.0
Δ*tolA*	+	0.3	0.8
	–	1.0	0.6
Δ*tolB*	+	0.1	$<3.0 \times 10^{-7}$
	–	1.0	1.0
Δ*tolC*	+	1.0×10^{-2}	$<3.0 \times 10^{-7}$
	–	0.5	1.0
Δ*tolQ*	+	8.0×10^{-2}	4.0×10^{-7}
	–	1.0	1.0
Δ*tolR*	+	6.0×10^{-2}	$<3.0 \times 10^{-7}$
	–	0.7	0.7
Δ*pal*	+	0.7	$<3.0 \times 10^{-7}$
	–	0.8	1.0

[a] The Rex$^+$ plasmid is pHA1, a pUC-derived high-copy plasmid carrying the P_M-*cI-rexA-rexB-*t_{imm} cassette.

[b] All assayed strains are isogenic derivatives of Keio collection wild-type BW25113. Relative efficiencies of plating are determined by dividing the number of plaques for the sample by the number of plaques on the 100% control: BW25113 as the 100% control for T4*rII* plating and BW25113[pHA1] as the 100% control for Rex activity. All results represent the average of three independent plating assays. Strains (*rex*$^+$) attenuated for Rex activity showed pinpoint plaques compared to *rex*$^-$ counterparts.

[c] Pinpoint plaques were visible but only under high-density plating conditions, which did not make quantification at reasonable dilutions possible.

[d] The Δ*ompC* mutant is a host range mutant that precludes T4 adsorption, so neither phage can infect this strain.

control (Table 2). Additionally, Δ*ompX* exhibited $>10^2$-fold reduced viability on T4*rII*-infected derivatives carrying the *rex*$^+$ plasmid compared to the *rex*$^+$ control, likely due to compromised T4*rII* exclusion in these *rex*$^+$ mutants (Table 5). Like OmpA, OmpX has similarly been implicated in cell defense against virulence (Vogt and Schulz 1999). Deletion of *ompX* sensitizes cells to stressful environments including phage infection and killing (Otto and Hermansson 2004). Otto and Hermansson (2004) found that Δ*ompX* causes significant alterations in cell surface hydrophobicity and negative charge that may also increase phage-bacterial interaction. Small RNAs MicA and RybB regulate expression of *ompX* and some other membrane proteins (Valentin-Hansen *et al.* 2007). Their transcripts (*micA* and *rybB*), expressed under the control of σ^E, further downregulate *ompA* and *ompC* translation. The consequent disruption of Omp formation enhances σ^E-dependent cell lysis, as noted by the resultant high protein density in the culture medium (Kabir *et al.* 2005). Underproduction of OmpX has been noted to reduce σ^E activity (and vice versa) in *E. coli* as a "strain-dependent" phenotype (Mecsas *et al.* 1993), which may help explain the reduction in Rex activity (Figure 3A) in these *rex*$^+$ mutants.

Table 3 Other factors that attenuate the Rex phenotype

Mutation	Rex plasmid[a]	Relative efficiency of plating[b] T4 (*rII*$^+$)	T4*rII* (*rII*$^-$)
–	+	1.0	$<3.0 \times 10^{-7}$
	–	1.0	1.0
Δ*mglA*	+	7.0×10^{-2}	$<3.0 \times 10^{-7}$
	–	0.7	0.7
Δ*mglB*	+	0.7	$<3.0 \times 10^{-7}$
	–	0.7	1.0
Δ*mglC*	+	0.1	2.0×10^{-5}
	–	0.7	1.0
Δ*galM*	+	1.0	$<3.0 \times 10^{-7}$
	–	0.6	1.0
Δ*galK*	+	7.0×10^{-2}	$<3.0 \times 10^{-7}$
	–	0.6	1.0
Δ*galT*	+	8.0×10^{-2}	$<3.0 \times 10^{-7}$
	–	0.7	1.0
Δ*galE*	+	1.0×10^{-2}	$<3.0 \times 10^{-7}$
	–	1.0	1.2
Δ*galU*	+	$<3.0 \times 10^{-7}$	$<3.0 \times 10^{-7}$
	–	$<3.0 \times 10^{-7}$	$<3.0 \times 10^{-7}$
Δ*galF*	+	1.0	$<3.0 \times 10^{-7}$
	–	0.7	1.0
Δ*galP*	+	1.0	$<3.0 \times 10^{-7}$
	–	0.7	1.0
Δ*galS*	+	0.3	$<3.0 \times 10^{-7}$
	–	0.7	1.0
Δ*galR*	+	1.0	$<3.0 \times 10^{-7}$
	–	0.7	1.0
Δ*rpoS*	+	$<3.0 \times 10^{-7}$	$<3.0 \times 10^{-7}$
	–	0.7	0.7
Δ*rssB*	+	0.5	3.0×10^{-3}
	–	1.0	1.0
Δ*rssA*	+	3.0×10^{-3}	0.2
	–	1.0	1.0
Δ*iraP*	+	3.0×10^{-2}	$<3.0 \times 10^{-7}$
	–	1.0	1.0
Δ*iraM*	+	0.5	$<3.0 \times 10^{-7}$
	–	1.0	1.0
Δ*iraD*	+	0.5	$<3.0 \times 10^{-3}$
	–	1.0	1.0

[a] The Rex$^+$ plasmid is pHA1, a pUC-derived high-copy plasmid carrying the P_M-*cI-rexA-rexB-*t_{imm} cassette.

[b] All assayed strains are isogenic derivatives of Keio collection wild-type BW25113. EOPs are determined by dividing the number of plaques for the sample by the number of plaques on the 100% control. EOPs of T4/T4*rII* were determined using BW25113 as the 100% control for T4*rII* plating and BW25113[pHA1] as the 100% control for Rex activity. All results represent the average of three independent plating assays. Strains (*rex*$^+$) attenuated for Rex activity showed pinpoint plaques compared to *rex*$^-$ counterparts.

