

**Regulation, Mechanism of Action, and Function of a Small Toxin
Protein in EHEC**

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DEDICATION

To Mom, Dad, and my Mentors

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During this incredible journey over the past six years, many hands have guided my path, and many hearts have encouraged me to reach here. I am so fortunate to have received the opportunity to work with Dr. Elizabeth Fozo, who has been an amazing Ph.D. advisor. She believed in me, corrected my silly mistakes with a smile, trained me to become a better scientist each day, and ensured that I maintained a good work life balance. Thank you so much for all your guidance and support. If I were to start this journey again, I'd once more work with you.

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ABSTRACT

The *zor-orz* locus identified in the chromosome of *Escherichia coli* O157:H7 EDL933 is a type I toxin-antitoxin system that consists of two homologous gene pairs *zorO-orzO* and *zorP-orzP*. The *zor* genes encode for small toxin proteins and the *orz* genes encode for small RNAs. Previously it was shown that overproduction of ZorO results in cell growth stasis whereas co-expression of *orzO* rescues cellular growth. Within, I demonstrate that in addition to growth inhibition, ZorO overproduction results in membrane depolarization and ATP depletion but does not impact the gross morphology of *E. coli*. *In vivo* translation and subsequent impacts of ZorO is significantly increased if the 5' untranslated region (UTR) of the *zorO* mRNA is processed.

When the *zor-orz* locus or the *zorO-orzO* gene pair was cloned on a medium copy plasmid and transformed into *E. coli*, it significantly improved bacterial growth in the presence of several aminoglycoside antibiotics. An increase in the minimum inhibitory concentration (MIC) for gentamycin and kanamycin was observed linking, for the first-time, a type I toxin-antitoxin system with an increase in MIC. The transcription profile of EDL933 cells harboring pBR-*zor-orz* after kanamycin treatment showed upregulation of nitrogen metabolism and anerobic respiration genes whereas flagella associated genes were downregulated.

Mutation of the charged residues within ZorO revealed that several charged residues are critical for ZorO induced toxicity. Using one of the non-toxic mutants of ZorO (arginine at the 23rd position replaced by leucine), we tested its impact on cell growth in the presence of kanamycin. Unexpectedly, this non-toxic mutant was also able to improve cell growth, indicating that toxic effects observed during ZorO overproduction are unrelated to protection against kanamycin. Ongoing work in the lab challenges the hypothesis that *zorO-orzO* mediated improved cell growth is due to the ZorO protein. Additional work on this can change our perspective as to how type I toxin-antitoxin systems function natively and better appreciate their complex nature.

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CHAPTER I

Introduction

Publication note

My contribution to this chapter is literature review and writing.

I. Toxin-antitoxin systems

Toxin-antitoxin (TA) systems are gene pairs encoded in the chromosome and plasmids of the bacteria and archaea (1,2). They consist of a stable toxin, which characteristically targets an essential cellular function such as translation, DNA metabolism, energy production or membrane homeostasis, and an antitoxin, which counteracts the activity of its cognate toxin (3–5). These gene pairs were first identified in plasmids and serve in plasmid maintenance (6). This function is often conferred by the difference in stability of the toxin and antitoxin as plasmid loss rapidly decreases cellular antitoxin levels and no new antitoxin can be produced, enabling the stable toxin to kill plasmid-free segregants (6,7). The discovery of TA systems in prokaryotic chromosomes and accessory genomes increased as novel bioinformatics approaches allowed for their identification through exhaustive homology searches in sequence databases (8–11). Additionally, some TA systems have been discovered serendipitously (12), while others have been unveiled through the search for small, untranslated transcripts (13). However, the function of these gene pairs in bacterial chromosomes is not well understood (8,14). Co-expression of antitoxins prevent self-intoxication upon overproduction of the toxins. The antitoxins are often dispensable for chromosomally-encoded systems in a strain where the toxin gene has been deleted (15). Furthermore, deletion of the entire TA locus seldom produces a measurable phenotype, thus making their study challenging (16). Despite these drawbacks, the contribution of TA systems in bacterial fitness in specific growth environments is increasingly being appreciated.

Previous and ongoing studies over the last twenty years have gathered evidence linking TA systems to the formation of persister cells, biofilms, and virulence (17–21). Persister cells are a sub-population of bacterial cells that can survive lethal doses of antibiotics and can later restore the population when the antibiotic pressure is removed. Persister cells were first described in 1944 for staphylococci treated with penicillin (22,23). Unlike genetically acquired antibiotic resistance, bacterial persistence is a transient phenotypic switch and is increasingly recognized as a cause of antibiotic treatment failure (24). Clinical examples of chronic and recurrent infections due to persister cell formation include urinary tract infections with uropathogenic strains of

Escherichia coli; recalcitrant infections with *Mycobacterium tuberculosis*; or opportunistic infections of implanted devices and open wounds with *Pseudomonas aeruginosa* and *Staphylococcus aureus* (18,24). The implication of TA systems in the formation of persister cells, together with the revelation of association between growth inhibition by TA systems and induction of persister phenotypes has become of great interest (3,4,25).

Based on the nature of the antitoxin, and the neutralization mechanisms impressed on its cognate toxin, TA systems are primarily classified into six types (26,27) (Table 1.1). The toxin components in all TA systems are proteins whereas the antitoxins are small RNAs (sRNAs, type I and type III) or proteins (type II, IV, V and VI). The sRNA antitoxin of a type I system neutralizes the toxin-encoding mRNA via complementarity base pairing. In a type III system, the antitoxin sRNA directly interacts with the toxin protein and neutralizes it. The protein antitoxins in type II systems function by directly binding to their toxin proteins, whereas those categorized as type IV function indirectly (without interacting with the toxin protein) by reversing the effect of the toxin on its targets. The antitoxins in type V systems act as ribonucleases, cleaving the toxin mRNA, while those of type VI systems act as proteolytic adapters, enhancing degradation of the toxin. Recently, two other TA systems have been proposed, a type VII system where the antitoxin (protein) chemically modifies the toxin protein (28) and a type VIII where the toxin is a sRNA, and is neutralized by anti-sense binding by the antitoxin (29). Another example resembling type VIII TA systems has also recently been reported where the *creT* mRNA sequesters tRNA^{UCU}, halting protein translation; this toxicity is prevented by the antitoxin sRNA CreA through base pairing to the toxin mRNA (30).

II. Type I toxin-antitoxin system

In a type I TA system, the antitoxin is a sRNA with sequence complementarity to the cognate toxin mRNA. Upon base pairing, there is inhibition of translation and/or

Table 1.1 Types of toxin-antitoxin systems and the interaction between the toxin and antitoxin

Type	Toxin	Antitoxin	Interaction	Founding member
I	Protein	RNA	Toxin mRNA neutralized by antitoxin RNA	Hok/Sok(33)
II	Protein	Protein	Toxin protein neutralized by antitoxin protein binding	CcdB/CcdA* (6)
III	Protein	RNA	Toxin protein neutralized by antitoxin RNA binding	ToxI/ToxN(34)
IV	Protein	Protein	Indirect; antitoxin interferes the toxin's interaction with the target	YeeU/YeeV (CbeA/CbtA)(35)
V	Protein	Enzyme	Toxin mRNA neutralized by an RNase antitoxin	GhoT/GhoS(36)
VI	Protein	Protein	Toxin protein binding to antitoxin protein triggering toxin degradation by proteases	SocB/SocA(5)
VII	Protein	Enzyme	Toxin protein is chemically modified by enzymatic activity of antitoxin	Hha/TomB (37)
VIII	RNA	RNA	Toxin RNA neutralized by antitoxin RNA	SdsR/RyeA (29)

*the first TA system described in 1983

activation of degradation of the target mRNA (31,32). With few exceptions, the toxin gene encodes a small protein (less than 60 amino acids). The first type I TA system was discovered in a plasmid (*hok/sok* gene pair in R1 plasmid of *E. coli*) and their homologs or novel type I gene pairs, encoded in the bacterial chromosome, were quickly identified (33,38). For example, the *hok/sok* system of plasmid R1 has up to 15 homologs in the chromosomes of differing *E. coli* strains (11,39). In comparison, other systems such as Ldr/Rdl, lbs/Sib, TisB/IstR1, ShoB/OhsC, ZorO/OrzO, SymE/SymR are, to date, exclusive to the bacterial chromosomes (14,39–41). The first type I TA system identified in Gram positive bacteria, Fst/RNall in the pAD1 plasmid of *Enterococcus faecalis*, has hundreds of homologs in the genomes of the Firmicutes (encoded in mobile genetic elements and chromosomes; (38,42)). In addition to their presence in plasmids and/or chromosomes, type I TA systems can be categorized in several ways- distribution of the gene pairs across bacterial groups, genetic organization of the toxin encoding gene in relation to the antitoxin, cellular target of the toxin protein, or the function conferred by the TA system.

Of the six types of TA systems, types I and II remain the most identified and broadly studied. Compared to the type II system, most known type I gene pairs show a narrow phylogenetic distribution and many occur within a small proportion of species (<5%) within a phylum (43). This might indicate the lineage specificity of type I TA systems, but may alternatively suggest the need for more robust search engines for their identification across bacterial sequence databases. The small size and hydrophobic nature of type I toxins make them easy to miss in conventional homology searches. For instance, the homolog of the lbs toxin family (18-19 amino acids) in *E. coli* was not identified in *Helicobacter pylori* by both BLAST and PSI-BLAST, but was identified in a search for sRNAs that were expressed from regions antisense to small ORFs (open reading frames; (9,44)). Some type I toxin such as SymE, however, are identified in both Gram positive and Gram negative genomes (45). The accumulation of new sequencing results and novel bioinformatic tools designed to identify small ORFs and sRNAs might extend the narrow distribution of type I TA systems. Further novel

approaches such as ribosome profiling with stalled initiation complexes helps to experimentally validate the occurrence of these small proteins (46,47).

Type I TA gene pairs, to date, are encoded on the opposite strands of the DNA and are transcribed from their own promoter (41). Their genetic organization, however, can vary: the antitoxin sRNA can be arranged either as overlapping, convergently transcribed (*cis*-encoded antisense) or divergently transcribed (*trans*-encoded) from its toxin counterpart (14) (Table 1.2). The *cis*-encoded TA systems consist of extensive perfect base pairing potential between the toxin and antitoxin RNA whereas the *trans*-encoded systems often have a limited complementarity. The antitoxins presently studied in Gram positive bacteria often are encoded antisense to the 3' end of their toxin mRNAs (48) while there are more diverse arrangements observed in Gram-negative bacteria. Regardless of their organization, the antitoxin sRNAs share sequences that are complementary to the antisense toxin mRNA and prevent translation of the toxin mRNA.

With the exception of TimP (a type I toxin in *Salmonella* Typhimurium) and SymE of *E. coli* MG1655, detection of native production/expression of a type I toxin from the chromosome has not been shown (45,49). The reliance on artificial overproduction in order to study TA systems led to the speculation that they are selfish genes and cell suicidal factors (50), or genomic “junk” which was accidentally acquired from plasmids or alternate sources (51), with no true biological function. However, most type I TA systems are highly conserved in relation to bacterial species and have been proposed to be less mobile than other TA systems (43). The protein coding sequences of toxin genes do not contain premature stop codons or frame shifts (40), indicating that toxin proteins have the potential to be endogenously expressed. It is likely that toxin translation occurs only when the cell is exposed to a specific environment. For the *tisAB/istR* gene pair the deletion of either or both toxin and antitoxin genes did not affect cell growth, but the attempt to transfer the *tisAB* gene into an SOS-on strain (*lexA51-Def* with a constitutive SOS response and deleted for *tisAB/istR*) was only successful if the SOS-on strain had *istR* expressed on a plasmid (52). This demonstrates the toxin can function in a specific condition. The number and concentration of proteins expressed in

Table 1.2 Genetic organization of type I toxin-antitoxin systems

TA system	Toxin mRNA (nt)	Antitoxin RNA (nt)	Protein (aa)	Genetic organization*
HokB/SokB(11,53)	357	61	49	
LdrD/RdlD(53,54)	368	67	35	
TisB/IstR1(52)	354	75	29	
ShoB/OhsC(12)	313	95	26	
DinQ/AgrB(55)	331	84	28	
ZorO/OrzO(56)	264	81	29	
TimP/TimR(49)	295	95	38	
IbsC/SibC(12)	160	145	18	
SymE/SymR(45)	454	77	113	
AapA1/IsoA1(57)	250	80	30	
RalR/RalA(58)	195	179	64	
BsrG/SR4(59)	294	180	38	
Fst/RNAlI(42,60)	~230	~40	33	
TxpA/RatA (61)	270	220	59	
SprA1/SprA1-as(62)	208	60	30	
SprG1/SprF1(63)	439	142	44	
YonT/as-YonT (61)	~239	101	58	

*Blue arrows denote toxin mRNAs (with coding region in box). Grey box denote confirmed/predicted leader peptide. Red arrows denote antitoxin RNAs.

a bacterial cell at any given time is highly dependent on its genetic and environmental context to ensure efficient use of energy, survival, and growth (15). Using *E. coli* as an example, 620 genes were identified as essential when grown on nutrient-rich media (64) but this number increased by hundreds of genes when grown on nutrient-limited media (15). Given that type I toxin proteins are often regulated at multiple levels, this further influences their translation (65,66). It is likely that the translation of toxin proteins during endogenous expression can reach high levels under specific condition(s) the bacterial cell encounters. Since these definitive condition(s) for many type I toxins are yet to be identified, overproduction studies are important available tools that allow for the investigation of these systems (67).

Upon translation, type I toxin proteins can target several cellular components. Based on their sub-cellular localization, type I toxins can be categorized as cytosolic (such as RalR, SymE) or membrane-associated (such as HokB, TisB, LDr, Ibs, ShoB, ZorO, Fst, BsrG) (68). Membrane-associated toxins are small, hydrophobic, and have a predicted transmembrane domain and show the toxic effect during overproduction by pore formation in the membrane resulting in ion leakage or impacting cell morphology, whereas cytosolic toxins can act as nucleases (cleaving DNA or RNA) (68).

III. Toxicity-mediated by small proteins of type I toxin-antitoxin systems

Type I toxin translation can occur after the toxin mRNA escapes regulation by the antitoxin sRNA and other repressive mechanisms such as long, inhibitory 5' untranslated regions (UTRs) (41,65,66). Since this translation event may occur only during a specific growth environment for bacterial cell(s), it is challenging to detect the natively translated type I toxin proteins using conventional methods. Translated type I toxin has been detected for SymE in a strain whose antitoxin promoter was mutated (-10 *symR*) but no growth defect was observed (45). Another type I toxin, TimP, (in *Salmonella* Typhimurium) was detected using western blot in a strain deleted for the antitoxin (TimR) encoding gene (49). This strain grew comparable to the parental strain, indicating little to no toxicity from de-regulation of *timP* (49). There is therefore no compelling evidence to suggest that TA systems result in bacterial cell death under

physiological conditions (69) and most of the reported toxic effects of type I toxins are based on the ectopic overexpression. Overall, the impacts of the type I toxin can range from no effects to cell lysis depending upon its presence in the chromosome, elimination of the repressor elements, multi-copy plasmid vector or inducible system (proposed via schematic in Fig. 1.1) along with the growth conditions chosen for the study.

The cytosolic type I toxins SymE and RalR are unusual, having characteristic biochemical activity and function as endonucleases cleaving RNA and DNA respectively (53,58). SymE (SOS-induced *yjiW* gene with similarity to MazE) belongs to the AbrB superfamily: a group that includes transcription factors and antitoxins of classical type II TA systems. SymE shares key hydrophobic residues with classical AbrB members but has evolved to function like a toxin (45). SymE is larger than other type I toxins, and its overexpression affects bacterial survival and greatly impacts overall translation (45). It functions as an endoribonuclease, degrading several mRNAs and noncoding RNAs and functions both independently as well as through ribosomal association (45). RalR acts by non-specifically cleaving DNA, but not RNA (58). This toxin is able to function in a similar way as DNase I and its overproduction results in filamentous growth due to the SOS response to DNA degradation (58).

Overproduction studies have illustrated that many other characterized type I toxins are membrane-associated and can directly affect the cell membrane by forming pores, increasing membrane depolarization, or can instead result in cell morphology changes as a primary effect (68). For example, HokB and TisB have been established as pore forming type I toxins whereas ShoB, IbsC, DinQ, LdrA, ZorO, PepA1, PepA2 etc. were shown to increase membrane depolarization and/or permeabilization (68,70–72). The toxins Fst, LdrD, BsrG and AapA1 primarily impact cell morphology after associating with the membrane without an immediate membrane permeabilization effect (70–72). For some of these toxins, it is difficult to distinguish whether membrane associated effects (depolarization) are the primary toxic effects or changes in cell morphology as seen with DinQ.

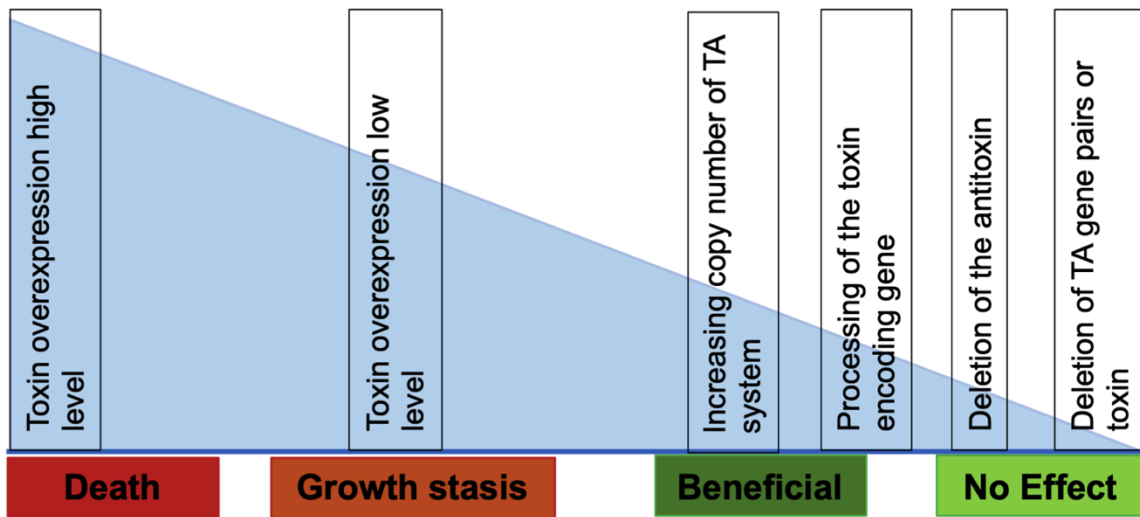


Figure 1.1. Schematic showing the effects of type toxin-antitoxin system. Expression from the chromosomal copy or deletion of the toxin-antitoxin locus may not have any observed physiological consequences whereas overexpression using ectopic vectors can result in cell growth stasis and cell death.