Table 4 Rex activity is influenced by *E. coli* proteins that affect membrane integrity and maintenance

Mutation	Relative EOP of T4*rII*-infected centers[a]
–	1.0
– (λ)	$<3.0 \times 10^{-7}$
Δ*ompT*	$<3.0 \times 10^{-7}$
Δ*ompX*	4.5×10^{-4}
Δ*ompF*	3.3×10^{-5}
Δ*ompA*	5.0×10^{-4}
Δ*ompW*	1.0×10^{-3}
Δ*ompG*	$<3.0 \times 10^{-7}$
Δ*ompN*	$<3.0 \times 10^{-7}$
Δ*ompC*[b]	$<3.0 \times 10^{-7}$
Δ*ompR*	$<3.0 \times 10^{-7}$
Δ*ompL*	$<3.0 \times 10^{-6}$
Δ*rpoS*	0.7
Δ*tolA*	1.0
Δ*rssA*	0.9
Δ*rssB*	1.0
Δ*iraM*	0.1
Δ*iraP*	0.1
Δ*iraD*	1.0

[a] All assayed strains are *rex*$^+$ isogenic derivatives of Keio collection wild-type BW25113 lysogenized by λ. EOPs of T4/T4*rII* were determined using BW25113 as the 100% control for T4*rII* plating. All mutant-infected centers were permitted to adsorb T4*rII* for 15 min before washing the infected cells three times, diluting, and plating on the relevant test strain. All results represent the average of three independent plating assays.

[b] The Δ*ompC* mutant is a host-range mutant that precludes T4 adsorption, so neither phage can infect this strain.

OmpW: Another significant attenuator of Rex activity was Δ*ompW*, increasing T4*rII* plating in a *rex*$^+$ context by $>10^3$-fold (Table 2). OmpW forms porins in the outer membrane, generating long, narrow, hydrophobic channels that serve as ion channels in the transport of small hydrophobic molecules across the membrane (Hong *et al.* 2006). Δ*ompW* also exhibited 10^2-fold reduced viability on T4*rII*-infected derivatives carrying the *rex*$^+$ plasmid (Table 5). Interestingly, OmpW, along with OmpA and OmpF, has been documented to protect *E. coli* against environmental stressors including viral superinfection (Wu *et al.* 2013). The reduction in the viability of *ompX*, *ompW*, and *ompF* mutants (Table 5) supports our observations that these *rex*$^+$ mutants were attenuated for Rex activity (Figure 3A) and were therefore more sensitive to T4*rII* infection. As such, they were more readily lysed.

OmpR, OmpC, and OmpF: As expected, the Δ*ompC* mutants demonstrated complete resistance to T4 and T4*rII* infections, and therefore serve as the negative controls for T4 infection due to direct prevention of T4 adsorption. Deletion of *ompR* did not significantly affect T4*rII* plating, while deletion of *ompF* increased T4*rII* plating by $>10^2$-fold (Table 2). Therefore, Rex activity is more heavily affected by Δ*ompF* than Δ*ompR* (Figure 3A). The *ompF* and *ompC* genes are differentially expressed based on changes in medium osmolarity, as sensed by the membrane-bound EnvR sensor (Srividhya and Krishnaswamy 2004) and carried out by the cytoplasmic transcriptional regulator OmpR, reviewed in depth elsewhere (Mizuno and Mizushima 1990).

The ionic environment is crucial for the Rex phenotype, where monovalent cations are essential for the onset and divalent cations can abrogate the phenotype (Garen 1961; Brock 1965; FerroLuzzi-Ames and Ames 1965; Sekiguchi 1966). OmpF and OmpC function as cation-selective diffusion channels that control cell osmolarity in response to changes to extracellular osmolarity (Cowan *et al.* 1992; Apirakaramwong *et al.* 1998). Under high osmotic pressure, *ompC* expression is upregulated; on the other hand, under low osmotic pressure, *ompF* expression is upregulated to

Table 5 Omp proteins influence *rex*⁺ host viability following T4*rII* infection

Mutation	Rex plasmid	EOP[a]	Cell viability[b]
–[1]	–	3.0×10^{-7}	0
–[1]	+	1.0×10^{-3}	1.0
Δ*ompA*	+	5.0×10^{-4}	0.5
Δ*ompC*[c]	+	0.2	–[3]
Δ*ompF*	+	2.0×10^{-5}	1×10^{-2}
Δ*ompG*	+	6.0×10^{-4}	0.6
Δ*ompL*	+	1.0×10^{-3}	1×10^{-3}
Δ*ompN*	+	2.0×10^{-4}	0.5
Δ*ompR*	+	0.7	–[3]
Δ*ompT*	+	5.0×10^{-4}	0.5
Δ*ompW*	+	1.0×10^{-5}	1×10^{-2}
Δ*ompX*	+	1.0×10^{-5}	1×10^{-2}

[a] Efficiency of plating showing CFU arising at 30° after infection by T4*rII* at an MOI of 3. Average of three trials. A 100% control of T4*rII* infectivity was BW25113 (*rex*⁻); BW25113 (wt) carrying [pHA1] plasmid was employed as the 100% positive control of Rex activity.

[b] Rex-mediated protection of host cells from T4*rII* challenge. Calculated using BW25113 carrying pHA1 (*rex*⁺) plasmid as 100% viability control following T4*rII* challenge.

[c] The deletion of *ompC* precludes T4 phage adsorption protecting this strain against infection. Similarly, the *ompR* mutation inhibits *ompC* expression precluding T4 phage adsorption protecting this strain against infection by T4.

stabilize cellular osmosis. OmpR regulates transcription of the small RNAs MicC and MicF that stimulate degradation of the *ompC* and *ompF* mRNA transcripts, thus reducing their expression in an OmpR-deprived environment (Guillier 2006). Importantly, the expression of *ompC* can also be stimulated independently of OmpR by sucrose, previously shown to powerfully suppress Rex activity (Schnaitman and McDonald 1984). OmpC also functions as the primary adsorption receptor for T4 (Yu and Mizushima 1982; Washizaki *et al.* 2016), which we similarly confirmed in this study as Δ*ompC* results in the loss of T4's ability to infect (Table 2). Because of the prohibitive state of *ΔompC* toward T4 plating, we cannot definitively conclude any involvement of OmpC in Rex activity. However, based on previous observations of OmpC, OmpF, and OmpR regulation, potential scenarios can logically be envisioned for their roles in Rex activation: (1) as the adsorption receptor for T4, OmpC makes a very logical Rex activation target or sensor, triggered by T4 superinfection; (2) poor T4 infectivity in the *ompR* mutant could be attributed to reduced expression of *ompC*, that again, is essential for T4 adsorption; (3) OmpF likely plays an indirect role in Rex, perhaps through osmotic dysregulation and stimulation of a stationary phase-like state; and (4) as OmpC and OmpF are expressed under inverse osmolarity conditions, reduced *ompF* expression would stimulate *ompC* transcription leading to increased T4 infection (Srividhya and Krishnaswamy 2004).

OmpL and OmpT: Rex activity was moderately reduced in the absence of *ompL* or *ompT* (Figure 3A). In the presence of *rex*, T4*rII* plating increased by 10^2-fold in the *ompL* mutant, while no significant changes were observed in the *ompT* mutant (Table 2). In the absence of *rex* however, we observed reductions in T4*rII* plating on both *ompT* (10^4-fold reduction) and *ompL* (10^2-fold reduction). This is in contrast to T4 (*rII*⁺), whose plating does not appear significantly affected by Δ*ompT* or Δ*ompL* regardless of *rex* presence. It is not currently known what roles, if any, OmpT and OmpL may play in bacteriophage infection. OmpL functions as a low-molecular-weight diffusion porin (Sardesai 2003), while OmpT is a protease that cleaves foreign peptides encountered within the *E. coli* cell (Stumpe *et al.* 1998). Absence of *ompT* or *ompL* has not previously demonstrated any detriment to bacterial cell growth. However, we did observe that deletion of *ompL* reduced cell viability 10^3-fold in a *rex*⁺ context (Table 5). T4*rII* (*rII*⁻) plating in general appears to be compromised in *ΔompT* or *ΔompL* contexts (Table 2). It is possible that either OmpT or OmpL may serve alternative functions to RII and support T4*rII* infection in the absence of RII.

Rex and other membrane proteins:

We also examined Rex activity in knockouts of other critical membrane proteins (Figure 4). In general, knockout of *tolA*, *lamB*, and to a marginal degree, *lpp*, *tolR*, and *tolQ*, improved T4 (*rII*⁺) plating specifically under *rex*⁺ conditions. This phenotype was not observed in *rex*⁻ conditions (Table 2), indicating some connection between these genes and RII. Below, we discuss the effect of these gene knockouts in further detail.

LamB: While knockout of *lamB* did not improve the plating efficiency of T4*rII* in a *rex*⁺ context, it did greatly reduce the plating efficiency of T4 (*rII*⁺) by 10^6-fold (Table 2). In other words, RII's ability to circumvent Rex is impaired in absence of *lamB* (Figure 4A). LamB is mainly responsible for maltose uptake, but also acts as a receptor for λ adsorption (Randall Hazelbauer and Schwartz 1973). A highly abundant protein, LamB, exhibits dynamic spatial localization throughout the outer membrane (Gibbs *et al.* 2004). Like OmpA, its expression is modulated by σ^E and by the small RNA MicA. Along with the *mal* operon, *lamB* can also be upregulated by σ^S. Mutations in *lamB* are very harmful to the cell, causing major defects in the inner membrane, disrupting the proton motive force, and unfolding Omps (Death *et al.* 1993). It is interesting that Δ*lamB* showed almost complete abrogation of T4 (*rII*⁺) and T4*rII* plating in a *rex*⁺ context, but full plating efficiency in the absence of *rex* (Table 2). Hence, LamB seems to interact with RII and/or Rex.

Tol, Pal: Knockout of *tolA* almost completely abrogated Rex activity (Figure 4A), leading to complete plating of T4*rII*. Knockouts of *tolQ*, *R*, and *B* showed no effect (Table 2), suggesting the direct involvement of TolA with Rex and/or RII. Interestingly, we observed full plating efficiency with pinpoint plaques of T4*rII* compared to wild-type T4 on a Δ*tolA* mutant, but not Δ*tolB* (Table 2), as previously noted in a different study (Rolfe and Campbell 1977). The Tol proteins play critical roles in maintaining cell membrane stability and integrity (Lazzaroni *et al.* 1999; Lloubès *et al.* 2001), including roles in assembly of outer membrane porins and lipopolysaccharide synthesis. Tol normally forms a complex with the membrane-bound Pal lipoprotein (Tol-pal complex) (Cascales *et al.* 2002). However, knockout of *pal* surprisingly

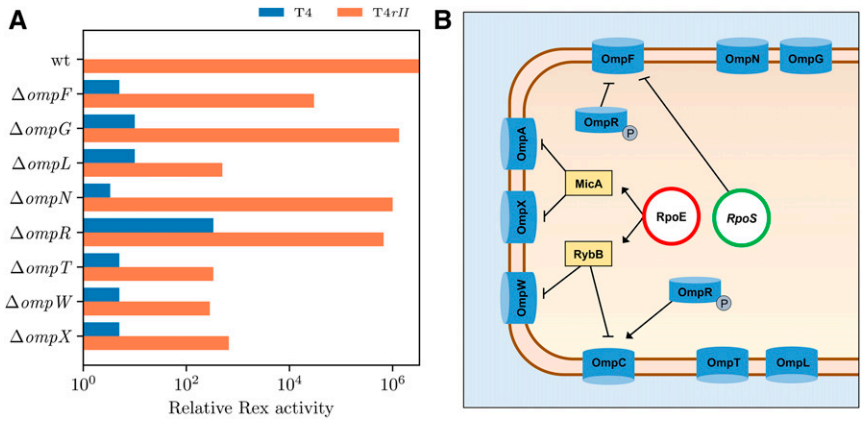

Figure 3 Omps may play a role in Rex, as their deletions affect Rex activity. In particular, Δ*ompF*, Δ*ompL*, Δ*ompT*, Δ*ompW*, and Δ*ompX* attenuate Rex activity. (A) Rex activity is the ability of pHA1-delivered *rex* genes to reduce plating in the presence or absence of RII. Activity is derived by taking the inverse of the relative EOP of the sample in *rex*$^+$ conditions (plasmid) compared to *rex*$^-$ conditions (no plasmid). Maximal Rex activity is the complete attenuation of T4*rII* (*rII*$^-$) plating in presence of the *rex* pHA1 in *E. coli* BW25113 (wt). Absence of Rex activity is full plating of T4 (*rII*$^+$) in presence of the *rex*$^+$ pHA1 in *E. coli* BW25113 (wt). (B) Omps mediate ion transport as regulated by *rpoE* and *rpoS*.