The Hok/Gef family (*hok*, *hok A-E*, *pndA1*, *srnB*) is widely distributed in plasmids and chromosomes of Gram-negative bacteria (73–75). HokA, HokC and HokD overexpression in *E. coli* results in ghost cell formation and cell lysis (75). More research has been conducted with the HokB toxin, showing that it forms pores and leads to membrane depolarization and ATP depletion (76,77). HokB dimerizes via disulfide bonding to form pores resulting in cellular ATP leakage (76,78). This loss of membrane potential and ATP from the cell results in bacterial cell death. Overproduction of HokB in *Erwinia amylovora* affects its morphology by forming multiple “blebs” and results in cellular leakage (79). Membrane localization of TisB and pore formation has been confirmed using a combination of *in vivo* and *in vitro* assays. In a membrane mimicking environment, TisB forms an antiparallel dimer, stabilized by an electrostatic charge zipper that further cross links to form a tetramer (80). This tetramer resembles a polar “tunnel” which extends from the aqueous phase through the peptide complex and across the entire thickness of the lipid bilayer (80,81). The stable tetramer form was confirmed *in vivo* by SDS-PAGE (80). Upon overexpression, TisB results in a loss of membrane potential and reduction of intracellular ATP levels, which affects global cellular transcription, translation, and DNA replication, ultimately resulting in cell death (82).

Overproduction of IbsC or ShoB rapidly impacts membrane integrity with at least 97% of the cells becoming depolarized within 20 minutes of induction, which subsequently impacts cellular viability (12). DinQ localizes to the bacterial inner membrane and its overexpression demonstrates similar effects on membrane depolarization (55). The impact of DinQ when its antitoxin (*agrB*) was deleted resulted in a cell having only approximately 50% of the ATP concentration compared to wild type (55). To date, however, pore formation and the subsequent size of the pores has not been experimentally confirmed for these toxins. LdrA overexpression has similar impacts on ATP depletion, which inhibits major cellular processes such as transcription, translation and replication, and results in growth inhibition of the cells (83). ZorO overexpression also results in loss of membrane potential and impacts cellular ATP levels by targeting the bacterial inner membrane (84). Furthermore, it increases reactive

oxygen species (ROS) which leads to the permeabilization of the membrane and subsequent cell death (84). Other type I toxins such as TisB, DinQ-V and HokB are also linked to ROS formation in *E. coli* upon overproduction (85).

The TxpA toxin encoded in the chromosome of *Bacillus subtilis* contains a transmembrane domain and is predicted to affect the membrane, thus resulting in cell death (13). Based on homology with *txpA*/RatA, the SprG toxins (with four homologs SprG1-SprG4) were identified in *S. aureus* and were shown to disrupt the integrity of the bacterial membrane (63,68).

While membrane effects appear at first examination to be the major cause of toxicity, careful studies with specific type I toxins suggest that for at least some, the primary target is not the membrane. For example, Fst is a member of the Fst/Ldr superfamily of type I toxins. Fst overproduction is toxic not only in *E. faecalis* that harbors it, but also in *B. subtilis* (60,86). Overexpression of Fst results in nucleoid condensation and an uneven distribution of chromosomal DNA between daughter cells during replication (86). Effects on cell division and DNA segregation were followed by effects on membrane permeability, indicating that membrane disruption is likely a secondary effect (86). A synergistic effect of Fst and nisin (cell wall inhibiting lantibiotic) was observed against *E. faecalis*, indicating cell membrane alteration as one of the effects of Fst (72). This could be another secondary effect by Fst as it impacts cell division and therefore the cell envelope. Other members of the Fst/Ldr superfamily, PepA1, PepA2, *fst*-Sm, Lpt, LdrA and LdrD, result in cell death when overproduced in their native host, or in some instances, heterologous hosts (42). Both Lpt and LdrD also have been observed to cause nucleoid condensation in *E. coli* (54,87). In addition to nucleoid condensation, LdrA and Fst overexpression inhibit macromolecular synthesis and ATP production in the cells (72,83). While membrane damaging effects of overproduction of PepA1 (encoded by *sprA1*) in *S. aureus* are speculated to be the result of either pore formation or interference with membrane-associated functions (88), there are no reports examining its role in DNA condensation to our knowledge.

Like Fst, the BsrG toxin in *B. subtilis* also does not permeabilize the bacterial membrane but results in nucleoid acid condensation and morphological alterations (70).

In addition, BsrG also inhibits transcription and translation and therefore appears to possess a broader cellular effect. Although localized to the membrane, BsrG does not form large pores, affect membrane potential, or membrane fluidity, and therefore has no impact on cellular ATP levels (70). Instead, BsrG overexpression impairs cell wall biosynthesis, which causes invagination of the membrane, and leads to small, irregularly shaped bacteria (70). Membrane invagination leads to MreB-cytoskeleton delocalization which further impacts other protein complexes associated with cell wall synthesis. The combined effects on cell wall biosynthesis by BsrG, along with the activity of bacterial autolytic enzymes, leads to cell lysis (70).

Overproduction of another membrane localizing toxin, AapA1, in *Helicobacter pylori* alters its bacterial morphology from a spiral shape to the coccoid form and modifies the mucopeptide composition of the peptidoglycan in response to toxin production (71). Interestingly, AapA1 does not significantly impact membrane potential and intracellular ATP content (71).

Overall, by employing overexpression systems, the toxicity of most type I toxins has been established, demonstrating their effects on vital cellular processes. Although many identified type I toxins have been shown to target the cytoplasmic membrane, other cellular targets such as RNA, DNA and cell envelope are not uncommon. Ectopic overproduction systems have been useful tools for the study of cellular target(s) of toxins, as well as their mechanism of action to cause cell growth inhibition and/or cell death. However, the similarity of those effects compared to when the toxin is produced endogenously remains to be determined. The lack of evidence of cell death by chromosomal expression for many of the characterized toxins, unlike in overproduction, clearly confirms that the effects observed during overproduction cannot be extrapolated to infer effects of toxin expression under physiological conditions. Therefore, it is important to investigate the effects and biological roles of type I toxins when expressed from their native regulatory elements. Detailed mechanistic studies of type I toxin action, their endogenous translation, and their direct and downstream effects, together with the strategies utilized by bacteria to “rescue” themselves from these effects, should be

expanded. This will lead to a better understanding of the cellular pathways affected by these toxins, as well as their true biological roles in bacteria.

IV. Regulation of translation of type I toxin proteins

The immediate effects on cell growth inhibition and/or cell lysis after overexpression of type I toxins demonstrates the potentially harmful nature of these proteins. This demands tight, controlled regulation of toxin gene expression. The lack of severe effects at the endogenous level of toxin expression could be the result of condition specific translation and a multi-layered regulation for translation of these proteins. Some type I toxin-encoding genes such as *tisB*, *symE* and *dinQ* are shown to be controlled by a transcriptional regulator (53,55,82). For the *tisB*/IstR-1 gene pair, the IstR-1 antitoxin is constitutively expressed whereas the promoter of *tisB* is repressed by LexA and is transcribed upon DNA damage (66). LexA is a major SOS response regulator and has also been observed to repress the transcription of *symE* and *dinQ* (53,55). For some of the other toxin encoding genes, such as *shoB*, *zorO*, *zorP*, *ibsC*, *txpA*, *bsrG* and *yonT*, transcripts have been detected by northern blot analysis in varying levels depending on the growth phase and laboratory growth conditions (9,12,59).

Coupling of transcription with translation is common in prokaryotes as they lack nuclear compartmentalization (89). This is problematic in the instance of a toxin-encoding mRNA since the translated protein can be detrimental to the cell. The coupled transcription/translation of the toxin protein would also allow toxin encoding mRNA to escape regulation by the antitoxin sRNA (41). The toxin encoding transcripts usually have a long untranslated region (UTR) with sequence complementarity that can base pair to sequester the ribosomal binding site (RBS) of the mRNA and block translation initiation. Thus, transcription-translation coupling is often prevented by controlling ribosomal access to the transcript (65,66,90).

As is evident by the definition of a type I TA system, another repressive mechanism of toxin translation is via the antitoxin sRNA. These sRNAs are encoded antisense to their cognate toxins and have nucleotides that can base pair with their toxin

mRNA (31). The complementarity base pairing to an antitoxin can stimulate its degradation by ribonucleases and/or prevent translation.

While in most instances type I toxin effects cannot be prevented, for the type I toxin protein, SymE, if it escapes the tight post-transcriptional regulation and is translated, post-translational regulation is observed: specifically, it is degraded by Lon protease (53). Lon protease has been shown to play a significant role in type II TA systems. However, degradation of “traditional” type I toxins, *i.e.*, those under 60 amino acids and highly hydrophobic, has not been well-documented nor have the potential effects of toxin protein post-translation, and thus requires further investigation.

a. Translation inhibition mediated by untranslated regions

The type I toxin mRNAs are usually far longer than the coding region with additional UTRs at the 5' or 3' ends (56,67,91). These long UTRs will form secondary structures via complementary base pairs and often sequester the Shine Dalgarno (SD) sequence. The role of these UTRs in keeping the toxin encoding mRNA translationally inert has been experimentally validated in many type I TA systems encoded in Gram-negative and Gram-positive bacteria. These UTRs can also function to maintain the stability of the toxin encoding mRNA as their processing has been shown to reduce mRNA stability (65).

The full-length *hok* mRNA is not translated due to sequestration of its RBS by an interaction between the translational activator element at the 5'-end and the fold-back-inhibitory element located at the 3'-end (92). This long distance interaction between the two ends of the mRNA is disrupted after RNase III and polynucleotide phosphorylase (PNPase, via 3'-5' exonuclease activity) processes the 3' end that leads to mRNA structural rearrangements at the 5' UTR (92,93). Similar 3' processing (cleaving 25 nucleotides) is required for the activation of *aapA1* mRNA in *H. pylori* (57). The processing event opens the sequestered SD sequence in *aapA1* and makes it available to interact with 16s rRNA and to initiate translation.

The 5'UTR of *tisB* makes it translationally inert and requires processing of the first 41 nucleotides (94,95). This processing however does not open up the SD

sequence but changes the structure, thus opening the so-called ribosome standby site, and making it available for the 30S ribosomal subunit to bind (90). This binding is further enhanced by a pseudoknot at the 5' end of the processed mRNA (+42) and ribosomal protein S1, therefore enabling the standby-dependent translation of the TisB toxin (90,94). The 5' UTR processing of *zorO* ($\Delta 28$) also does not open the SD sequence but exposes a probable standby region as observed with *tisB* (65). Transcripts of different sizes, indicating processing at 5' ends, have also been observed with *dinQ* and *shoB* (55,96). An *in vitro* translation of *dinQ* was rapid and robust with the +44 *dinQ* mRNA compared to that from the +1 mRNA (97). In case of *shoB*, of the two transcripts that were mapped 40 nts apart at the 5' end, the expression was observed only from the shorter 5' UTR indicating translational regulation and need of 5' processing (12,96). The nucleases involved in the 5' end processing for any of these mRNAs remain unknown.

UTRs that sequester the SD sequences of toxin mRNAs in Gram-positive bacteria have been observed, however, the processing events and their significance in these bacteria require further examination (41). RNA I that encodes the Fst toxin (part of *par* locus of the pAD1 plasmid in *E. faecalis*), inhibits its own translation via an interaction between an upstream helix and a small stem-loop at the 5' UTR, restricting access of the SD sequence to the ribosomes (98). The stem-loop region was later discovered to be critical in preventing translation as its disruption leads to an increase in Fst translation *in vitro* (99). The *bsrG* mRNA in *B. subtilis* forms a highly structured double-stranded regions with multiple stem-loops and one of these stem-loops blocks the SD sequence, thus preventing its translation (100).

b. Translation inhibition mediated by antitoxin sRNA

The antitoxin of a type I TA system is encoded antisense to the toxin and can be organized at different positions relative to its toxin target (Table 1.2). While other sRNAs from Gram-negative bacteria require an RNA chaperone (such as Hfq) to effectively bind to their targets, it is not required for the function of most antitoxin sRNAs studied to date. Using overexpression systems, the growth effects observed from the toxin encoding gene does not appear if the antitoxin is co-expressed (9,49,56). This

illustrates the robustness of antitoxin mediated neutralization of their cognate type I toxins. To prevent toxicity, several of the described antitoxin sRNAs (such as IsoA1, SymR, SibC, RalA) base pair such that they occlude the ribosome binding site of the toxin mRNA (45,54,57,58). In some cases, however, sRNA base pairing can occur outside the RBS, such as within the toxin encoding mRNA or in other locations of the 5' or 3' UTR (7,52,53,65). Some toxins require translation of an upstream leader sequence and thus their translation can be prevented by antitoxin sRNAs binding to these leader sequences. This has been observed in *hok/sok* and *ldrD/rdlD* where the antitoxin prevents translation of the leader peptide (7,53).

For the *tisB/istR-1* gene pair, the *tisB* mRNA is not available to ribosomes because of a stem structure formed at the RBS (101). A preloading event of ribosomes occurs at the nucleotide sequence upstream of RBS known as a “standby region.” An initial binding to this region is essential in this case before the ribosomes can bind to the RBS and initiate translation. This “standby region” also consists of sequence complementary to the antitoxin IstR-1. The 5' UTR processing of *tisB* mRNA ($\Delta 1-41$) opens the “standby region” for ribosomes and the antitoxin sRNA (101). IstR-1 binding prevents the preloading of ribosomes on the toxin-encoding mRNA, and this binding is followed by cleavage of the double stranded RNA by RNase III (90,101). The regulation by OrzO sRNA also occurs by base pairing at the region distal to the RBS and is proposed to follow a similar mechanism- cleavage of the “standby region” within the *zorO* mRNA to prevent its translation (65).

The *aapA1* mRNA in *H. pylori* is available for interaction with the antitoxin IsoA1 after the mRNA is processed at the 3' end and undergoes structural arrangement allowing IsoA1 to form a duplex (57). This results in translation inhibition of the AapA1 and RNase III mediated degradation of the *aapA1*/IsoA1 complex (57).

Translation of the leader peptide encoded within and overlapping the toxin gene is required for the translation of some type I toxin proteins: these studies have demonstrated that the antitoxins function by preventing the translation of such leader peptides (53). The Sok antitoxin of the *hok/sok* TA system base pairs with the RBS and part of the coding sequence of a leader peptide Mok (102). This prevents ribosomal

access to the *mok* RBS. Since *hok* translation is coupled with *mok* translation, inhibition of Mok translation subsequently prevents translation of the Hok toxin. The *ldrD*/*rdlD* system also consists of an additional open reading frame (*ldrX*) that starts upstream of the LdrD coding sequence (103). Although not experimentally validated, LdrD is proposed to be co-translated with the LdrX, and the RdlD antitoxin, which contains the sequence that overlaps the *ldrX* RBS. This stops translation of LdrD by binding to and preventing the translation of LdrX (53,54,103).

The type I antitoxins studied in Gram-positive bacteria to date are often encoded antisense to the 3' end of their toxin mRNAs (48). However, they can have sequence complementary to the 5' ends of their respective toxin (58,60). The Fst/RNAl system was observed in both the plasmid pAD1 and the chromosome of *E. faecalis* (38,42). The RNA II antitoxin interacts with *fst* (also referred to as RNAl) via several complementary regions and is accomplished in two subsequent steps- i) formation of an initial reversible “kissing complex” as a result of interactions between the complementary regions at the 3' end followed by ii) base pairing of two direct repeats at the 5' end that sequesters the translation initiation site (60). Interestingly, the disruption of the stem-loop region within the *fst* mRNA (RNA I; that regulates its own translation), prevented the neutralization of the Fst toxicity when both toxin (RNAl) and antitoxin (RNAlI) were expressed in *E. coli* (99). The RNA II from the pAD1 encoded Fst/RNAlI system cannot neutralize *fst* mRNA when produced from the chromosomal copy and is presumably the result of differences in the sequences within the direct repeats (104). The antitoxin of the Fst homolog in *S. aureus*, SprA_{AS} also has overlapping bidirectional transcriptional terminator sequence. However, the interaction occurs between the non-overlapping regions of the 5' UTR and represses the translation of SprA1(PepA1) toxin (105).

Other Gram-positive antitoxins located antisense to the 3' end of their toxin mRNAs are SR4, RatA, and BsrH. SR4 is unique as it binds to the 3' end of the *bsrG* toxin mRNA leading to structural rearrangement of RBS at the 5' region (100). Before SR4 base pairing, the secondary structure of *bsrG* forms a 4nt-stem at the RBS and inhibits its translation. This 4nt-stem is extended to 8 nt upon formation of the *bsrG*/SR4

complex. Binding of the SR4 sRNA not only inhibits translation by sequestering the SD sequence, but also affects *bsrG* stability as the *bsrG*/SR4 duplex is degraded by RNase III (100). The *txpA* and *bsrH* toxin mRNAs are also cleaved by exoribonucleases after RatA and as-BsrH respectively bind to their 3' end (13,100,106). RNase III is also critical to prevent type I toxin translation in *B. subtilis*: deletion of *rnc* (encodes RNase II) is lethal due to the inability of the antitoxins (such as RatA and YonT) to effectively neutralize their cognate toxin mRNAs (13).

Repression of most type I toxin gene by the cognate antitoxin sRNA is specific. In the *zor-orz* locus which consists of two homologous gene pairs, *zorO-orzO* and *zorP-orzP*, the OrzP sRNA is unable to neutralize the *zorO* mRNA, showing very high specificity (56). Similar specificity of the TA interactions are also maintained in the *H. pylori aapA/IsoA* and *S. aureus sprA/sprA_{AS}* systems (57,105). The *txpA/ratA* homologous locus *sprG/sprF* system (with 4 homologs in *S. aureus*), however, exhibits *in vitro* interaction of *sprG3* with SprF1 and *sprG2* with SprF4 indicating a possible *in vivo* cross-talk (107). Research addressing the *ibs/sib* system, which has five homologs, suggests some evidence of cross-talk as *ibsA* overexpression was not toxic in *sibA* deleted strains (12). SibA has identical domains with SibE, which likely neutralized the toxicity of *ibsA* (108).