did not show any effect on Rex (Figure 4A). Alongside Omps, Tol proteins are involved in the import and export of micro- and macromolecules. TolA has three domains that extend to the outer membrane, bridging interactions between outer- and inner-membrane proteins (Levengood-Freyermuth *et al.* 1993; Derouiche *et al.* 1996). Transcription of *tolA* is positively regulated by σ^E through the activation of the RcsC/B sensor kinase system in response to envelope stress (Dam *et al.* 2018). Activation of RcsC/B would also stimulate the activation of σ^S and σ^E.

TolA indirectly regulates OmpF by increasing *ompF* expression through downregulation of *ompC* expression; as such, *ompF* expression is downregulated in *tolA* mutants (Lazzaroni *et al.* 1986; Derouiche *et al.* 1996). Deletion of *tolA* also reduces LamB levels, resulting in the onset of cell stress responses (Derouiche *et al.* 1996). Mutations in *tolA* changes cell morphology, making bacterial cells very leaky and sensitive to external stresses, including infections, but it also renders them resistant to colicins (Meury and Devilliers 1999; Lloubès *et al.* 2001; Lazzaroni *et al.* 2002). Many colicins require the TolQRAB complex, OmpA, and the pore-forming OmpF and OmpC, to translocate into cells. We noted the deletion of any of these demonstrated at least some increase in T4*rII* plating compared to the *rex*$^+$ control. There appears to be a strong analogy between the colicin and Rex systems.

Lpp: In the presence of *rex*, Δ*lpp* improved T4*rII* plating 10^4-fold compared to the *rex*$^+$ control. In contrast, T4 plating was reduced by 10^4-fold (Table 2). In addition to the Tol-Pal complex, Lpp is a major prolipoprotein on the inner face of the outer membrane that protects and maintains the structural and functional integrity of the cell membrane (Ozawa and Mizushima 1983). Lpp maintains the network between outer membrane and peptidoglycan layer. Transcription of *lpp*, like other membrane proteins, is under the regulation of σ^E, emphasizing its role in stress responses. Mutation of *lpp* results in loss of the structural link between envelope membranes, consequently releasing periplasmic proteins into the medium and forming vesicles, which is phenotypically similar to mutations in *tol-pal* (Bernadac *et al.* 1998). Transcripts of *lpp* are rapidly degraded after T4 infection (Qi *et al.* 2015); its deletion does not appear to affect T4 or T4*rII* plating in the absence of *rex* genes (Table 2). In contrast, we observed medium plating efficiency for both in a *rex*$^+$ context, indicating some Lpp involvement in Rex. Since it is not on the outer surface, Lpp might not participate in the onset of Rex, but it may maintain activated Rex throughout the exclusion mechanism. Mutations in *lpp* do not affect expression of Tol-Pal, but *tolA* mutations decrease *lpp* expression (Cascales *et al.* 2000). Lpp may be an important link between TolA and Omps. Although no direct interaction between TolA and OmpA has yet been found, TolA indirectly interacts with a TolB-Pal-Lpp-OmpA complex as well as the rest of Omps (Lloubès *et al.* 2001).

Rex and sigma factors:

We next examined Rex activity in sigma factor knockouts (Figure 5) as key Rex-involved membrane proteins are regulated by σ^S. Most importantly, knockout of *rpoS* completely abrogated T4 (*rII*$^+$) and T4*rII* plating alike in a *rex*$^+$ context (Table 3). Below, we discuss these knockouts in detail.

rpoS and regulators: Knockout of *rpoS* completely abrogated T4 (*rII*$^+$) and T4*rII* plating alike in a *rex*$^+$ context (Table 3). Knockouts of small RNA regulators of σ^S expression also demonstrated rescue of T4*rII* plating to various degrees: Δ*rssA*, Δ*rssB*, and Δ*iraD* notably increased T4*rII* plating (10^6-fold for Δ*rssA*, $>10^4$ for both Δ*rssB* and Δ*iraD*). However, little to no effect on Rex activity was observed in the Δ*iraM* and Δ*iraP* mutants in the presence of high *rex*$^+$ expression (Figure 5A). *E. coli* cells enter stationary phase upon exposure to extrinsic or intrinsic stress initiating σ^E activation, where all growth phase genes are switched off and stress response genes are switched on (Chen *et al.* 2004). σ^S, encoded by *rpoS*, is essential for cell survival in stationary phase. It regulates the expression of >10% of all *E. coli* genes during this phase (Weber *et al.* 2005). During growth phase, the small RNA protease ClpXP rapidly degrades levels of *rpoS* mRNA by binding to the *rpoS* transcript adaptor RssB (Schweder *et al.* 1996; Battesti *et al.* 2015). In contrast, the anti-adaptor small RNAs IraD, IraM, and IraP stabilize the *rpoS* transcript via interactions with RssB during different stress conditions such as the stationary phase. During

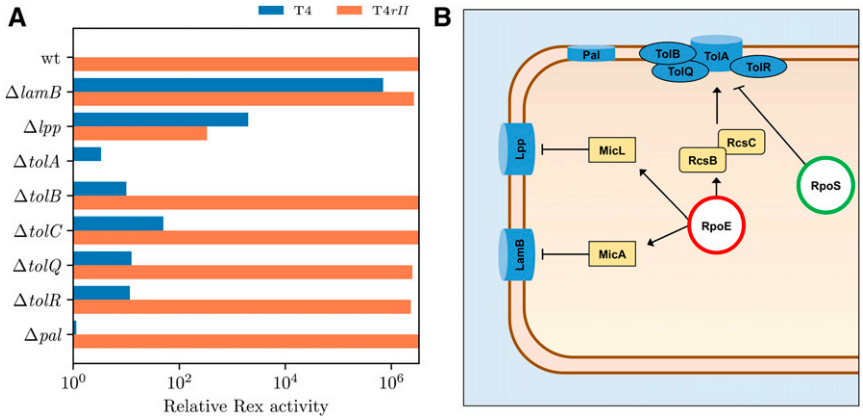

Figure 4 Several other key membrane proteins may play a role in Rex, as their deletions affect Rex activity. In particular, Δ*tolA* completely restores T4*rII* plating, thereby eliminating Rex activity. (A) Rex activity is the ability of pHA1-delivered *rex* genes to reduce plating in the presence or absence of RII. Activity is derived by taking the inverse of the relative EOP of the sample in *rex*$^+$ conditions (plasmid) compared to *rex*$^-$ conditions (no plasmid). Maximal Rex activity is the complete attenuation of T4*rII* (*rII*$^-$) plating in presence of the *rex* pHA1 in *E. coli* BW25113 (wt). Absence of Rex activity is full plating of T4 (*rII*$^+$) in presence of the *rex*$^+$ pHA1 in *E. coli* BW25113 (wt). (B) These major membrane proteins are upregulated by *rpoE*.

stationary phase, ClpXP instead increases the expression of *rpoE*, thereby stimulating the σ^E response, leading to alteration of Omp stability, which, in turn, leads to altered cell morphology. In a Δ*rpoS* context, the absence of *rpoS* transcripts frees ClpXP to elevate σ^E expression.

Interestingly, while *ompF* expression is positively controlled by σ^E through OmpR, its expression is negatively controlled by σ^S through the small RNA MicF. MicF's own expression is also downregulated by σ^S (Pratt and Silhavy 1996; Patten *et al.* 2004). Expression of *ompF* has been found to be elevated in *rpoS* mutants without effect on OmpC levels (Pratt and Silhavy 1996; Battesti *et al.* 2015). In this study, *rpoS* mutants were able to fully exclude T4 and T4*rII* from plating in the presence of *rex* (Table 3), which might be explained by the overexpression of σ^E-controlled TolA, LamB, and OmpF and their direct effect/interaction with Rex. Wild-type levels of plating by both T4 and T4*rII* alike were observed in the absence of *rex* (Table 3). This super-Rex phenotype was also seen in *ompA* mutants (Table 2), which could indicate the involvement of *rpoS* in "triggering" Rex.

rpoE: Based on our observations of the effect of *omps*, *tolA*, and *rpoS* in Rex activity (Figures 3–5), we believe that the σ^E pathway may also be intimately connected to Rex. σ^E is an essential factor that governs transcriptional activation of many downstream genes, including the periplasmic proteins and degradation factors. It has been implicated in a cell lysis pathway entailing small RNAs MicA and RybB that reduce levels of Omps, leading to the eventual disintegration of the outer membrane (Murata *et al.* 2012). A strikingly similar scenario is when *E. coli* cells enter stationary phase, where elevation of active σ^E leads to the decrease in expression of OmpA, OmpC, and OmpW (Kabir *et al.* 2005). *E. coli* sigma factors σ^E and σ^S share homology with T4 gp-55, the T4 late sigma factor that is responsible for the degradation of the periplasmic peptidoglycan layer 5 min following infection (Kassavetis and Geiduschek 1984; Arisaka 2005). This timing is very close to that recorded for the onset of Rex (7–10 min), which potentially positions σ^E as a major regulator of Rex activity.

Assessing activity of host genes with natural rex gene dosage

We sought to confirm these effects with the natural gene dosage for T4*rII* exclusion. We expected that with wild-type *rex* expression levels, attenuation of Rex would be intensified in absence of key genes. Selected mutants were lysogenized by phage λ and assayed for Rex activity (Table 4). As expected, with reduced gene dosage, attenuation of Rex activity was generally exacerbated. Notable reductions in Rex activity were observed in the following groups of lysogenized knockouts (Table 4): *omps*, *tolA*, *rpoS*, and small RNAs. Deletion of *ompL*, *ompF*, *ompA*, *ompX*, and *ompW* improved T4*rII* plating up to 10^4-fold compared to the *rex*$^+$ control. Almost complete abrogation of Rex activity was observed upon lysogenization of the Δ*tolA* mutant by λ where pinpoint plaques of T4*rII* were again observed. This was also exhibited in the Δ*rpoS*(λ) mutant, supporting our suspicion that σ^S acts in a Rex-dependent manner. Interestingly, almost complete attenuation was observed for λ lysogens of Δ*rssA*, Δ*rssB*, and Δ*iraD*. Together, these findings again suggest that there is a gene dosage-dependent interplay between Rex and small RNAs.

Mutagenesis and isolation of E. coli mutant(s) that influence Rex activity

We also employed a one-step mutagenesis plasmid system (pHA2; Figure 2) that transposes randomly into the *E. coli* genome while simultaneously expressing λ *rexA-rexB*. This would ensure expression of *rexAB* and simultaneously knock out nonessential host genes that could influence Rex. Candidates would be genotypically *rex*$^+$, but phenotypically Rex$^-$ or attenuated for Rex activity. Transposition of the Tc^R marker into recipient cells (Km^R) allowed for selection of successful transposition and integration of the Tn*10* transposon carrying the *cI857-rexA-rexB* cassette. We generated Rex$^-$ integrants using conjugative transposition of the pHA2 plasmid into recipient cells by growing exconjugants directly on plates seeded with T4*rII* as part of the one-step selection for integrants while simultaneously screening for Rex$^-$. We anticipated that Rex$^+$ integrants able to exclude