Besides UTRs and antitoxins, there are other factors that can determine the fate of toxic gene translation. The two main factors impacting this are the abundance of transcripts for each of the TA gene pairs and their relative half-lives. The abundance of toxin and antitoxin RNAs can vary based on growth conditions and growth phase. For some of those examined, the antitoxins are constitutively transcribed and are less stable with shorter half-lives compared to that of the toxin mRNA (57,92). For example, *hokB* and *ldrD* mRNA have been experimentally shown to have longer half-lives than their cognate antitoxins (32,54). The half-life of *hok* mRNA from the *parB* locus of plasmid R1 was 20 minutes as compared to 3-5 minutes for the *sok* sRNAs (11,109), and that of *ldrD* from the chromosomal expression was 30 minutes compared to its antitoxin RdlD which was only 2 minutes (54). In an unusual type I TA system, *symE/symR*, the antitoxin sRNA levels were 10-fold higher than the toxin mRNA when expressed from

the native chromosomal locus, as observed via northern blot analyses in *E. coli* MG1655 (45).

It can be concluded that the cell has equipped itself with multiple ways to regulate the translation of the toxin, which suggests the occurrence of their translation in a specific environment is to adjust cellular physiology accordingly. While using overexpression systems to establish the toxic effects of these genes, the field should allocate increased focus on the impacts observed during the expression at the chromosomal level, together with the function of these chromosomally encoded type I TA systems.

V. Function and global impacts of type I TA systems

Although type I toxins encoded in plasmids function for plasmid maintenance in the cell via the post-segregational killing mechanism (33,110), the biological function of only a few chromosomally encoded type I TA systems has been determined. Homologs of plasmid encoded TA systems in the chromosome have been noted for a few type I TA systems. For example, the plasmid encoded *hok/sok* system has five homologs in the *E. coli* MG1655 chromosome (up to 15 in some strains), three of which are inactivated by insertion elements (11,111). This led investigators to suggest that these systems are just remnants from plasmid integration. However, most type I TA systems are highly conserved in relation to bacterial species, and have been proposed to be less mobile than other TA systems (43). The protein coding sequences of the toxin do not contain premature stop codons or frame shifts (40), suggesting that the toxin proteins are endogenously expressed. The presence of multiple layers of regulation for toxin translation reduces potential of leaky expression for these toxins when not essential.

Chromosomally encoded type I toxins (which lack homology to mobile elements), can have multiple homologs that encode similar small proteins. Bacterial chromosomes can encode up to ten *ldrd/rdl* and up to seven *ibs/sib* homologs (12,54). Similarly, six homologs for *aapA/isoA* have been identified in *H. pylori* and two for *zor/orz* in *E. coli* (44,56,57). This evidence leads to a question of the function of their redundancy. It remains unknown whether these homologs work together in a specific growth

environment or have separate functions, and if one antitoxin homolog has the capability to neutralize multiple homologs of the toxin mRNA. The specificity of the sRNA to the toxin mRNA has been determined for the *ibs/sib*, *zor-orz* and *aapA/isoA* homologs (12,56,57). For the *ibs/sib* gene pair, the deletion of the antitoxin genes halted growth of cells expressing the toxins from plasmids, whereas the chromosomal copy of the antitoxin (*sib*) was sufficient to repress toxic effects of low *ibs* expression from plasmids (12).

Using the conventional method of identification and characterization of TA systems, overproduction of toxins clearly demonstrates bacterial cell growth inhibition and/or cell lysis. We questioned why a bacterial cell would maintain such self-toxins encoded in the chromosomes (as observed with most type I TA systems). Unlike our laboratory conditions with toxin overproduction, bacterial cells are expected to rely on the chromosomal regulation of these genes. The deletion of the TA systems does not show differences in the optical density during growth in laboratory conditions (49). In the *tisAB/istR* system, the transfer of the *tisAB* gene via P1 transduction into an SOS-on strain was not successful, indicating its ability to be lethal to the cells in specific growth conditions (52). The P1 transduction was achieved if the SOS-on strain had *istR* expressed on a plasmid (52). No other evidence has been reported which demonstrates cell death caused by toxin from the TA system when endogenously translated from the chromosome (69). In the *txpA/ratA* system, the antitoxin (RatA) deleted mutant have been shown to accumulate the toxin gene (*txpA*) suppressor mutations (13). Some overproduction studies have linked the type I toxins to benefit bacterial cells in a variety of ways. These benefits include protection from bacteriophages (Hok), increase in virulence by cytotoxic effects (PepA1, PepA2) or improved survival in intracellular environments (TisB, HokB, Ldr), cleavage and recycling of RNA (SymE), cleavage of DNA (RalR), defense against competing bacteria (SprG), chromosomal stabilization (BsrG, YonT) and persistence (TisB, HokB, RalR) (112). While the role of some type I TA systems in increasing the number of persister cells has been examined in detail, an unusual case of TA system reducing the number of persister cells has been reported with the *fst-Sm/sr-Sm* gene pair in *Streptococcus mutans*. Specifically, a significant

decrease in persister cells occurred upon treatment with the antibiotics vancomycin, cefotaxime, and oxacillin after mild-overproduction of the entire *fst-Sm/sr-Sm* (113).

The type I toxins which have been determined to confer a role in persistence are TisB and HokB. TisB is capable of inducing persistence by causing membrane depolarization and ATP depletion, thus leading the cells into a transient dormancy which is tolerant to the antibiotics. Deletion of the *istR1* antitoxin significantly increased (~4-10-fold) the number of persister cells against ciprofloxacin, while deletion of *tisB* showed a consistent (~10-fold) decrease in persister cell number (66,114). Furthermore, deletion of the regulatory element in the 5' UTR ($\Delta 1-41$; note this processed form is translationally active (66)) increased persister cells by 11-fold, and deletion of both the 5'UTR and antitoxin *istR* (double deletion) increased that by more than 100-fold against ciprofloxacin (66). The double deletion strain also had an observed increase in lag time with a delayed appearance of colonies, which is often associated with persister cells as they are metabolically "slow." The persistence phenomenon as seen in the double deletion strain was also very distinct (increase by >200-fold) in response to the cell wall inhibiting antibiotic, ampicillin (66). Unlike *tisB*, deletion of *hokB* does not decrease the number of persister cells. Overexpression of HokB, however, results in an increase in the persister cells against ofloxacin and tobramycin (3).

HokB also follows a similar mechanism of growth stasis by membrane depolarization and ATP depletion. HokB monomers are dimerized by the enzyme DsbA, that forms a disulfide bridge between the cysteine residues (C46) in the periplasm (78). This results in pores within the membrane, which affect membrane potential and even causes ATP leakage into the extracellular medium, which leads the cells into dormancy and persistence (76,78). The awakening of persister cells has also been studied with the HokB toxin. Another periplasmic enzyme DsbC, acts on disulfide bridges and disassembles the pore formed by HokB, after which HokB monomers are degraded by the protease DegQ (78). This results in the repolarization of the cell so that it can function normally and grow. All these analyses on the HokB toxin, however, have been conducted by using overexpression plasmids.

Many studies have investigated the possible target-based effects of type I toxins, while few of them have assessed the global impacts. Incorporation of omics-based experiments in an effort to determine global impacts of a specific toxin may help specify the cellular pathways that it impacts within the cell. Using microarrays, global transcription was assessed after overexpression of LdrD (54). Several genes were found to be differentially expressed. Genes involved in purine metabolism were upregulated at least 3-fold, which can be linked to the nucleoid condensation observed during LdrD overproduction. Another study investigated the cellular global effects by observing changes in whole genome expression after overexpression of TisB, IbsC, LdrD, or ShoB (12). Several genes were induced during overexpression of each of these toxins. A superoxide stress response regulator *soxS* was induced by all four of the studied toxins (12). Other significantly upregulated genes which were common to four toxins included those encoding membrane proteins, those involved in heat shock or cell envelope stress-related genes (12). There were genes, however, that were induced in specific response to the overexpression of each of the toxin. As an example, only IbsC overexpression (no other toxins) showed induction of phage shock proteins (*psp*) (12).

The proteome (both intracellular and extracellular) of *S. aureus* in relation to the TA system *sprG1/sprF1* (homolog of the *txpA/ratA*) was recently studied using the wild type, a strain with the gene pair deleted and the deletion strain complemented only for the antitoxin (*sprF1*). While the strain with *sprG1/sprF1* had an increased number of proteins secreted into the extracellular space, the *sprF1* complemented strain had its protein expression moderately down regulated in compared to the deletion strain (Δ *sprG1/sprF*) (115). This kind of investigation helps in the generation and testing of research questions that aimed to determine whether SprG1 has role in the secretion of specific proteins onto the extracellular space. It also further helped elaborate on the mechanisms by which SprF1 modulates the intracellular downregulation of specific proteins.

VI. Conclusions

Type I TA systems are increasingly identified in bacterial chromosomes as well as in accessory genomes such as phages and plasmids. The existence of multiple mechanisms to regulate translation of the toxin protein demonstrates that the bacterial cell would likely only express the protein when essential. The overexpression of toxin and antitoxin genes from ectopic plasmid vectors is an important tool in understanding the impacts of the toxins and the interaction of the toxin and antitoxin genes. However, caution must be exhibited in extrapolating findings from the overexpression system to the native level expression from the chromosome. Multi-copy plasmid vectors with native regulatory elements can be used as an alternative when chromosomal deletion constructs do not show a strong phenotype. This might be helpful in elucidating the identification of the specific conditions in which the toxins are translated with their own regulatory elements, and the function they provide to the bacterial cell.

VII. Summary

This dissertation work combines the commonly employed overexpression tools to study the effects of a type I TA system (*zor-orz* locus) and to determine the role of a long 5'UTR in the regulation of *zorO* translation and subsequent impacts of ZorO overproduction within the cell. While ZorO overproduction shows immediate and significant impacts in the cells, the chromosomal mutants Δ *zor-orz* (deletion strain) and Δ 28-*zorO* (strain deleted for two regulatory elements, antitoxin *orzO* and the first 28 nt at 5'UTR of *zorO* gene) do not show significant differences in growth and physiology (in the laboratory conditions tested). The results from the overexpression system, therefore, cannot be extrapolated to infer the effects during native level expression. To address this problem, we incorporated a novel approach of utilizing a medium-copy plasmid vector cloned with the *zor-orz* locus under its own regulatory elements. Using these plasmid vectors, we implemented a commonly used disc diffusion antibiotic susceptibility assay to screen if the *zor-orz* locus is protective. In fact, we found that the cells with multiple copies of *zor-orz* locus were more resistant to aminoglycoside antibiotics. As a preliminary step to investigate the mechanism of kanamycin resistance

via *zor-orz* locus, we compared the transcriptome after kanamycin treatment. We also investigated the role of the specific charged amino acids within the ZorO sequence towards ZorO mediated toxicity in the cells. Finally, we identified that even the non-toxic mutant of ZorO that does not show effects on growth inhibition, membrane depolarization and ATP depletion is able to protect cells against kanamycin antibiotics.

Combining the results from the works in this dissertation, we conclude a possible role of the *zor-orz* locus in protection of the cells against specific antibiotics. Further, the results from the non-toxic mutants and other preliminary result suggests a possible expansion of the role of type I toxin-encoding RNA in bacterial cells.

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CHAPTER II

Improved growth of *Escherichia coli* in aminoglycoside antibiotics by the *zor-orz* toxin-antitoxin system

Publication note

A version of this chapter was originally published by Bikash Bogati, Nicholas Wadsworth, Francisco Barrera, and Elizabeth M. Fozo:

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Bikash Bogati performed mutant constructions, 5' rapid amplification of cDNA ends, western blot, dot blot, flow cytometric analysis, ATP assay, scanning electron microscopy, survival assay, tolerance detection assay, minimum inhibitory concentration assay and growth curves. Nicholas Wadsworth and Francisco Barrera performed works in Figure 2.4 and Figure 2.7B. Jia Wen did the growth curve in Fig 2.1A and primer extension assay in Figure 2.1C. Bikash Bogati, Francisco Barrera and Elizabeth M. Fozo wrote the manuscript.

Abstract

Type I toxin-antitoxin systems consist of a small protein (under 60 amino acids) whose overproduction can result in cell growth stasis or death, and a small RNA that represses translation of the toxin mRNA. Despite their potential toxicity, type I toxin proteins are increasingly linked to improved survival of bacteria in stressful environments and antibiotic persistence. While the interaction of toxin mRNAs with their cognate antitoxin sRNAs in some systems are well characterized, additional translational control of many toxins and their biological roles are not well understood. Using an ectopic overexpression system, we show that the efficient translation of a chromosomally encoded type I toxin, ZorO, requires mRNA processing of its long 5' untranslated region (UTR; $\Delta 28$ UTR). The severity of ZorO induced toxicity on growth inhibition, membrane depolarization, and ATP depletion were significantly increased if expressed from the $\Delta 28$ UTR versus the full-length UTR. ZorO did not form large pores as evident via a liposomal leakage assay, *in vivo* morphological analyses, and measurement of ATP loss. Further, increasing the copy number of the entire *zor-orz* locus significantly improved growth of bacterial cells in the presence of kanamycin and increased the minimum inhibitory concentration against kanamycin and gentamycin; however, no such benefit was observed against other antibiotics. This supports a role for the *zor-orz* locus as a protective measure against specific stress agents and is likely not part of a general stress response mechanism. Combined, these data shed more insights into the possible native functions for type I toxin proteins.

I. Introduction

Within the past two decades, numerous toxin-antitoxin (TA) systems have been identified encoded on bacterial chromosomes. A TA system consists of a relatively stable toxin which typically targets an essential cellular function such as translation, DNA metabolism, energy production, or membrane homeostasis and an antitoxin which counteracts the activity of its cognate toxin (1–4). Based on the nature of the antitoxin *i.e.* either a small RNA (sRNA) or a protein and the mechanism used by the antitoxin to neutralize the toxin, a TA system is primarily classified into one of six types (type I – type VI) (4, 5). A type VII TA system has been proposed and an unorthodox TA system whereby an RNA is the toxin have recently been described, indicating further expansion of the variety of TA systems (6, 7). The toxin components of type I TA systems, which were first identified on plasmids, are small proteins usually under 60 amino acids (8, 9). In a type I system, the sRNA antitoxin neutralizes the toxin mRNA *via* complementary base pairing which in turn leads to degradation of the toxin mRNA, physical prevention of its translation, or both (4, 10). This repression in translation (in laboratory growth conditions) and the small size of the toxin protein makes the study of type I toxins difficult. Another challenge is that the deletion of one or even multiple TA gene pairs does not always produce an obvious phenotype (11–13). An ectopic overexpression system, therefore, is commonly used to investigate the interaction between the toxin and antitoxin and the impacts of the toxin protein on a bacterial cell.

ZorO is a type I toxin (29 amino acids) and one of the toxin components of the *zor-orz* locus. The *zor-orz* locus, first identified on the chromosome of *Escherichia coli* O157:H7 EDL933 (EDL933), encodes two highly homologous type I TA systems in tandem, *zorO-orzO* and *zorP-orzP* (*zorO* annotated as z3289 and *zorP* as z3290 in EDL933, GCA_000006665.1) (14, 15). Considering the *zorO-orzO* gene pair, *orzO* encodes a sRNA that specifically base pairs with the *zorO* mRNA, preventing translation and triggering its cleavage (14, 15). Increasing the amount of *zorO* mRNA *via* artificial overproduction from a plasmid allowed it to escape OrzO repression and cause cell stasis or death (14, 15). These toxic effects of ZorO may be due to its high hydrophobicity, similar to some other type I toxin proteins such as TisB (16), HokB (17),

and AapAI (18). Overproduction studies indicated an inner membrane localization of ZorO that leads to membrane depolarization and ATP depletion (19).

The secondary structures of many toxin-encoding mRNAs, including *zorO*, are either experimentally validated or predicted to inhibit their own translation by intramolecular sequestration of the ribosome binding sequence, providing an additional layer of toxin regulation (20). For those examined, processing of the untranslated region (UTR) of the mRNA is required for efficient translation. As an example, the full-length *tisB* mRNA is translationally inert and requires processing of the first 41 nucleotides of the 5' UTR for its translation (21). For the *zorO-orzO* gene pair, translation of the *zorO* mRNA is also repressed by a long 5' UTR (174 nucleotide), and the processed form ($\Delta 28$) was translated nearly 10-fold higher in an *in vitro* system (15, 22). Interestingly, for TisB, deletion of its antitoxin, *istR*, and the first 41 nucleotides of the mRNA which normally inhibit its translation, resulted in a strain with over 200-fold and 100-fold increase in the number of cells that survived lethal doses of ampicillin and ciprofloxacin respectively, as compared to the wildtype (23).

While ZorO overproduction is toxic, the role of the long 5' UTR in ZorO-induced cellular physiology has not been investigated. Herein, we show that the long 5' UTR of the *zorO* mRNA regulates its effects on translation *in vivo*, cellular growth inhibition, membrane depolarization, and ATP depletion in cells. In addition, we demonstrate the role of the *zor-orz* locus in improving growth in the presence of aminoglycoside antibiotics kanamycin and gentamicin. This is significant as improved growth and increased MIC (minimal inhibitory concentration) to specific antibiotics has not been attributed to a type I TA locus and indicates that the protective effects of a type I toxin may be towards specific stress agents.

II. Materials and methods

Strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 2.1. The sequences of all oligonucleotides are listed in Table 2.2.