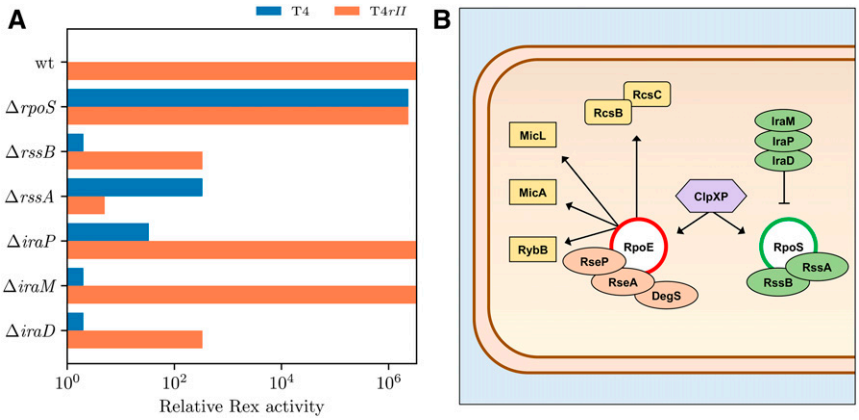

Figure 5 Regulatory small RNAs and *rpoS* may interact with Rex, as rex genes are able to inhibit T4 (*rII*$^+$) plating if *rpoS* is deleted. (A) Rex activity is the ability of pHA1-delivered *rex* genes to reduce plating in the presence or absence of RII. Activity is derived by taking the inverse of the relative EOP of the sample in *rex*$^+$ conditions (plasmid) compared to *rex*$^-$ conditions (no plasmid). Maximal Rex activity is the complete attenuation of T4*rII* (*rII*$^-$) plating in presence of the *rex* pHA1 in *E. coli* BW25113 (wt). Absence of Rex activity is full plating of T4 (*rII*$^+$) in presence of the *rex*$^+$ pHA1 in *E. coli* BW25113 (wt). (B) *rpoE* and *rpoS* are regulated by ClpXP activity and respective regulatory small RNAs.

T4*rII* would be unaffected by the presence of phage and should form regular, circular colonies, while mutants compromised for Rex activity (*i.e.*, able to propagate T4*rII*) would be characterized by irregular, "bitten" colonies (Gussin and Peterson 1972). Transposition frequency of the *rex*$^+$ element under inducing conditions generally ranged from 10^{-3}-10^{-7} of exconjugants (results not shown). The bitten:regular colonies ratio averaged ~1:1500 in JW0427-1 recipient cells, which is expected considering the >3000 nonessential *E. coli* protein-encoding genes, most of which would not be expected to influence the Rex phenotype.

We were able to isolate 13 candidate mutants confirmed to be genotypically *rex*$^+$, while also strongly attenuated or fully abrogated for Rex activity (Table 6). Although these mutants were confirmed to be genotypically *rex*$^+$, the noted improvement in T4*rII* plating indicates insertional mutation of a host gene that is either directly or indirectly involved in Rex phenotype. In all cases, abrogated or attenuated Rex activity was not due to a faulty *rex* cassette, as each isolate was subsequently lysogenized by λ (*rex*$^+$) to deliver yet another copy of the *rex* genes and then tested again for Rex activity (Table 6).

Sequencing of candidate mutants (results not shown) indicated that JW-HA 11 was a 95% match to the nonessential gene *mglC*, a hydrophobic ABC transporter permease that, when mutated, rescues T4*rII* plating by >10^3-fold (Table 6). High expression of *rex* in a Δ*mglC* knockout corroborated this finding, demonstrating at least 10^2-fold improvement in T4*rII* plating (Table 3). MglC is part of a complex operon expressing Mgl-Gal proteins (Boos *et al.* 1971; Death *et al.* 1993; Death and Ferenci 1994). While *mglC* clearly influences Rex activity, coexpression of *mglA* may, in tandem, influence RII activity, since mutation of *mglA* reduces T4 (*rII*$^+$) plating by >10-fold in a *rex*$^+$ context (Table 3). Deletion of *galK*, *T*, or *E* reduced T4 (*rII*$^+$) plating efficiency by up to two orders of magnitude, which may mean that the presence of this operon is needed to enable RII function. Gal proteins may also enhance RII activity in the presence of Rex proteins (Table 3). MglC is likely located on the internal face of the cytoplasmic membrane (Harayama *et al.* 1983) and is involved in the methyl-galactoside transport system (Hogg *et al.* 1991). Interestingly, it would therefore share this location with RexB. Furthermore, its internal orientation has the potential for interaction with the cytoplasmic RexA, suggesting much potential for MglC's role in the activation or mechanism of Rex.

A proposed model for Rex and RII suppression of Rex

Based on the data derived in this work, we attempt to link membrane proteins, sigma factors, and their regulators to the activation, manifestation, and the exclusion mechanism itself (Figure 6). Overall, we propose that in the absence of RII proteins, Rex-mediated abrogation of T4 replication arises from the interplay between σ^S- and σ^E-governed regulons.

T4 infection activates host stress responses:

Upon infection, T4 first adsorbs to OmpC on the outer membrane and injects its DNA into the host cell, causing a disturbance in periplasmic and cytoplasmic osmolarity (Arisaka 2005) and activating membrane sensors such as OmpX and TolA. A σ^E-mediated stress response is triggered in response to envelope stress (Dam *et al.* 2018). Membrane and osmotic stress also triggers σ^S activation and induction of stationary phase genes (Hengge-Aronis *et al.* 1991, 1993). During growth phase, ClpXP normally outcompetes anti-adaptor small RNAs IraD, IraM, and IraP to bind RssB, minimizing active σ^S levels (Schweder *et al.* 1996; Battesti *et al.* 2015). During T4 infection, activation of σ^S regulons would instead increase the supply of IraD, IraM, and IraP to outcompete ClpXP, thereby elevating active σ^S levels (Bryan *et al.* 2016). Free ClpXP would then be available to elevate σ^E levels. Generally, in absence of Rex, T4 infection would lead to cell lysis within 15 min (Bryan *et al.* 2016). Not surprisingly, phage infection is a major stress stimulator; overall, the start of T4 infection is expected to activate stress response pathways, resulting in high expression levels of both σ^E and σ^S in absence of Rex.