Table 2.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<u>Bacterial strains</u>		
<i>E. coli</i> MG1655	<i>E. coli</i> F ⁻ λ <i>ilvG</i> <i>rfb</i> -50 <i>rph</i> -1	Lab stock
<i>E. coli</i> MG1655- Δ 28- <i>zorO</i>	Δ 28- <i>zorO</i> integrated in the chromosome	This study
UTK007	DJ480 PCP- <i>araE</i>	(12)
UTK102	UTK007 <i>lepB</i> -SPA	This study
UTK105	UTK007 <i>lepB</i> -SPA <i>pgm</i> -SPA	This study
<i>E. coli</i> O157:H7 EDL933	Wildtype EHEC, O157:H7	Lab stock
EDL933- Δ <i>zor</i> - <i>orz</i>	<i>zor</i> - <i>orz</i> locus deleted	This study
EDL933- Δ 28- <i>zorO</i>	<i>zorP</i> - <i>orzP</i> & <i>orzO</i> deleted and first 28 nts of <i>zorO</i> 5' UTR	This study
<u>Plasmids</u>		
pAZ3	Cm(R)	(12)
pAZ3-full-length- <i>zorO</i>	Cm(R)	(12)
pAZ3- Δ 28UTR- <i>zorO</i>	Cm(R)	(19)
pAZ3-No-UTR- <i>zorO</i>	Cm(R)	This study
pAZ3-FLAG- <i>zorO</i>	Cm(R)	This study
pAZ3- <i>zorO</i> -FLAG	Cm(R)	This study
pAZ3- Δ 28UTR- <i>zorO</i> -FLAG	Cm(R)	This study
pAZ3- <i>zorO</i> -UTR- <i>gfp</i>	Cm(R)	(19)
pAZ3- Δ 28-UTR- <i>gfp</i>	Cm(R)	(19)
pBR322	Amp(R), Tet(R)	(51)
pBR- <i>zor</i> - <i>orz</i>	Amp(R)	This study
pCP20	Amp(R), Cm(R)	(53)
pKD3	Cm(R)	(55)

Table 2.2. Oligonucleotides used in this study

Name	Oligonucleotide sequence, 5'→3' (Restriction sites underlined)	Use
A1	GUTUTGCGCGUUTCCTGTUGUUCGUUCUCTUGUUGU UU	RNA adapter
A4	GATATGCGCGAATTCCTGTAGAACGAACACTAGAAGAAA	5' RACE
EF510	GGTTTACGTATGTTGGTACGAAAC	5' RACE
EF 524	GTTGGTACGAAACGTTGCTCTCCG	Primer extension
EF1325	GCTCCTTAATAAGGAGAGCGGA <u>AGGACACGCTGACACA</u> AAAGTTAAC	Site directed mutagenesis of <i>zorO</i> ATG start to AAG
EF1326	GTAACTTTTGTGTCAGCGTGTCTTCCGCTCTCCTTATT AAGGAGC	Site directed mutagenesis of <i>zorO</i> ATG start to AAG
EF910	GAATAAAAGCTTCTAGATAATTAAAGTCGCAGCAC	pBR- <i>zor-orz</i>
EF912	GTTTGAGGATCCAATATCTGGGAAGCAGATC	pBR- <i>zor-orz</i>
EF1065	CAGTGAGTGAGATTTATAATCGAATTCGTTGGGAG	pAZ3-P _{BAD} - <i>zorO</i> -FLAG
EF1066	GATAATTAAGCTTGCAGCACATGCAACTTGAAG	P _{BAD} -No-UTR- <i>zorO</i>
EF1141	GGTTGTGCCGGATCGGAATTCCTTTAAGTCCTGGCTGC	P _{BAD} -Δ28- <i>zorO</i> -FLAG
EF1279	CACAATCGCTCAAGACAAGCTTCTATTTATCATC	pAZ3- P _{BAD} - <i>zorO</i> -FLAG pAZ3- P _{BAD} -Δ28- <i>zorO</i> -FLAG
EF1465	GTAAGGTGCTGGAATTCGCGAATGAATAAGGAGAGCG GATGGATTATAAAGATGATGATGATAAAGACACGCTGAC ACAAAAG	P _{BAD} -FLAG- <i>zorO</i>
EF1611	GTAAGGTGCTGGAATTCGCGAATGAATAAGGAGAG	P _{BAD} -No-UTR- <i>zorO</i>
EF585	GAAGTATGACGAGTATAGCTTTCTTAAAGGGGGAAACG GTGTGTAGGCTGGAGCTGCTTC	Δ <i>zor-orz</i>

Table 2.2 (continued)

Name	Oligonucleotide sequence, 5'→3' (Restriction sites underlined)	Use
EF645	CCTAACACCACAGCCTGTGCAACATCGTGTTCCAAC GAACGAATTAATGGGAATTAGCCATGGTCC	<i>Δzor-orz</i>
EF1572	CGCATTAGTTGTTTGAGCGAAAAATATCTGGGAAGCA GATCACAGTAAATAAAAAAACCTGCTGGTGAGGC	MG1655- <i>Δ28-zorO</i> , EDL933- <i>Δ28-zorO</i>
EF1573	CCAAGAAATAGAATAATATACTCTAGATAATTAAAGTC GCAGCACATGCAACTTGAAGTATGACGAGTATAGC	MG1655- <i>Δ28-zorO</i> , EDL933- <i>Δ28-zorO</i>
EF1795	GGAATACCGTTTTCCCCCTTTAAGAAAGTGTAGGCTGG AGCTGCTTCGTGTAGGCTGGAGCTGCTTCG	EDL933- <i>Δ28-zorO</i>
EF1796	GAATAATATACTCTAGATAATTAAAGTCGCAGCACAT GCAACTTGAAGTATGACGAGTATAGCCATATGAATAT CCTCCTTAG	EDL933- <i>Δ28-zorO</i>
EF1869 A	CTCTAAATAATTCAAATTGGTCCGATCCGGCGCAACG TCCCCATCGTCATCCTCGGCACCGGATCC	pBR- <i>zorO</i>
EF1870 A	GGAGTGTGGATATGGTTATAATTTATTCGGATCCGGT GCCGAGGATGACGATG	pBR- <i>zorO</i>

Growth conditions. Cultures for overproduction of ZorO were grown as described previously (15). Briefly, *E. coli* UTK007, a derivative of MG1655 with constitutive *araE*, was transformed fresh (not older than 10 days) with the indicated plasmid derivatives of pAZ3 using either electroporation or chemical transformation (15). The resulting transformants were grown overnight in 5 mL LB (with 25 µg/ml chloramphenicol final concentration) at 37°C with shaking and diluted in LB with appropriate antibiotics (chloramphenicol 25µg/ml for pAZ3 plasmids) to an OD₆₀₀ of 0.01. When the OD₆₀₀ reached ~ 0.3, arabinose was added to a final concentration of 13.32 mM (0.2%), 6.67 µM (0.0001%), 3.33 µM (0.00005%), 1.33µM (0.00002%), or 0.67 µM (0.00001%) as indicated in the text. *E. coli* UTK007 carrying empty vector (pAZ3) or uninduced (no arabinose) was used as a control. OD₆₀₀ was measured as shown in the figures and cells were harvested at indicated time points after overexpression. Shown are averages ± standard deviations for a minimum of three replicates.

Microplate growth curves. Overnight cultures were grown either in LB medium or M9 (1X M9 salts, 2mM MgSO₄, 0.1mM CaCl₂, 1µg/mL thiamine, supplemented with carbon source as indicated). Ampicillin (100 µg/ml final concentration) was added when testing cells transformed with pBR322 (24) or pBR-*zor-orz* plasmids. Overnight cultures were diluted to an OD₆₀₀ of 0.2 in 1 mL sterile phosphate buffer saline or sterile growth medium. A 96 well microplate was prepared by adding 190µl of the culture media (LB or M9) containing the antibiotics as indicated. Then 10 µl of 0.2 OD₆₀₀ culture (diluted in PBS from overnight culture) was added to obtain a 200 µl total volume of a 0.01 OD₆₀₀ culture. Absorbance was recorded on a Gen5™ Microplate reader (BioTek Instruments, Inc.) every 30 min after a 15 sec of shaking for 24 hrs at 37°C. Shown are averages ± standard deviations for a minimum of three replicates. Note, as we observed variation even in control strains emerging from lag phase when grown in M9 minimal media with fructose, we used 0.4% fructose as the final concentration.

Plasmid and strain construction. The overexpression plasmid P_{BAD-zorO-FLAG} (full-length 5'UTR) was generated by amplifying C-terminal FLAG tagged *zorO* using the oligonucleotides EF1065 and EF1279 from the EDL933 genome. To generate P_{BAD-}

$\Delta 28$ -*zorO*-FLAG, the insert was amplified with oligonucleotides EF1141 and EF1279 from the plasmid P_{BAD}-*zorO*-FLAG. To construct P_{BAD}-No-UTR-*zorO*, the insert was amplified from *E. coli* O157:H7 EDL933 genomic DNA with oligonucleotides EF1066 and EF1611. The N-terminus FLAG tagged *zorO* (with 5'UTR similar to that of P_{BAD}-No-UTR-*zorO*) was constructed by amplifying the insert with oligonucleotides EF1465 and EF1066. The inserts were digested and ligated into the EcoRI and HindIII sites of the high-copy plasmid pAZ3 (25) under control of the P_{BAD} promoter. To generate P_{BAD}-*zorO*-AAG, site directed mutagenesis (15) was performed on the plasmid P_{BAD}-*zorO* (22) using primers EF1325 and EF1326.

The plasmid pBR-*zor-orz* (with native regulatory elements) was constructed by amplifying the insert from EDL933 genomic DNA with oligonucleotides EF910 and EF 912, restriction digested and ligated into the HindIII and BamHI sites of medium copy pBR322 plasmid. The plasmid pBR-*zorO* was constructed by amplifying the pBR-*zorO-orzO* plasmid using oligonucleotides EF1869A and 1870A (to remove *orzO*) and was assembled using NEB Gibson Assembly Master Mix.

E. coli MG1655 UTK102 was derived from MG1655 UTK007 (15). P1 transduction was used to replace the *lepB* with SPA (sequential peptide affinity) and kanamycin tagged version of *lepB* (inner membrane localization control) (26). The kanamycin resistance gene (flanked by FRT sites) was removed using pCP20 (27) and confirmed via PCR to generate UTK102. *E. coli* MG1655 UTK105 was subsequently derived from *E. coli* MG1655 UTK102 to replace *pgm* with a SPA tagged *pgm* (cytoplasmic localization control) and confirmed via PCR.

To construct *E. coli* MG1655- $\Delta 28$ -*zorO*, $\Delta 28$ -FLAG-*zorO* linked to a chloramphenicol cassette (flanked with FRT sites) was amplified from a pBR322 plasmid construct using the oligonucleotides EF1572 and EF1573. The amplified insert was recombinated into *E. coli* NM1100 utilizing the mini- λ -Red recombination system (28, 29). This was then moved into *E. coli* MG1655 using P1 transduction. The antibiotic cassette was removed using pCP20 (27, 30). The strain was verified via sequencing of the genetic region.

EDL933- Δ 28-*zorO* was constructed by amplifying Δ 28-FLAG-*zorO*-Kan using the oligonucleotides EF1572 and EF1573 from a plasmid vector that was synthesized (Genescript). The amplified product was recombineered in EDL933, replacing the *zor-orz* locus as described previously (28). The kanamycin resistant gene was then replaced with a chloramphenicol resistance gene amplified from pKD3 using primers EF1795 and EF1796. The chloramphenicol marker was then removed via pCP20 treatment (30). The strain was PCR screened and confirmed via sequencing of the genetic region.

Strain EDL933- Δ *zor-orz* was generated via recombineering as outlined previously using primers EF585 and EF645 (28). The *zor-orz* locus was replaced with a chloramphenicol resistance marker (flanked with FRT sites). The chloramphenicol marker was then removed via pCP20 treatment (30).

RNA isolation. *E. coli* cells harboring pAZ3-*zorO* were grown to $OD_{600} \approx 0.2$ – 0.3 and arabinose (13.32 mM) was added (time 0). Cells were harvested 15 and 30 min post-arabinose induction. For cells harboring pBR-*zorO*, *E. coli* was grown to OD_{600} of ~ 0.3 and cells were harvested. Total RNA for all conditions was isolated via direct lysis as described previously (31).

Primer extension assay. Total RNA (5 μ g) was isolated 30 min after arabinose induction (in LB medium) and was separated on a denatured 8% polyacrylamide-urea gel. Primer extension was performed as described previously using labeled primer EF524 (14, 32).

5' RACE analysis. 5' rapid amplification of cDNA ends (RACE) assay was performed as described previously (25). Isolated RNA was ligated to the RNA adapter A1. Reverse transcription was carried out with *zorO* 5' UTR specific primer EF510. Amplification of *zorO* cDNA was performed with Taq DNA polymerase and primers EF510 and A4. Amplified cDNA fragments were cloned into pCR $\text{\textcircled{R}}$ 4-TOPO $\text{\textcircled{R}}$ vector (Invitrogen) and then sequenced.

Cell survival after ZorO overproduction. Cellular aliquot of 100 μ l were collected at mid-log ($OD_{600} \sim 0.3$, before arabinose induction) and 1h post-arabinose induction. The aliquots were serially diluted in 900 μ l of sterile PBS of which 100 μ l was plated onto LB

medium (with 25µg/ml chloramphenicol). The plates were incubated at 37°C for 18-24 hrs and the colony forming units were counted. The log ratio of survivors was calculated by comparing the CFUs obtained after 1h arabinose induction to that before induction.

Western blot and dot-blot analysis. Total protein was extracted from a 50 ml culture (after overexpression for 30 minutes as indicated in the text) via bead beating. Protein concentration was measured using a Bradford Protein Assay. Protein samples (20µg) were separated on a NuPAGE Bis-Tris gel, transferred to the immobilon-FL membrane, and probed with a rat derived α -FLAG tag primary antibody and an α -IgG secondary antibody (LI-COR Biosciences) that fluoresces at 680 nm. LepB was tagged with SPA tag (UTK102) to serve as a loading control. For GFP detection, blots were probed with a rabbit derived anti-GFP primary antibody (Invitrogen) and an α -IgG secondary antibody (LI-COR Biosciences) that fluoresces at 800 nm.

Subcellular fractionation was performed as described previously with modifications (26). Total protein lysate was ultracentrifuged (100,000 g, 4°C, 45 min) to yield supernatant (cytoplasmic proteins) and a pellet (membrane proteins). For separation of inner and outer membrane proteins, the pellet was washed with PBS and resuspended in 500µl of 0.5% sodium-lauryl sarcosinate, incubated at room temperature for 30 min, and ultracentrifuged (100,000 g, 4°C, 15 min). The supernatant and pellet corresponded to the inner and outer membrane fractions respectively. Then inner membrane fraction was transferred and acetone precipitated while the pellet was washed with 0.5% sodium-lauryl sarcosinate and then resuspended in PBS.

Protein samples (20µg) were loaded onto Novex 10-20% Tricine gels and transferred to an immobilon-FL membrane. Blots were probed with α -FLAG (BioLegend) and α -OmpA primary antibodies (Antibody Research Corporation) and α -IgG secondary antibodies (LI-COR Biosciences) fluorescing at 680 nm and 800 nm respectively.

Dot blots were performed by spot inoculating a nitrocellulose membrane with 10µl of the sample containing 10µg or 20µg protein and processing the membrane as above (probed with α -FLAG primary antibody α -IgG secondary antibody) (33).

Microscopic image of colonies expressing GFP under different 5' UTR variants.

Overnight cultures of UTK007 cells transformed with either pAZ3-*zorO*-UTR-*gfp* or pAZ3- Δ 28-UTR-*gfp* were serially diluted and plated on LB with 25 μ g/ml chloramphenicol and 13.32 mM arabinose. The plates with 30-300 colonies were used to image under all-in-one fluorescence microscope BZ-X710 using bright field and green fluorescent channel with 2X magnification.

Membrane depolarization assay. Membrane potential sensitive dye Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄-3; Invitrogen™) was used to determine the effect of ZorO production on membrane depolarization. A 25 mg/mL stock solution was prepared in dimethyl sulfoxide. At indicated time points, 50-100 μ l of culture was taken, flooded with 4ml 1X phosphate buffer saline (1XPBS) and centrifuged for 10 min at 4°C. Cells were resuspended in 1 ml PBS and stained with DiBAC₄-3 (10 μ g/ml final concentration) for 20 min in the dark. After two steps of washing with 1XPBS, cells were re-suspended in 0.5 ml of 1XPBS and analyzed by flow cytometry in an LSR II flow cytometer (Becton Dickinson) with a 488-nm laser. Samples were run at ~3000 events per second and fluorescence was collected in the fluorescein isothiocyanate (FITC) channel. Data was analyzed using FlowJo software package (FlowJo LLC, Ashland, OR, USA) such that the cells showing at least 10³ abu or more fluorescence were gated and mean fluorescence intensity was obtained. All tests were done with a minimum of three biological replicates.

ATP measurements. ATP levels were measured using BacTiter Glo ATP Assay System Bioluminescence Detection Kit for ATP Measurement (Promega) according to the manufacturer's instructions. The observed relative luciferase unit (RLU) values were normalized to the OD₆₀₀ at the time of cell harvest. A minimum of three biological replicates were performed per strain/condition. To determine ATP leakage, ATP measurements of the spent medium were determined. Cells were pelleted at indicated time points after ZorO overproduction and 100 μ l of the supernatant was used to measure the ATP concentration. As a control, cells carrying empty vector were treated with 5% (final concentration) of butanol for 5 min and ATP measurements of the spent medium were determined as above.

Circular dichroism (CD). The ZorO peptide was synthesized using Fmoc chemistry by ThermoFisher. The peptide sample had a purity of >95%, as assessed via HPLC. Peptide identity was determined by MALDI-TOF. Chloroform stocks of the synthetic lipids DOPE and DOPG (Avanti Polar Lipids) were dried and resuspended with a methanol solution of ZorO. The resulting solution was dried first with a stream of Argon, followed by overnight incubation under vacuum. Large unilamellar vesicles containing ZorO reconstituted in the lipid bilayer were formed by extrusion. Standard and oriented CD experiments were performed as described previously (34, 35).

Membrane leakage experiments in vesicles. Leakage experiments were performed after encapsulation of calcein as described elsewhere (36). ZorO was added from methanol stocks at a 1% v/v final concentration of methanol. No methanol induced leakage was observed. 1% (v/v) Triton X-100 was added to the sample to obtain total calcein release and the fluorescence corresponding to this sample was used for normalization.

Scanning Electron Microscopy (SEM). Cells harboring either empty vector pAZ3 or P_{BAD}-No-UTR-zorO were grown in LB until mid-log phase (OD₆₀₀ of ~0.3) and induced by adding 13.32 mM final concentration of arabinose. At 30 min post-induction, cells were harvested (50 ml) and washed twice with 1x PBS to remove media. Fixation was performed with glutaraldehyde (3% final concentration) for 1h. Cells were washed twice with distilled water and stored in 500µl of 1XPBS at 4°C overnight. The next day, samples were centrifuged, the supernatant was discarded, and 20µl of the pellet was transferred to silicon chip with poly-L-lysine. The cells were dehydrated by immersion of the silicon chip in an increasing concentration of ethanol (25%, 50%, 70%, 95% and 100 %) for 10 min each followed by a critical point dry. The silicon chips were mounted to an aluminum mount, coated with iridium, and observed under an Auriga Scanning Electron Microscope in Advanced Microscopy and Imaging Center, University of Tennessee, Knoxville. Images from at least five random microscopic fields were taken at 5,000X and 20,000X magnification. The cells producing ZorO were analyzed for differences in morphological characteristics as compared to the uninduced controls. Cell length was

measured using ImageJ (37). Cells (at least 30 per image) from each of the three biological replicates were analyzed.

MIC determination. Minimum inhibitory concentration was performed as previously described (38) with slight modification. An overnight culture was diluted in Mueller Hinton Broth to 0.01 OD₆₀₀ (with 100 µg/ml final concentration of ampicillin to maintain pBR plasmids). To a set of 10 sterile tubes, 1 mL of the diluted culture was added to 8 tubes; the first tube was added with 2 ml culture and the last tube with sterile media (media control). Antibiotic was added to the first tube (containing 2 ml culture) to obtain the maximum concentration that was tested (eg. 25.6 µg/ml for kanamycin), mixed properly, and serially diluted by transferring 1 ml. The tubes were incubated at 37°C with shaking for ~18 hrs and observed for visible growth to determine MIC. All tests were performed with a minimum of three biological replicates.

Statistical analysis. Statistical analyses were performed using *t* test or multiple unpaired *t* test with Welch correction (with $\alpha=0.05$) and presented within the text. A minimum of 3 biological replicates were tested.

III. Results

ZorO translation and toxicity are dependent upon its 5'UTR.

We previously demonstrated that overexpression of the *zorO* mRNA was toxic to *E. coli* when the mRNA was expressed from a multicopy plasmid behind the arabinose inducible promoter (P_{BAD}). To confirm that this toxicity was due to the predicted encoded 29 amino acid protein ZorO; we mutated the start codon from an ATG to an AAG. As shown in Fig. 2.1A, overexpression of the AAG mutant did not inhibit growth of *E. coli*, confirming the encoded protein is responsible for toxicity.

There are two dominant forms of the *zorO* mRNA: one possessing a 174 nucleotide (nt) 5' UTR (full-length) and a second, referred to as $\Delta 28$ -*zorO*, lacking the first 28 nt of the 5' UTR as observed by primer extension assay and 5' rapid amplification of cDNA ends (5' RACE; Fig. 2.1B and 2.1C) (22). The *zorO* mRNA levels upon artificial overproduction from these two different 5'UTRs under similar induction condition showed transcript levels equivalent to or even lower for $\Delta 28$ -*zorO* as compared to the

full-length 5' UTR (22). However, *in vitro* translation of the $\Delta 28$ -*zorO* mRNA was more robust than that of *zorO* possessing its full-length 5' UTR (22). The contribution of processing in regards to ZorO-induced toxicity though was not previously examined. To address the significance of the UTR in ZorO-induced phenotypes, we cloned *zorO* with a C-terminus FLAG tag with either its full-length 5'UTR (P_{BAD} -*zorO*-FLAG) or its processed form (P_{BAD} - $\Delta 28$ -*zorO*-FLAG), under control of an arabinose inducible promoter (P_{BAD}) on a high copy plasmid (Fig. 2.2A). The plasmids were transformed into a derivative of *E. coli* MG1655 (UTK007) that naturally lacks the *zor-orz* locus (14, 15). Induction of ZorO from either plasmid with high levels of arabinose, 13.32 mM, (0.2%), resulted in an immediate growth stasis (Fig. 2.2B) and loss of viable cells (Fig. 2.2E). When induced with either 6.67 μ M or 0.67 μ M (0.0001% or 0.00001% respectively) arabinose, cells harboring P_{BAD} - $\Delta 28$ -*zorO*-FLAG still resulted in nearly instantaneous growth stasis while those cells harboring P_{BAD} -*zorO*-FLAG were able to grow, although at a reduced rate to controls (Fig. 2.2C and 2.2D).

Considering the similar effects of P_{BAD} -*zorO*-FLAG and P_{BAD} - $\Delta 28$ -*zorO*-FLAG on growth inhibition when induced with 13.32 mM arabinose (Fig. 2.2B), we used this induction condition to compare production of ZorO from the differing 5' UTRs. Using dot blot analysis, we were able to detect ZorO from both the full-length UTR and $\Delta 28$ UTR (Fig. 2.2F). However, using western blot analysis, we were able to detect ZorO-FLAG only when overproduced from the $\Delta 28$ UTR but not the full-length 5' UTR (Fig. 2.3A). In addition, we confirmed the inner membrane localization of ZorO via western blot analysis after subcellular fractionation (Fig. 2.3B) as previously shown by Otsuka *et al.* (19). The cytoplasmic control Pgm was also observed in the inner membrane fraction, as was previously observed (39).

To further validate the translational effects were solely due to the differing 5' UTRs, we replaced the *zorO* coding sequence with that of green fluorescent protein (*gfp*) in a multicopy plasmid vector under control of the arabinose inducible promoter.

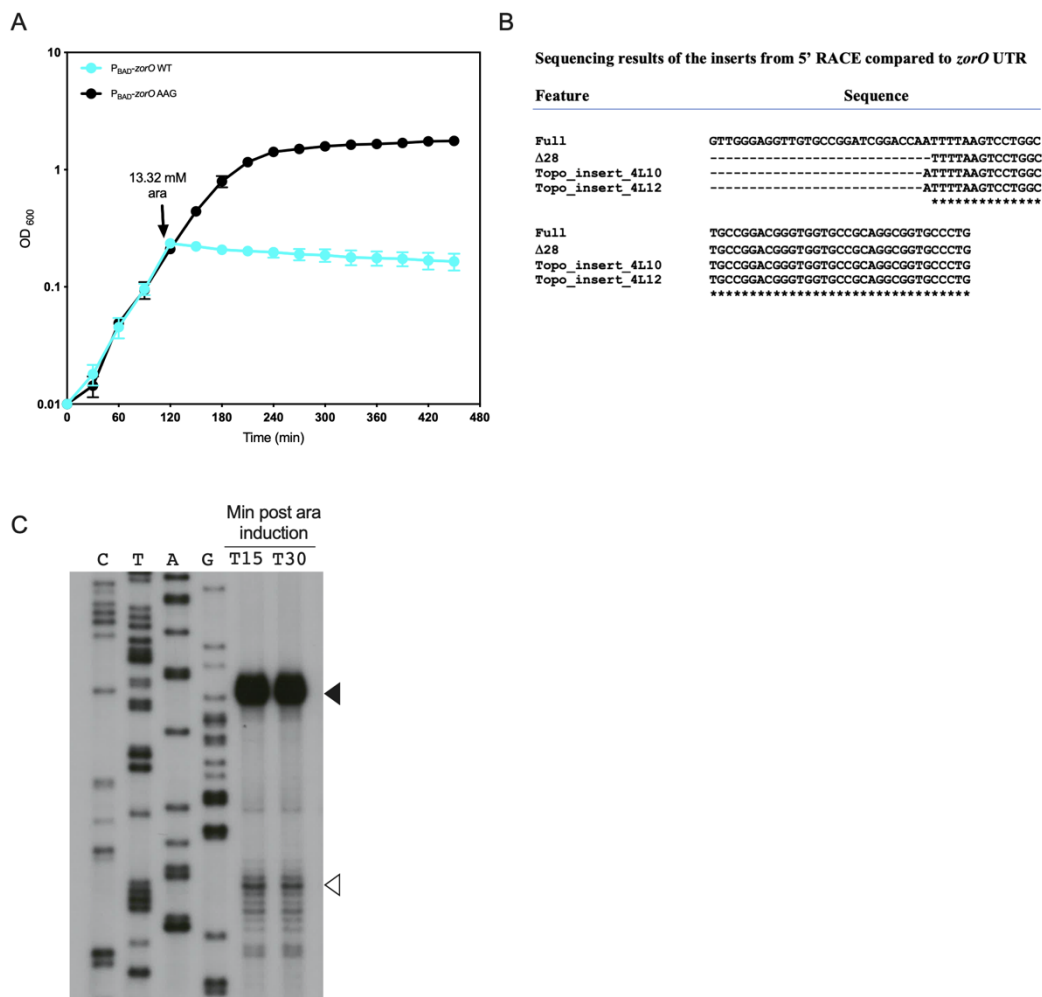


Figure 2.1. The ZorO peptide is needed for toxicity and there is processing of the 5' untranslated region of *zorO* mRNA. Growth curve of *E. coli* UTK007 strain carrying wild-type *zorO* or *zorO* with start codon mutated to AAG under control of the P_{BAD} promoter on a high-copy plasmid induced by 13.32 mM arabinose during mid-log phase. Shown are mean $\pm$ SD of $n=3$ (A). Processing of *zorO* mRNA when overproduced from P_{BAD} -*zorO*. Cells harboring P_{BAD} -*zorO* were grown to exponential phase as described in the main text and induced with 13.32 mM arabinose. Cells were harvested 15 and 30 min post arabinose induction, RNA was isolated and primer extension was performed to map *zorO* mRNAs. The two dominant species, full-length and $\Delta 28$, are indicated by closed and open triangle respectively (B). Rapid amplification of *zorO* cDNA ends of cells harboring pBR-*zorO*. RNA was isolated from the cells harboring pBR-*zorO* grown in LB until exponential phase. 5' RACE was performed to map the end of *zorO*, and shown are ends mapped (C).

Figure 2.2. The 5'UTR variant of *zorO* gene and its effect on growth, survival and translation. Schematic of the full-length (174 nts; P_{BAD}-*zorO*-FLAG) and processed (Δ 28; P_{BAD}- Δ 28-*zorO*-FLAG) UTR of *zorO* used in the study (A). Growth curve of MG1655 derived cells (UTK007) carrying respective plasmids after induction with arabinose concentration of 13.32 mM (B), 6.67 μ M (C) and 0.67 μ M (D) during mid-log phase (B-D, n=3, shown are mean $\pm$ standard deviation [SD]). Log survival (colony forming units) of cells after ZorO overproduction with indicated arabinose concentrations (E) (n=9, shown are mean $\pm$ SD). Dot blot assay after 30 min with 13.32 mM arabinose induction of FLAG tagged ZorO in *E. coli* UTK 007 cells from the two 5' UTR variants (10 μ g and 20 μ g of total protein loaded) (F); pAZ3- negative control, PC- positive control (No-UTR-FLAG-*zorO*).

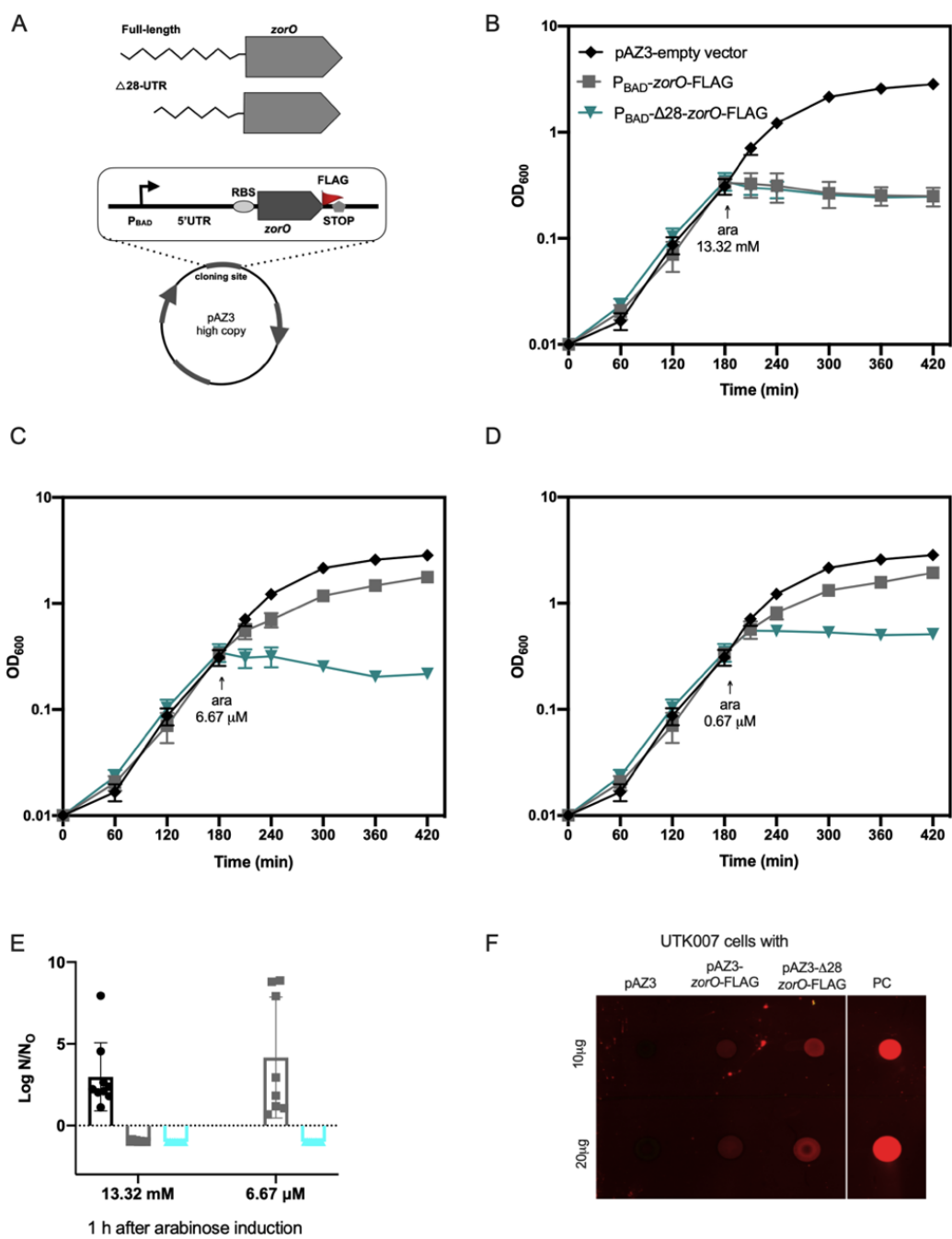
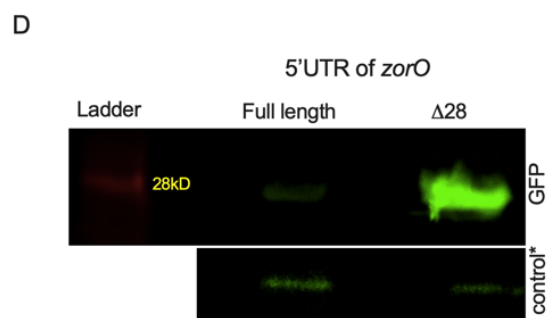
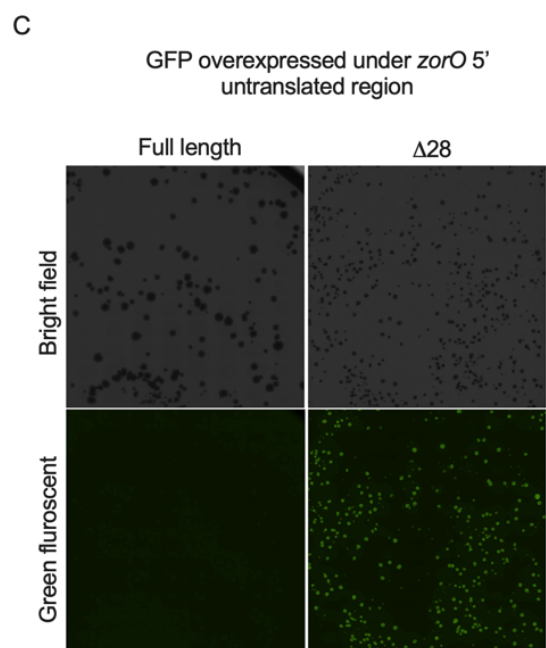
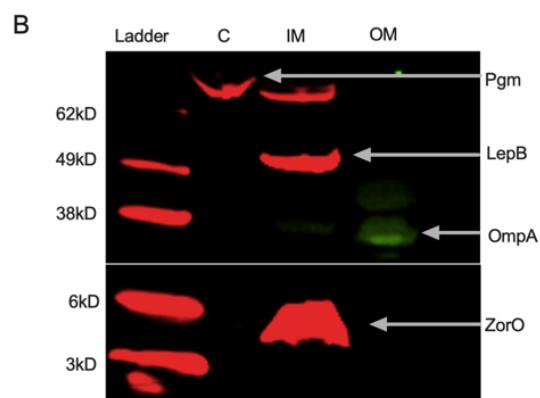
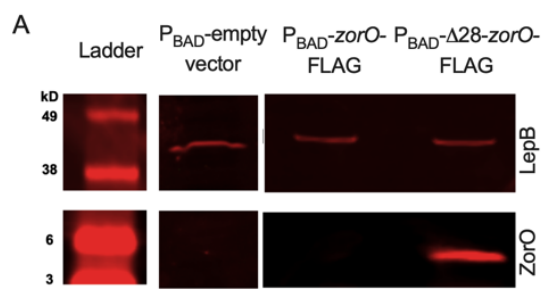


Figure 2.3. The 5' UTR controls translation. Western blot analysis after 30 min with 13.32 mM arabinose induction of FLAG tagged ZorO in *E. coli* UTK 102 from the two 5' UTR variants (20 µg of total protein loaded) (A); SPA tagged LepB is used as loading control. Western blot analysis of ZorO in cytoplasmic (C), inner membrane (IM) and outer membrane (OM) fraction after 30 min induction with 13.32 mM arabinose from P_{BAD}-No-UTR-FLAG-zorO (Fig. 2.5A) in *E. coli* UTK 105 (20 µg protein loaded) (B); SPA tagged Pgm and LepB are used as cytoplasmic and inner membrane control respectively; OmpA is used as outer membrane control. Translation of green fluorescent protein under different 5' untranslated region of *zorO*. Images of *E. coli* colonies on LB agar with 13.32 mM arabinose (C) harboring either pAZ3-zorO-UTR-*gfp* (left) or pAZ3-Δ28-UTR-*gfp* (right) using bright field (upper panel) and green fluorescent channel (lower panel); 2X magnification. Western blot analysis of GFP (20 µg of total protein loaded) after induction with 13.32 mM arabinose for 30 min under full-length (pAZ3-zorO-UTR-*gfp*) or processed 5' UTR (pAZ3-Δ28-UTR-*gfp*) (D); asterisk indicates a nonspecific signal and serves as a loading control.