As OmpA expression is directly linked to σ^E activation, depleted levels of OmpA in the cell may "prime" *rex*$^+$ cells for exclusion. The powerful inhibition of T4 and T4*rII* plating in Δ*ompA* mutants is heavily Rex-dependent as it was not

Table 6 Isolated integrant mutants attenuated for Rex activity

Strain	Wild-type level *rex*$^+$ expression[a,b]	Double *rex*$^+$ expression[b,c]
Controls		
JW0427-1[b]	1.0	1.0
JW0427-1(λ)[d]	$<1.0 \times 10^{-7}$	$<1 \times 10^{-7}$
Isolates		
JW-HA 1	2.3×10^{-5}	4.0×10^{-6}
JW-HA 2	7.0×10^{-6}	5.2×10^{-6}
JW-HA 3	1.5×10^{-5}	1.7×10^{-5}
JW-HA 4	1.0×10^{-3}	5.0×10^{-4}
JW-HA 5	1.7×10^{-5}	5.0×10^{-6}
JW-HA 6	1.4×10^{-4}	1.0×10^{-4}
JW-HA 9	5.0×10^{-6}	4.0×10^{-6}
JW-HA 11	1.6×10^{-5}	1.3×10^{-4}
JW-HA 12	1.3×10^{-5}	1.6×10^{-4}
JW-HA 19	2.0×10^{-4}	1.5×10^{-4}
JW-HA 20	8.0×10^{-6}	6.8×10^{-6}
JW-HA 21	1.6×10^{-5}	1.7×10^{-5}
JW-HA 25	1.8×10^{-5}	4.0×10^{-6}

[a] Random insertion of the *rexA-rexB* transposable cassette into the chromosome renders insertional mutants genotypically *rexA*$^+$*rexB*$^+$ (*rex*$^+$).
[b] *E. coli* strain JW0427-1 (Δ*clpP*::*kan*) (*rex*$^-$) used as negative control with relative T4*rII* EOP of 1.0. All values based on averaged EOP from three independent assays.
[c] Transponants[1] lysogenized with λ. Hosts carry two copies of *rexA-rexB*, one from transposon cassette and the second of λ lysogen.
[d] *E. coli* strain JW0427-1(λ) (Δ*clpP*::*kan*) (*rex*$^+$) used as positive control with relative T4*rII* EOP of 1.0.

seen in the *rex*$^-$ mutant (Table 2). This suggests that OmpA may be a target for inactivation by Rex protein(s) and that this inactivation may be competitively inhibited by RII proteins. Alternatively, RII suppression of Rex relies upon its interaction with OmpA. Either way, the incomplete abrogation of T4 plating in a *ΔompA* context suggests that *ompA* is not the only host gene involved.

Rex onset increases tol and rpoE expression, but reduces rpoS expression:

In a Rex$^+$ environment, onset of Rex is postulated to start with membrane-bound RexB binding with multiple RexA proteins (Parma *et al.* 1992). This enables RexB-mediated efflux of cations, which would result in further membrane destabilization and depolarization. Activated stress response pathways should replenish the depleted pools of Omps (including OmpX, OmpW, OmpF, or Lpp) in reaction to T4 infection. Moreover, membrane instability also stimulates *tol* expression to maintain cell integrity. TolA would further stimulate σ^E activity, leading to increased expression of *tol-pal*, *lamB*, *lpp*, and *ompA* to restore membrane integrity. We found that only the *tolA* mutant was able to completely abrogate Rex activity alone (Figure 4A). Thus, we suspect TolA must serve as one of the key controllers that maintains Rex activation and exclusion. Because of its transmembrane position facilitating interaction with both RexA and RexB, we hypothesize that direct TolA interaction with Rex proteins activates formation of the RexB-RexA active complex.

Based on their shared membrane-bound location, RexB could interact with RssB in place of σ^S, freeing σ^S from the RssAB-ClpXP complex. This would again free ClpXP to elevate σ^E levels and expression of the membrane stress regulon. Cytoplasmic RexA could also bind IraD preventing its upregulation of σ^S. We believe that the activation of Rex would therefore be associated with high σ^E levels, but growth phase levels of σ^S.

Rex manifestation is a stationary-like phase:

After Rex-mediated efflux of cytoplasmic cations (K^+ efflux), the cell must compensate by stimulating phosphatases and *ompF* to restore cell osmolarity. Given the observed modulation of Rex activity by both OmpA and OmpW, their regulator σ^E is expected to direct the harsh physiological cellular conditions associated with Rex via *micA* and/or *rybB* expression (Parma *et al.* 1992). Stress-induced upregulation of *ompF* would lead to heightened *ompA* expression and subsequent decreased *ompC* expression, which reduces T4 adsorption and superinfection. This could explain what has been previously observed upon activation of Rex: cells enter into a temporary growth arrest state, where they become resistant and protected against T4 superinfection (Slavcev and Hayes 2003). Lpp must play an important role in Rex exclusion, since, as we noted, disruption in Lpp expression greatly reduced Rex activity in T4*rII* (Figure 4A). We postulate that the membrane-bound RexAB complex could be maintained through Lpp, a structural membrane protein, whose own expression is maintained through σ^E.

This stationary-like phase resembles σ^E-dependent cell lysis during early stationary phase, where only about 10% of the cell population survives. Prolonged Rex activity eventually starves the cells of necessary nutrients and can eventually lead to cell lysis. From previous (Slavcev and Hayes 2002) and our own results, we see that Rex protects against T4 cell lysis for days, after which the harsh ionic environment could stop Rex, allowing T4 replication to resume and kill the host cell. Any interruption of the Rex system will also disrupt the delicately maintained balance between σ^E and σ^S, also leading to σ^E-mediated death. We postulate that as long as the balance between σ^E and σ^S is kept, some surviving Rex cells would eventually escape T4*rII* infection.