E. coli cells harboring these plasmids with *gfp* under *zorO* 5'UTRs were grown on LB agar with 13.32mM arabinose. We then examined colonies (2X magnification) for GFP production and observed green fluorescence only in the colonies expressing *gfp* under the processed 5'UTR (Δ 28 UTR) (Fig. 2.3C). Similarly, GFP detection via western blot analysis was greater from the processed 5' UTR as compared to the full-length UTR following induction with 13.32 mM arabinose for 30 min (Fig. 2.3D). When lower concentrations of arabinose (3.33 μ M and 6.67 μ M) were used to induce GFP production, we were only able to detect GFP from the Δ 28 UTR and not the full-length 5'UTR (data not shown). Thus, the *zorO* UTR is sufficient to control translation of a heterologous mRNA.

ZorO forms an alpha helix across a lipid bilayer.

Given the inner membrane localization of ZorO (19) (Fig. 2.3B), we determined the conformation that ZorO adopts in membranes. We used a reconstituted system to examine the structure of synthetic ZorO within lipid vesicles. To partially mimic the lipid composition of the *E. coli* inner membrane, we utilized vesicles composed of a 3 to 1 molar ratio of two lipids: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG). We first used circular dichroism (CD) to study the conformation that ZorO adopts in the lipid bilayer. Fig. 2.4A shows a CD spectra with minima at 208 and 222 nm, which is characteristic of an α -helical conformation (40), indicating that ZorO folds into an α -helix. To discriminate if the helix lies on the surface of the membrane, or it is inserted across the lipid bilayer, forming a transmembrane helix, we employed oriented CD (OCD), which allows distinguishing between these two possibilities. The OCD data shown in Fig. 2.4B indicate that ZorO forms a transmembrane helix, given the single minima at 225 nm (41).

Overexpression of *zorO* via its processed 5' UTR causes increased membrane depolarization and ATP depletion.

The overproduction of ZorO, like other type I toxins including TisB (23, 42), DinQ

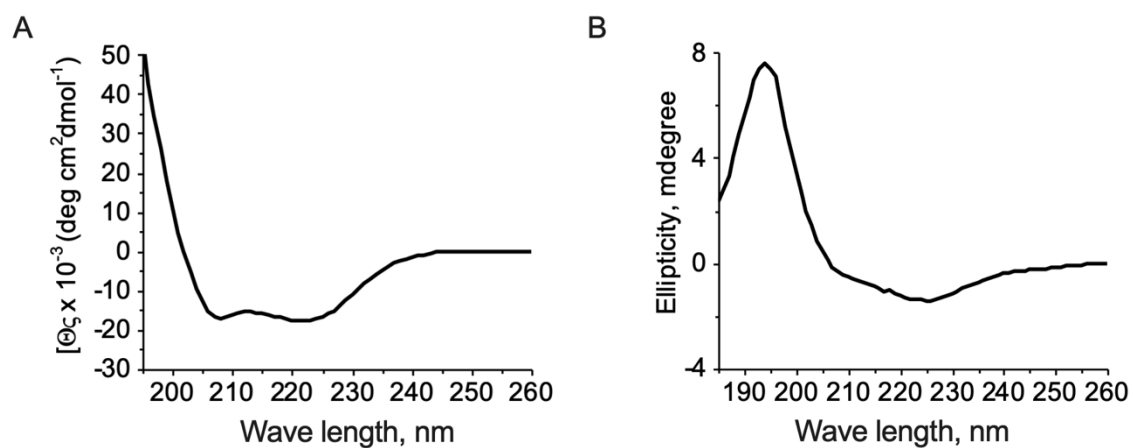


Figure 2.4. Synthetic ZorO peptide can insert across a lipid bilayer. Circular dichroism spectra of ZorO peptide in lipid composition partially mimicking *E. coli* membrane (A). Oriented circular dichroism of ZorO peptide on lipid bilayer (B).

(43), HokB (1), lbsC, and ShoB (25), caused membrane depolarization (19). The data in Fig. 2.3B and the work of others (19) indicated that ZorO targets the cytoplasmic membrane. However, we did note differences in ZorO production (Fig. 2.2F, 2.3A) dependent upon whether it was expressed from the full-length or $\Delta 28$ UTR and wanted to further investigate how expression from the differing UTRs may influence ZorO-induced physiology. We first assessed the differences in membrane depolarization using bis-(1,3-dibutylbarbituric acid) trimethine oxonol dye (DiBAC₄-3) which is a potential-dependent distributional fluorescent dye. This dye selectively enters cells upon membrane depolarization which are then measured using a flow cytometer (44).

We used plasmids harboring *zorO* with differing lengths of its 5' UTR (full-length and $\Delta 28$ UTR lacking FLAG tag). *E. coli* UTK007 cells transformed with these plasmids were induced with a final arabinose concentration of 1.33 μ M (0.00002%) and analyzed for membrane depolarization, as this arabinose concentration yielded growth inhibition of the P_{BAD}- $\Delta 28$ -*zorO* (Fig. 2.5B). Under this low level of arabinose induction, membrane depolarization occurred within 15 minutes of ZorO overproduction (Fig. 2.6A). We did observe that the percentage of cells positive for membrane depolarization was significantly lower ($p < 0.001$, $p < 0.02$) for those harboring the plasmid containing the full-length 5' UTR of *zorO* (~20%), as compared to the truncated ($\Delta 28$ -*zorO*) UTR constructs (~95%) (Fig. 2.6A). When the concentration of arabinose was raised to 3.33 μ M (0.00005%, Fig. 2.6B) or higher (data not shown), the impact on membrane depolarization was similar across all constructs examined with depolarization of most cells (>95%) within 15 min of ZorO overproduction (Fig. 2.6B). It is important to note that despite this severe impact on membrane depolarization when overproduced via the full-length 5'UTR, the translated ZorO protein was below our detection limit using western blot analysis (Fig. 2.3A) even when induced with higher (13.32 mM) arabinose concentration.

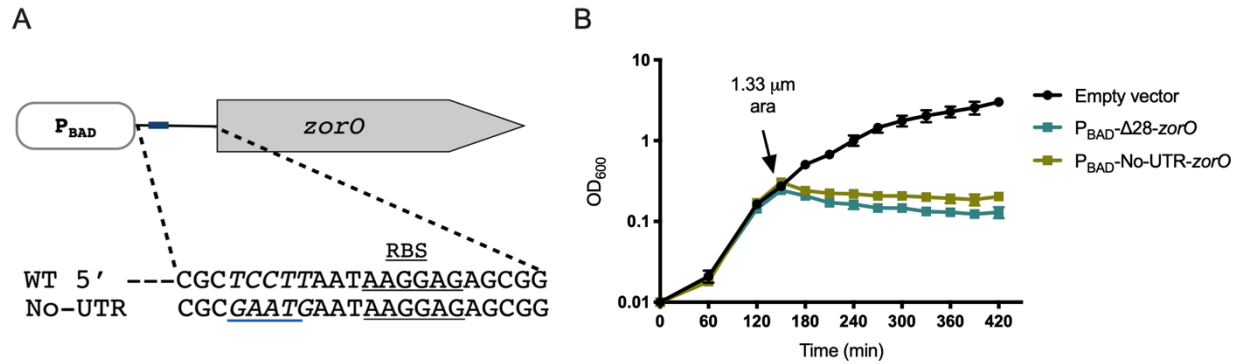


Figure 2.5. Truncation and modification of the 5' UTR of ZorO results in cellular toxicity similar to that induced by Δ 28-zorO. P_{BAD}-No-UTR-zorO construct (A). The long 5'UTR was removed leaving 22 nucleotides and mutated sequence predicted to form stem loop (italicized and underlined) with RBS (underlined). Effects of No-UTR-zorO as compared to the Δ 28-zorO on growth of *E. coli* UTK 007 strains when induced with 1.33 μ M arabinose (B), $n = 3$, shown are mean $\pm$ SD.

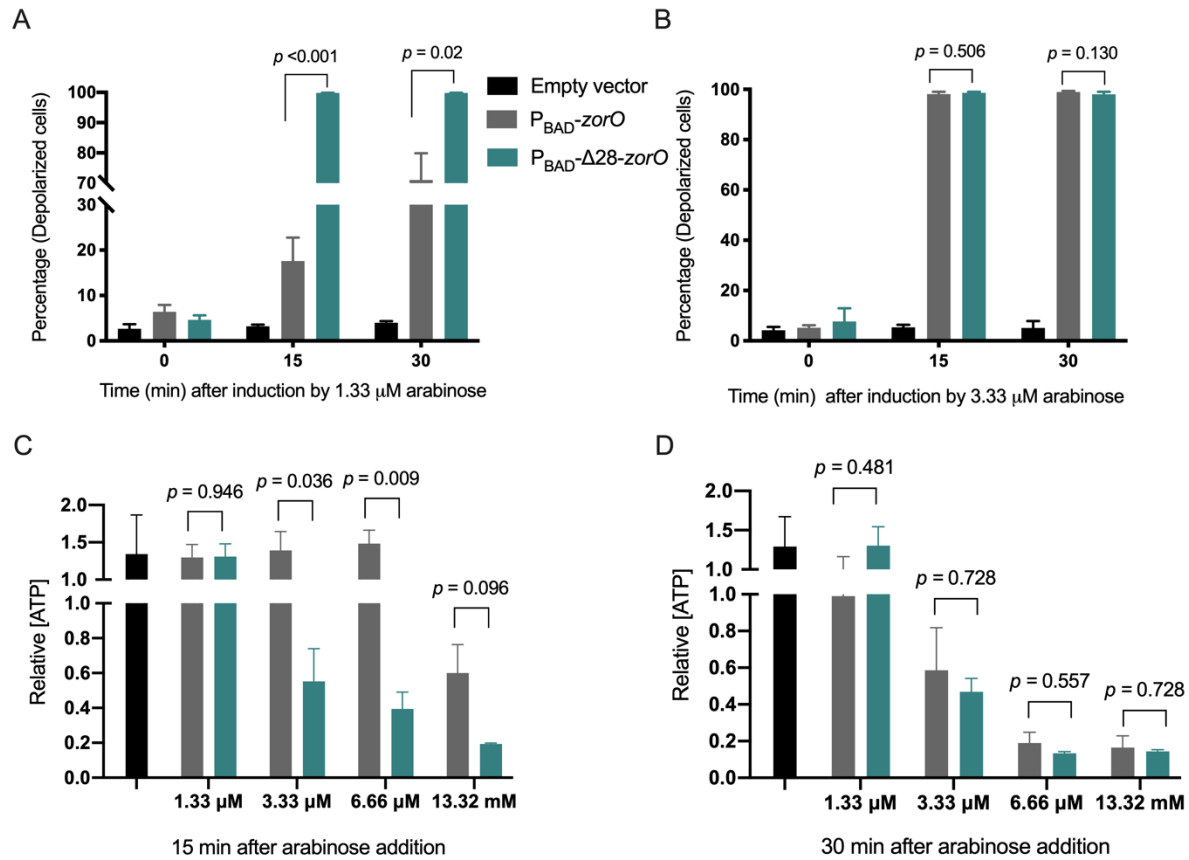


Figure 2.6. ZorO overproduction leads to membrane depolarization and ATP depletion. Cells overproducing ZorO under the control of the two 5' UTR variants; 15 and 30 min post-induction were analyzed via flow-cytometry after staining with DiBAC₄-3. Bar graphs quantifying percent of depolarized cells with 1.33 μ M (A) and 3.33 μ M (B) arabinose concentration ($n \geq 5$). Relative ATP levels after ZorO overexpression from respective 5'UTRs and arabinose induction after 15 min (C) and 30 min (D) ($n = 3$). For all panels shown are mean $\pm$ SD. The p values were calculated using a multiple unpaired t test with Welch correction.

Given the differences in membrane depolarization, we examined how the 5' UTR impacted ATP depletion using a quantitative luciferase-based assay (BacTiter-Glo™ Microbial Cell Viability Assay, Promega). In agreement with the membrane depolarization analyses, we observed that, at high levels of arabinose induction for 30 min, both 5'UTR variants depleted the ATP levels similarly (Fig. 2.6D). However, 15 min after addition of either 3.33 μ M (0.00005%) or 6.6 μ M (0.0001%) arabinose, overexpression of *zorO* from the Δ 28-UTR significantly reduced cellular ATP levels as compared to the full-length 5' UTR (Fig. 2.6C).

ZorO induced damage is not due to gross morphological changes.

Regardless as to whether its 5' UTR is processed or not, ZorO overproduction can still lead to severe membrane damage. The type I toxin protein HokB also causes membrane damage: it has been shown to insert in the cytoplasmic membrane and forms pores causing leakage of intracellular ATP (17, 45). We thus tested if ZorO formed large pores in the inner membrane that could result in ATP leakage. To enhance the overproduction of ZorO, we constructed the plasmid P_{BAD}-No-UTR-*zorO* by removing the 5'UTR and only leaving the ribosome binding site (Fig. 2.5A). The growth inhibition *via* this construct was similar to that of the P_{BAD}- Δ 28-UTR-*zorO* (Fig. 2.5B). We then tested if leakage of ATP occurred when ZorO was overproduced by measuring extracellular ATP but did not observe increase in ATP levels in the supernatant compared to that of the cultures with empty vector (Fig. 2.7A). We treated an aliquot of the cells carrying empty vector with butanol which resulted in a large amount of ATP detected in the supernatant.

We further validated these findings using a reconstituted system to examine if ZorO-permeabilized membranes allowed the release of medium-sized organic molecules. To this end, we performed a leakage assay where the fluorescent dye calcein was encapsulated in lipid vesicles. Calcein has a molecular mass of 622.5 Da, similar to that of ATP (507.2 Da). We performed a titration experiment and observed

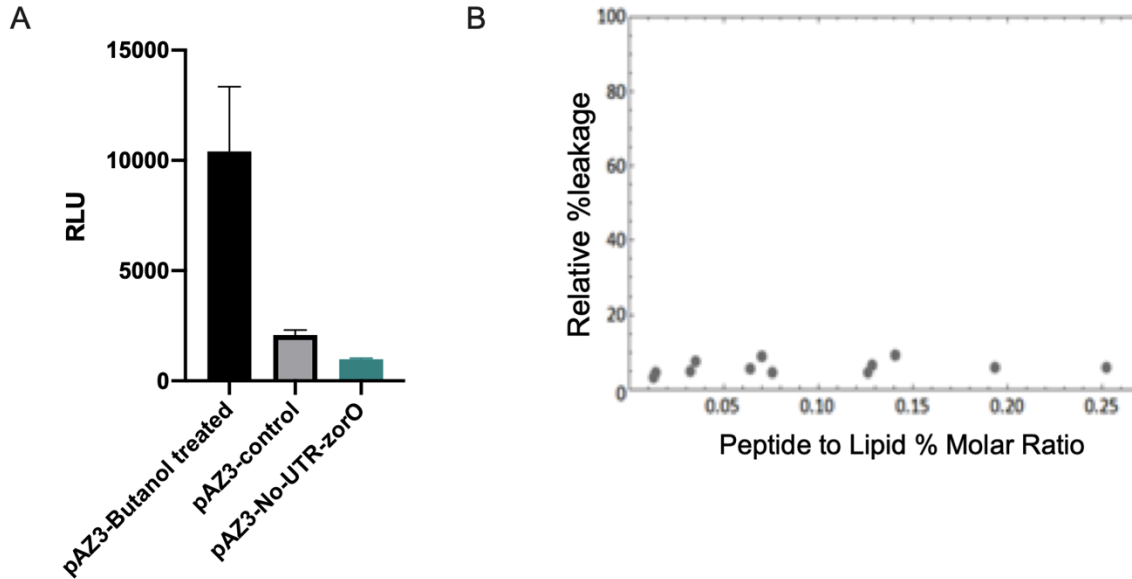


Figure 2.7. ZorO does not form large pores. Extracellular ATP levels after induction with 13.32 mM arabinose from P_{BAD} -No-UTR-zorO plasmid and empty vector in *E. coli* UTK007 (A); Cells (1 ml) harboring the empty vector were treated with 50 μ l of butanol for 5 mins to lyse cells and release ATP to use as positive control. $n = 4$, shown are mean $\pm$ SD. Relative percentage of calcein leakage compared to Triton X 100 treatment from lipid vesicles after ZorO peptide interaction (B).

that the addition of ZorO resulted in no significant release of the calcein molecules from the vesicles (Fig. 2.7B). TritonX-100 was used to obtain a total calcein release from the vesicles and the fluorescence corresponding to this sample was used to normalize the fluorescence observed after addition of ZorO. These results indicate that ZorO does not cause a large disruption of the membrane integrity, leading to calcein exiting the vesicle, in the concentration range studied.

We also performed scanning electron microscopy (SEM) of cells overproducing ZorO (using P_{BAD} -No-UTR-*zorO*; Fig. 2.5A) to determine if the toxin caused any morphological alterations. In contrast to the marked growth inhibition and cell death after ZorO overproduction, there were no overt differences in morphology (Fig. 2.8A) even when induced with a high concentration of arabinose (13.32 mM). Measurement of cell length (see Materials and Methods) revealed that ZorO induced cells were longer than uninduced cells ($p < 0.0001$) (Fig. 2.8B) but this was the only observable morphological difference.

Multiple copies of *zor-orz* locus contributes to survival in the presence of aminoglycosides. Virtually all antibiotics target active cellular processes, therefore active cellular metabolism is critical for effective killing by these drugs. ZorO overproduction caused membrane depolarization and reduced cellular ATP levels, which is expected to decrease metabolic activity. Reduced ATP levels in turn are linked to increased bacterial survival in the presence of antibiotics (1, 17, 46, 47). Further, type I toxins TisB and HokB are known to increase survival in the antibiotic ciprofloxacin or ofloxacin, we investigated whether the *zor-orz* locus impacted cell growth and inhibition by antibiotics (1, 48, 49).