RII attenuates Rex exclusion:

RII localizes to the inner membrane of the host ~10 min following superinfection (Takacs and Rosenbusch 1975; Mosig *et al.* 1984), where RexB is also localized (Parma *et al.* 1992). This coincides in timing with the interaction of small RNAs RssB and RssA with σ^S to prevent the binding of its anti-adaptors and σ^S degradation. As we found that both T4 and T4*rII* alike cannot plate on *rex*$^+$ Δ*rpoS* mutants (Table 3), we hypothesize that RII interactions with σ^S are necessary for T4 infection. In the absence of RII, the combination of RexA and RexB activity may stabilize σ^S levels, thereby leading to the exclusion phenotype. Conversely if RII is present, it could bind σ^S to activate it in a similar fashion to IraD,

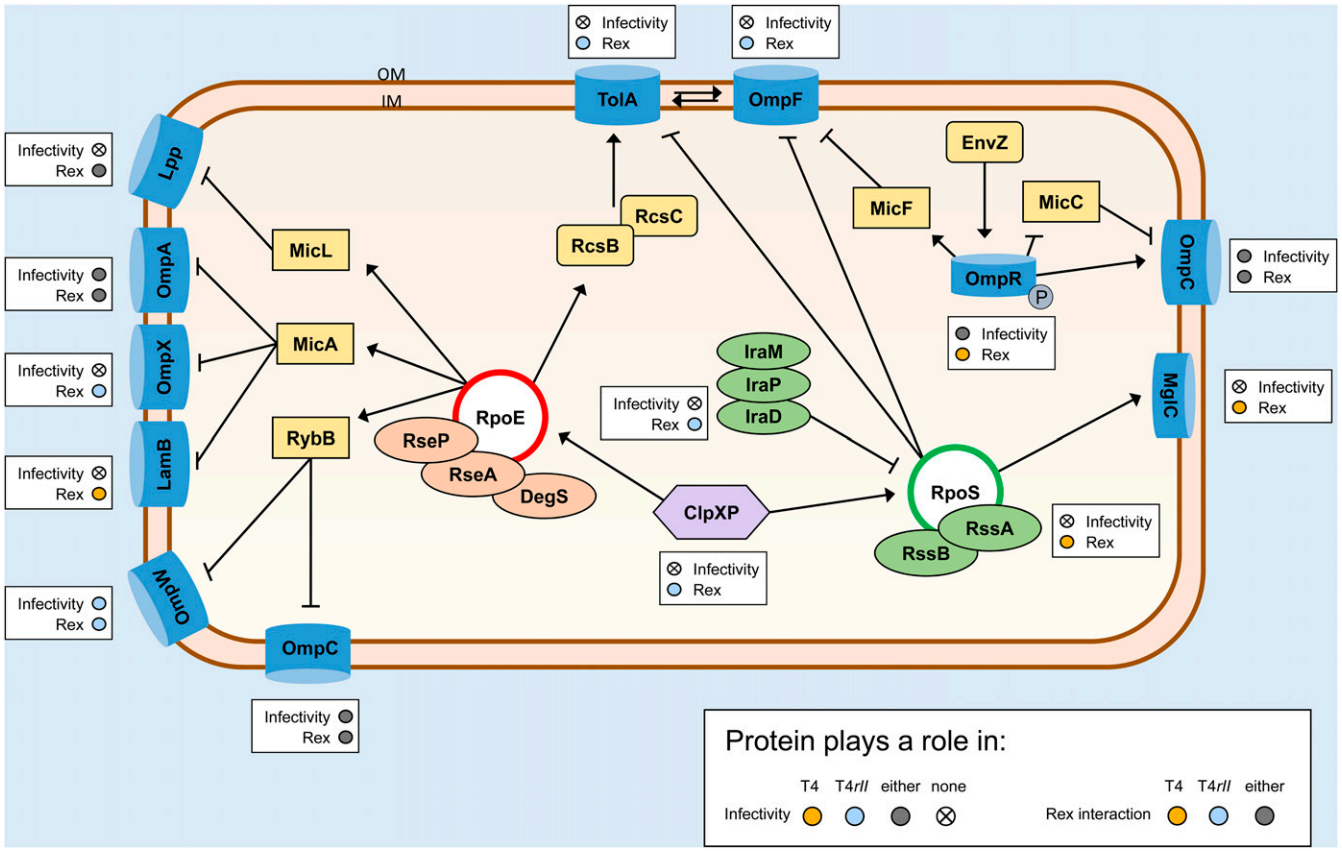

Figure 6 Model of T4 or T4*rII* infection in a *rex*$^+$ cell. Upon infection, *rpoE*/*rpoS* pathways are activated, depending on the presence of RII. Rex activation may be trigged by TolA, resulting in a physiological cellular shunt into a stationary-like phase. Blue cylinders indicate membrane proteins; green ovals indicate small regulatory RNAs involved in *rpoS* expression; red ovals indicate regulatory RNAs involved in *rpoE* expression; yellow boxes indicate small RNAs involved in the expression of membrane proteins. Small circles next to a protein indicate, firstly, its involvement in the infectivity of T4 (yellow), T4*rII* (blue), both (gray), or neither (X); secondly, postulated interaction with RexA and/or RexB only in the presence of RII (T4) (yellow), only in the absence of RII (T4*rII*) (blue), or regardless of RII presence (either phage) (gray). Pointed arrowheads indicate stimulation or upregulation, while bars indicate inhibition or downregulation. IM, inner membrane; OM, outer membrane.

leading to elevated σ^S pathways. As such, σ^S-RII interactions would be required to interrupt the onset of the Rex-mediated protective phase. Similarly, LamB, a σ^E-controlled porin localized in the outer membrane, was also implicated in Rex function in our results. RII may require both proteins to successfully inactivate Rex. As LamB is under the control of σ^E, it represents a vital link between RII, σ^E, σ^S, and Rex.

In conclusion, bacterial cells are highly capable of quickly perceiving and adapting to environmental changes and stresses to survive. The Rex exclusion mechanism is a powerful example of such a protective mechanism against superinfecting T4 mutants. We believe we have now assembled new pieces of the longstanding Rex puzzle. From our findings, we offer an updated model of the λ T4*rII* exclusion mechanism.

We believe that Rex exclusion arises from the balance of activated σ^E to σ^S pathways. Impairment to key players in either pathway attenuates the ability of Rex to exclude T4*rII* plating. A number of these players are membrane proteins, which is not that surprising given the plethora of structurally observable characteristics associated with the onset of the Rex phenotype. Disruptions in cell membrane permeability have been previously shown to render cells more sensitive to superinfecting T4. Understanding the individual functions of these factors and determining their precise interactions may help to finally unravel the Rex puzzle. Understanding Rex can ultimately provide important clues and strategies for use toward eukaryotic exclusion systems and control mechanisms.

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