Given that ZorO overproduction is toxic, we first examined how increasing the copy number of the entire *zor-orz* locus would influence growth in the presence of antibiotics. To this end we cloned the entire *zor-orz* locus, including all transcriptional and translational control elements, into pBR322 (pBR-*zor-orz*) and examined the effects on antibiotic sensitivity in *E. coli* UTK007 (that naturally lacks the *zor-orz* locus using a disc diffusion assay. Cells harboring *zor-orz* had statistically smaller zones of inhibition

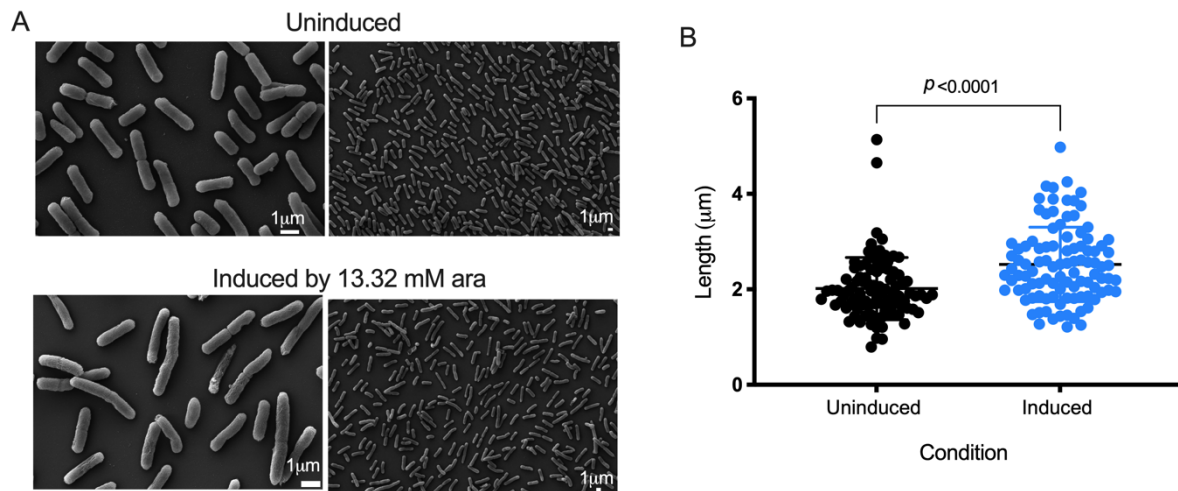


Figure 2.8. ZorO overproduction does not lead to gross alterations of cellular morphology. Scanning electron microgram of *E. coli* cells overproducing ZorO from $P_{\text{BAD}}\text{-No-UTR-zorO}$ plasmids with 13.32 mM arabinose induction for 30 min (A) compared to that of uninduced cells (B); right panels 20000X and left panels 5000X magnification. Comparison of cell length after ZorO overproduction. Shown are mean $\pm$ SD. The p values were calculated using t test with Welch correction.

upon exposure to the aminoglycoside kanamycin, a protein synthesis inhibitor, but not to ciprofloxacin (DNA gyrase inhibitor) (Fig. 2.9). *E. coli* with multiple copies of *zor-orz* also had higher minimum inhibitory concentration (MIC) to kanamycin and gentamicin but not to chloramphenicol or ciprofloxacin when grown in Mueller Hinton broth, as is standard in clinical laboratories (Table 2.3).

Given the increase in MIC, we then examined whether cells harboring multiple copies of the *zor-orz* locus could grow better in the presence of antibiotics than control cells. As *zorO* transcription may be sensitive to carbon source (data not shown), we investigated growth in rich media (LB) as well as minimal media with either glucose or fructose as a carbon source. Further, we utilized a microplate as opposed to glass tubes which were used for MIC determination (see Materials and Methods for details). We noted that cells harboring pBR-*zor-orz* grew far better in the presence of kanamycin in LB and also in M9 minimal media supplemented with 0.2% glucose, or 0.4% fructose (Fig. 2.10). Similar improved growth was observed with gentamicin (data not shown), but not in the presence of ciprofloxacin or chloramphenicol (Fig. 2.11).

To test the effect of multiple copies of the *zor-orz* locus in its native strain EDL933, we transformed these plasmids into an EDL933 strain in which we deleted the entire *zor-orz* locus (EDL933- Δ *zor-orz*). Similarly, there was improved growth of the cells harboring pBR-*zor-orz* plasmids in LB and minimal media (with glucose or fructose) in the presence of kanamycin (Fig. 2.12).

The uptake of kanamycin is dependent upon membrane potential and ATP (50). As artificial overproduction of ZorO can result in ATP depletion (Fig. 2.6), we wanted to determine if cells harboring multiple copies of the *zor-orz* locus had a reduced ATP levels. To test this, we grew EDL933- Δ *zor-orz* transformed with either pBR or pBR-*zor-orz* in LB until mid-log phase, split the culture, and treated half the culture as mock or

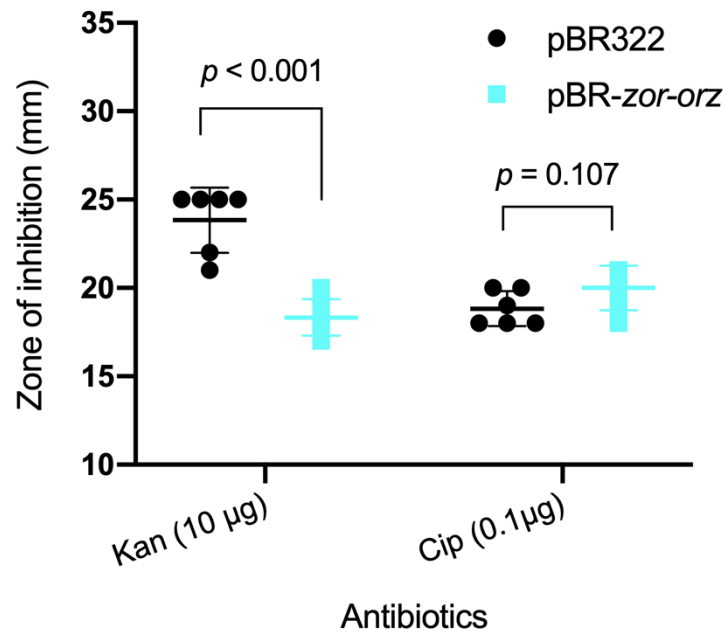
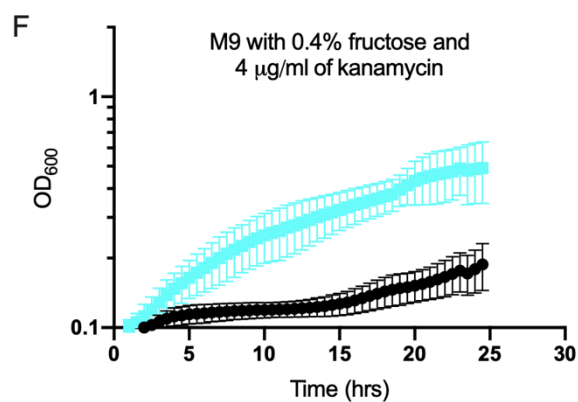
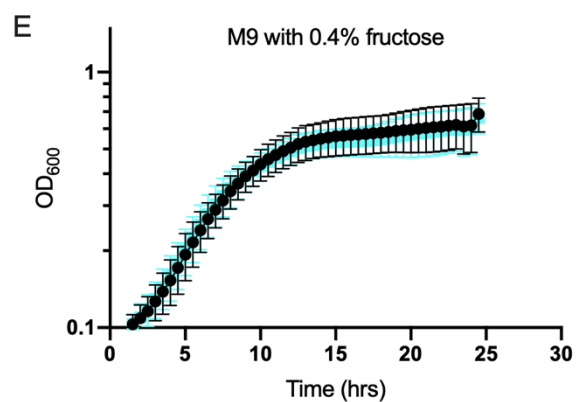
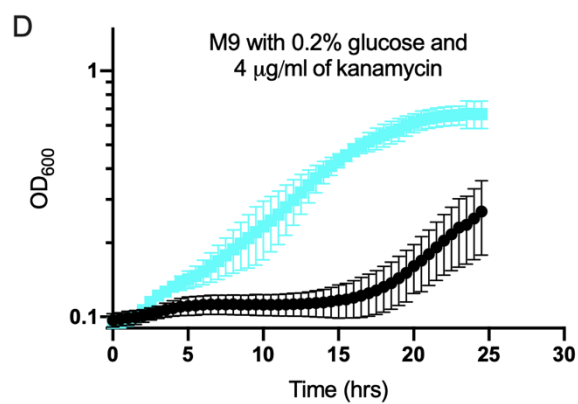
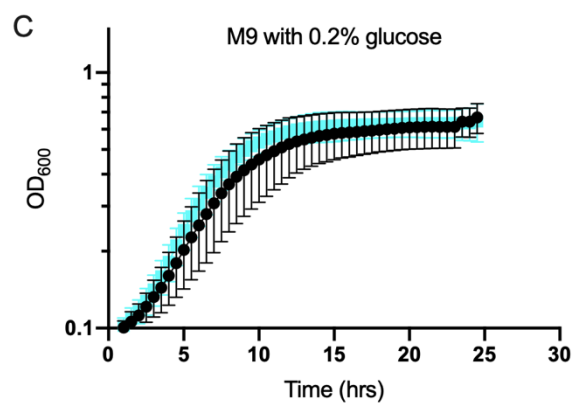
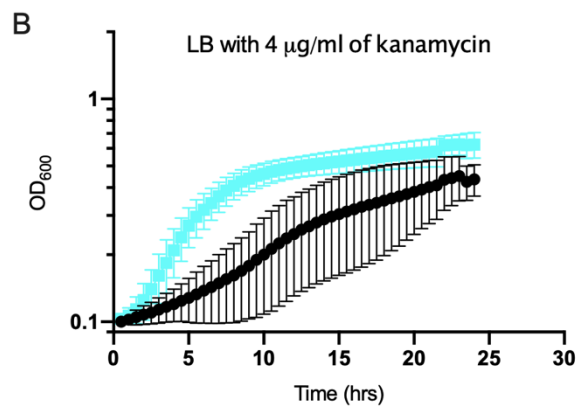
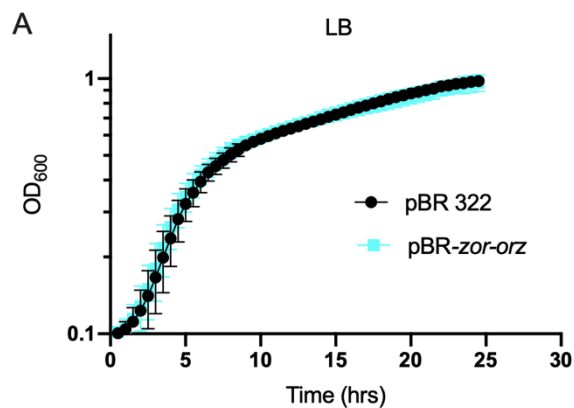


Figure 2.9. Role of *zor-orz* locus in antibiotic susceptibility. Zone of inhibition (mm) for *E. coli* UTK007 cells carrying indicated plasmid vectors for kanamycin (Kan) and ciprofloxacin (Cip) antibiotics. $n = 6$, shown are mean $\pm$ SD. The p values were calculated using a multiple unpaired t test with Welch correction.

Table 2.3. Median minimum inhibitory concentration for *E. coli* cells harboring pBR322 or pBR-*zor-orz* plasmids grown in Mueller Hinton ($n \geq 3$).

Antibiotic ($\mu\text{g/ml}$)	<i>E. coli</i> UTK007 strain transformed with					
	pBR322			pBR- <i>zor-orz</i>		
	Median	Upper limit	Lower limit	Median	Upper limit	Lower limit
Kanamycin	1.6	1.6	0.8	6.4	6.4	3.2
Gentamicin	0.4	0.4	0.4	0.8	1.6	0.8
Chloramphenicol	6.4	6.4	6.4	6.4	6.4	6.4
Ciprofloxacin	0.02	0.02	0.02	0.02	0.02	0.02

Figure 2.10. Multiple copies of the *zor-orz* locus allows for growth in the presence of kanamycin in MG1655. Growth curve of *E. coli* UTK007 harboring pBR322 (black) or pBR-*zor-orz* (turquoise) in LB (A), LB with 4 μ g/mL kanamycin (B), M9 minimal media with 0.2% glucose (C), M9 minimal media with 0.2% glucose and 4 μ g/mL kanamycin (D), M9 minimal media with 0.4% fructose (E), and M9 minimal media with 0.4% fructose and 4 μ g/mL kanamycin (F). $n \geq 9$; shown are mean $\pm$ SD. All growth conditions contained 100 μ g/mL ampicillin for plasmid maintenance.



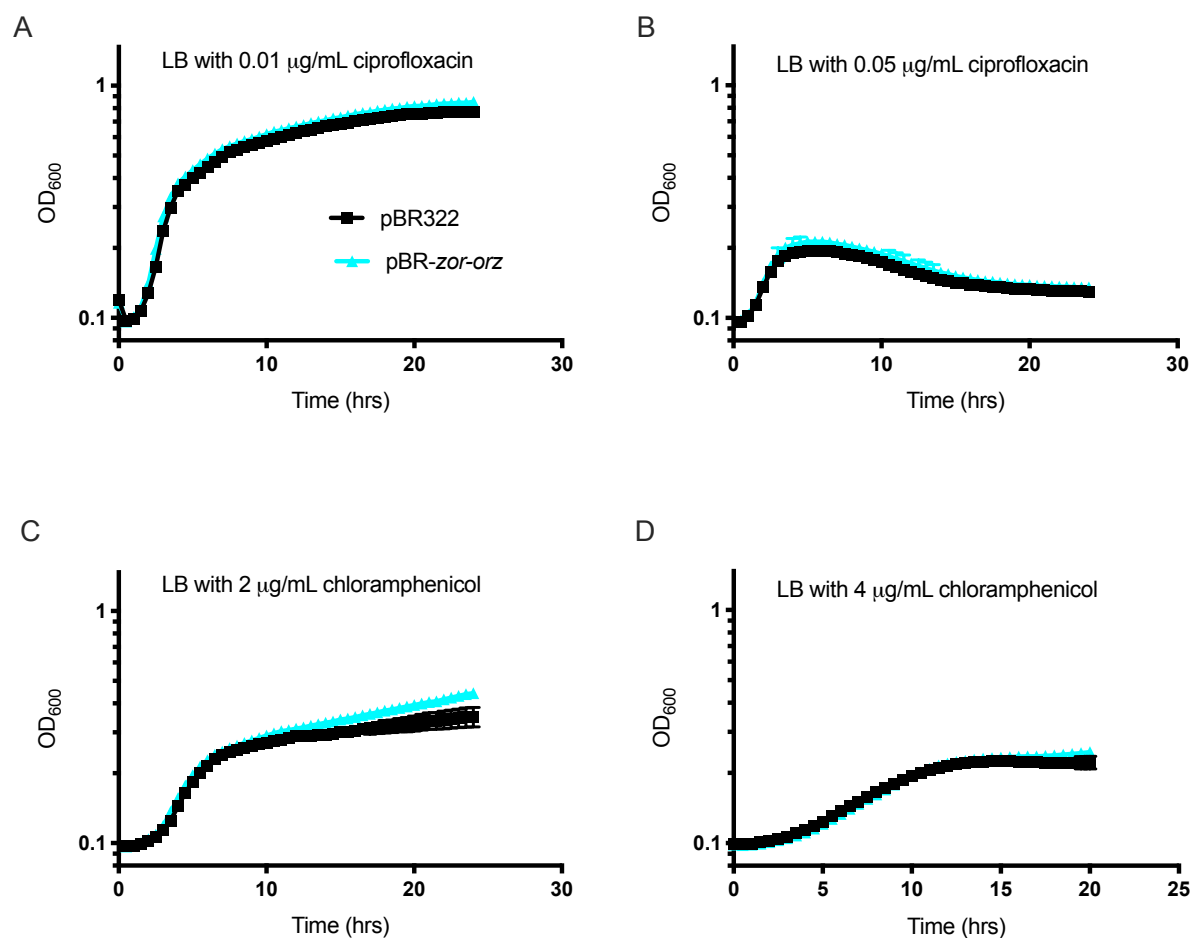
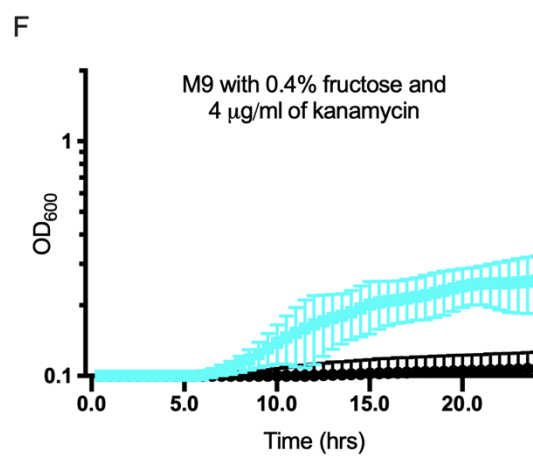
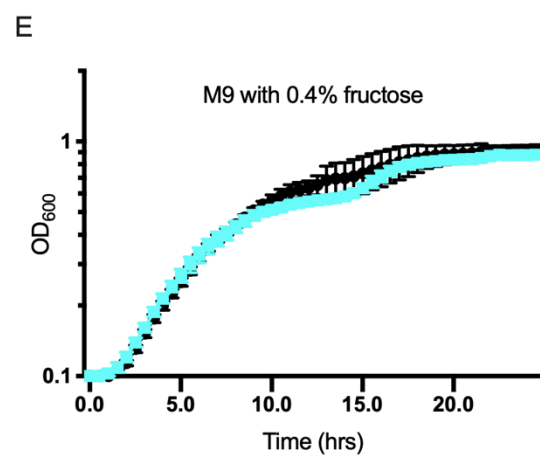
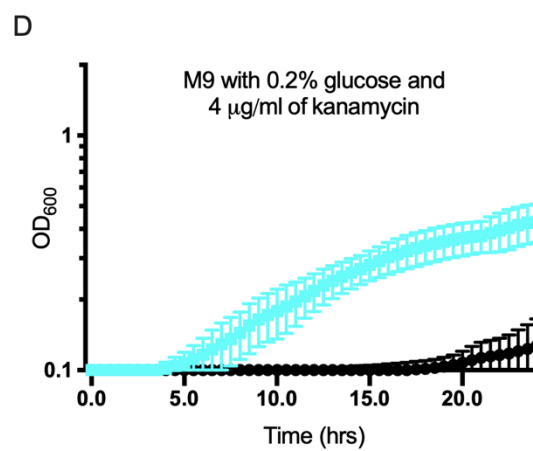
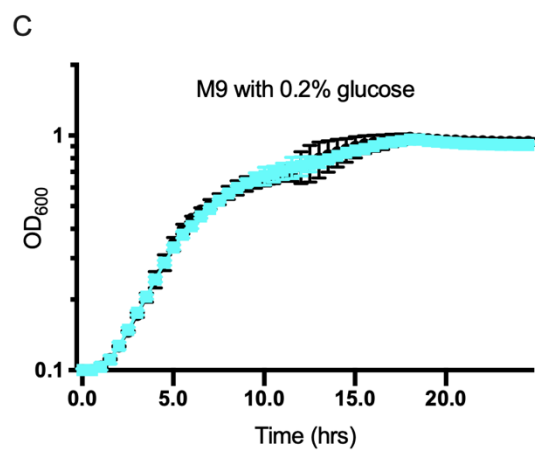
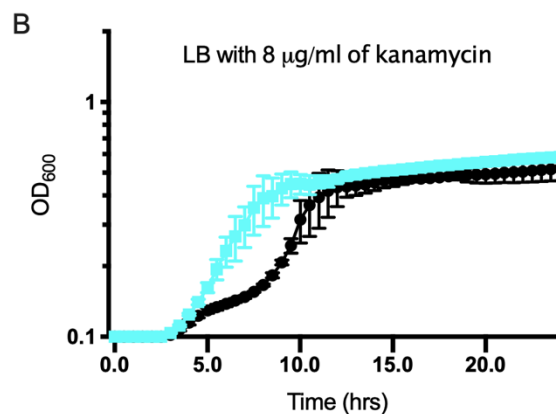
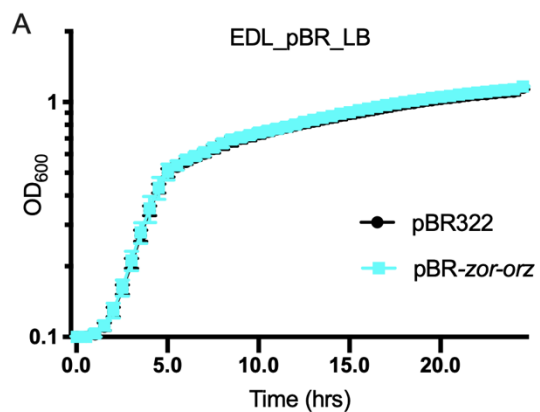


Figure 2.11. The *zor-orz* locus does not improve growth against ciprofloxacin or chloramphenicol. Growth curve of *E. coli* MG1655 derived cells (UTK007) harboring the indicated plasmids in LB medium with ciprofloxacin (A, B) and chloramphenicol (C, D) antibiotics. $n=3$, shown are mean $\pm$ SD.

Figure 2.12. Multiple copies of the *zor-orz* locus allows for growth in the presence of kanamycin in EDL933. Growth curve of EDL- Δ *zor-orz* harboring pBR322 (black) or pBR-*zor-orz* (turquoise) in LB (A), LB with 8 μ g/mL kanamycin (B), in M9 minimal media with 0.2% glucose (C), M9 minimal media with 0.2% glucose and 4 μ g/mL kanamycin (D) or M9 minimal media with 0.4% fructose (E), and M9 minimal media with 0.4% fructose and 4 μ g/mL kanamycin (F). All growth conditions contained 100 μ g/mL ampicillin for plasmid maintenance. $n \geq 3$, shown are mean $\pm$ SD.



added 4 µg/ml of kanamycin to the other half to better monitor the immediate cellular response to kanamycin. This also eliminated differences in the growth patterns observed above (Fig. 2.12). For our mock-treated cultures, the relative ATP levels were similar regardless if the strain harbored either the empty vector (pBR) or pBR-*zor-orz* during mid-log growth phase. For those cells treated with kanamycin, we observed no differences in relative ATP levels after 1 hour (hr) of treatment, but saw a relatively lower ATP levels in cells harboring pBR-*zor-orz* following 4 hrs of kanamycin treatment ($p = 0.013$, Fig. 2.13B). We also analyzed the effect on membrane depolarization upon treatment with kanamycin. There was no statistically significant difference in the percentage of cells with a depolarized membrane, but those cells harboring pBR-*zor-orz* did trend lower after 4 hrs of kanamycin treatment (Fig. 2.13C). We observed improved growth, however, of cells harboring pBR-*zor-orz* compared to those harboring the empty vector only after approximately 5-6 hrs after kanamycin addition (Fig. 2.13D).

To further test if this improved growth of *E. coli* cells in the presence of aminoglycoside antibiotics is maintained at a single copy level, *i.e.*, chromosomal level, we generated a derivative of *E. coli* MG1655 (which naturally lacks the *zor-orz* locus) in which we integrated into the genome a single-copy of *zorO* under control of its processed UTR ($\Delta 28$) and native promoter (this strain lacks the OrzO antitoxin). Unlike the effects observed with multiple copies of the *zor-orz* locus, the single copy of *zorO* integrated in the *E. coli* chromosome was not able to improve growth in the presence of kanamycin or gentamycin (Fig. 7B and 7C). We also constructed a similar EDL933 strain (EDL933- $\Delta 28$ -*zorO*) and compared its growth with the WT and the EDL933- Δ *zor-orz*. The growth patterns of the EDL933 strains were similar in LB (Fig. 7D) and M9 minimal media supplemented with glucose or fructose (data not shown). Like the above observations of our derived MG1655 *E. coli* strains, there was no significant differences in the growth pattern of these three EDL933 strains in the presence of kanamycin (Fig. 7E), gentamicin (Fig. 7F), chloramphenicol, or ciprofloxacin (data not shown).

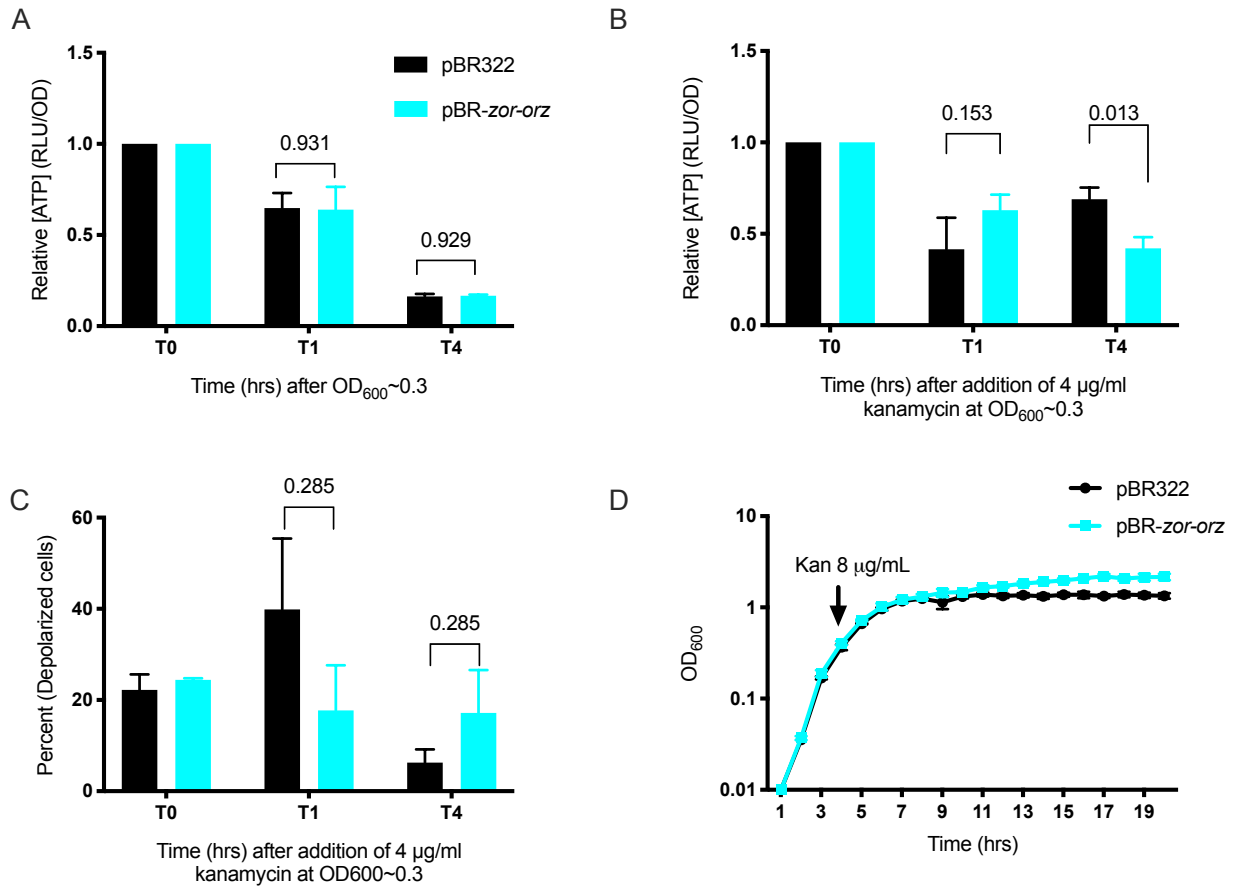
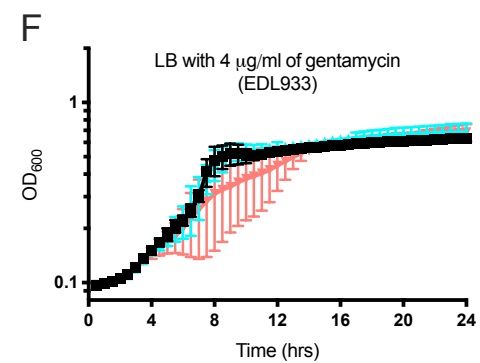
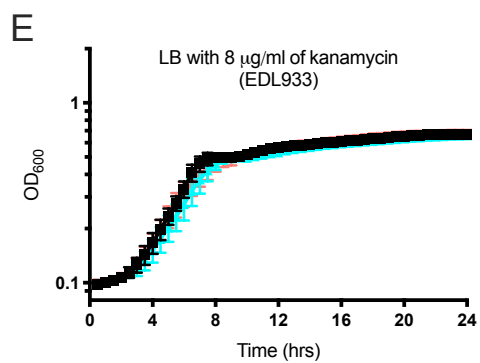
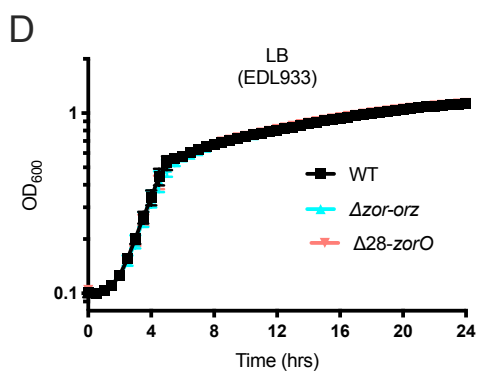
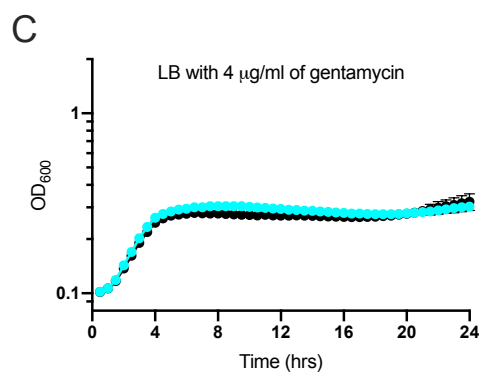
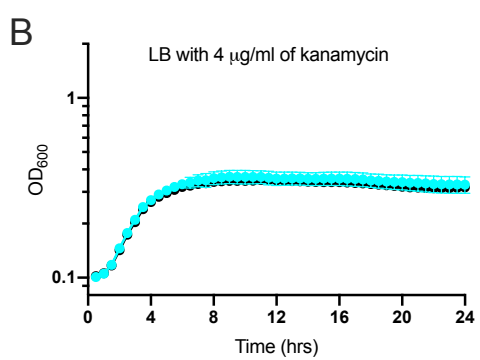
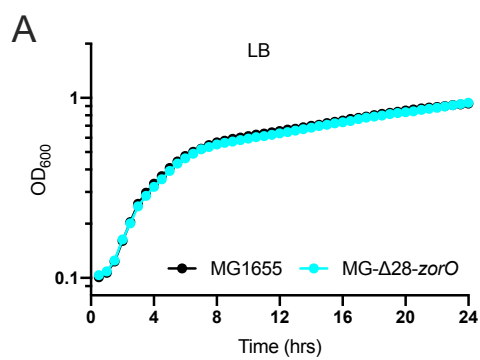


Figure 2.13. Impact of kanamycin spike during mid-log growth phase on ATP, membrane depolarization and growth. Relative ATP levels during growth of EDL933- Δ zor-orz with pBR or pBR-zor-orz plasmid in LB medium (A) and in LB after addition of 4 µg/ml kanamycin spike at mid-log phase (B). Membrane depolarization of the cells in LB after addition of 4 µg/ml kanamycin spike during mid-log phase. Growth curve after 8 µg/ml kanamycin spike during mid-log phase in LB medium (D). For all panels $n=3$, shown are mean $\pm$ SD. The p values were calculated using a multiple unpaired t test with Welch correction.

Figure 2.14. A single chromosomal copy of processed 5'UTR-zorO does not improve growth in the presence of aminoglycosides. Growth curve of MG1655 strains carrying *zorO* with a processed 5' UTR (turquoise) compared to the wild type MG1655 in LB (A), LB with 4 $\mu\text{g/mL}$ kanamycin (B) and LB with 4 $\mu\text{g/mL}$ gentamicin (C). Growth curve of WT EDL933 (black) compared to that of the EDL933- $\Delta\text{zor-orz}$ (turquoise) and EDL933- $\Delta\text{28-zorO}$ (salmon) in LB (D), LB with 8 $\mu\text{g/mL}$ kanamycin (E) and LB with 4 $\mu\text{g/mL}$ gentamycin (F). For all panels $n=3$; shown are mean $\pm$ SD.



IV. Discussion

In this study, we demonstrated that the physiological consequences of ZorO production in *E. coli* are directly regulated by the length of its 5' UTR. Previously, we characterized two forms of the *zorO* 5' UTR: a full-length (174 nt) that is prominent and a $\Delta 28$ form, that is the result of processing of the 5' end by an unknown ribonuclease (Fig. S1B and S1C) (22). Herein, we demonstrated that overproduction of $\Delta 28$ -*zorO* led to greater cell death, faster and more severe membrane depolarization, and ATP depletion. However, analyses of dye and ATP leakage, together with a lack of morphological changes, support that ZorO does not form large membrane pores, but instead, likely causes cellular death by forming small, possibly selective pores, resulting in ion imbalance preventing ATP production. An increase in the copy number of the entire *zor-orz* locus allowed for improved growth and increased MIC in the presence of aminoglycoside antibiotics without the complicating effects of the natural toxicity of ZorO overproduction.

Role of 5'UTR in subsequent effects of ZorO toxin protein. Unlike other TA systems, in which the two genes are transcribed from the same promoter, to date all identified type I TA genes are transcribed from their own promoters (20, 51) making it more challenging to balance the TA ratio. However, for those type I toxins that have been studied in depth, most possess extensive 5' or 3' UTRs that often play a role in translational regulation. The 3' processing of the *hokB* mRNA results in major structural rearrangements in the 5'-end, resulting in translational activation or inactivation via sRNA binding (52). Similarly, the *aapA1* toxin mRNA in *Helicobacter pylori* has also been shown to undergo 3' end processing via the 3'-5' endonuclease activity of the polynucleotide phosphorylase (53).

The control of ZorO translation *via* processing is most similar to *tisB*. In this case, the full-length *tisB* mRNA was not translated when using an *in vitro* system, but the processed form, referred to as $\Delta 41$ *tisB*, was translated readily and overproduction of this variant was far more toxic (21, 23, 54). However, unlike *tisB*, the *zorO* full-length

mRNA could be translated using an *in vitro* system, though not nearly as robustly as $\Delta 28$ (22). Whether or not the differences in the ability to translate the two toxins from their respective full-length 5' UTRs *in vitro* mirrors what occurs in an *E. coli* cell is not clear. The ribosome binding site (RBS) of both *tisB* and *zorO* are occluded in stem structures, preventing ribosomes from interacting. For *tisB*, processing allows for the interaction of ribosomal protein S1 to the standby site (upstream of the true RBS) of the mRNA; this is thought to stimulate structural unwinding, opening of the sequestered RBS, and eventually, binding of the ribosome to the RBS (54). While this mechanism has not been demonstrated for *zorO*, the similarities in structural requirements for translation suggest that the stand-by site model is likely true (22). Interestingly, *dinQ* and *shoB* also undergo a 5'-end processing, and which might be regulated similarly to prevent toxin translation (43, 55).

The combination of our current work and past findings (22) support the significance of the UTR for proper regulation of *zorO* given the potential detrimental effects of excess ZorO on cells. Further, previous and current work suggests that only a portion of the total mRNA population is subject to processing (Fig. S1) (22) and this likely leads to uneven production of ZorO across the population. Our physiological data support this as well: there is a clear delay in membrane depolarization and ATP depletion for cells overproducing ZorO under the full-length 5' UTR versus the processed form ($\Delta 28$, Fig. 2.6). This may help in “bet hedging” by having only a portion of the population reduced in growth from ZorO overproduction while positioning the population as a whole to deal with a sudden stressor that cells with higher metabolic activity would be more susceptible to. It may also be that cells accumulate full-length *zorO* mRNA that is not processed but then a processing event (note that the enzyme responsible for such processing is unknown) can be triggered by environmental stressors. In this manner as well, each cell may “bet-hedge” by accumulating the toxin mRNA to be poised for changing conditions.

ZorO: a membrane targeting toxin. The hydrophobic nature of the ZorO protein suggested it could be a membrane protein which was later confirmed to localize to the

inner membrane (Fig. 2.3B) (19). Reconstitution experiments showed that ZorO does not damage the integrity of the membrane bilayer (Fig. 2.7B). These results agree with the SEM data which did not show membrane damage or large morphological disruptions upon ZorO production (Fig. 2.8). Additional experiments in lipid vesicles showed that ZorO adopts a helical conformation. The magnitude of the helical signal is comparable to other peptides of similar size that adopt a TM topology (36, 56, 57). Finally, OCD experiments established that ZorO inserts into a TM configuration with a helical orientation close to perpendicular to the membrane plane (Fig. 2.4). This result agrees with the lack of gross membrane disruption and if ZorO indeed forms an ion channel, our data would support a closely tightened TM bundle.

Although many type I toxins are predicted to target and damage the membrane, only a handful of them have been experimentally verified (16–18, 58, 59). Apart from the membrane, some type I toxins target nucleic acid (60, 61) or cell envelope biosynthesis (62). It is currently unclear why some hydrophobic toxins appear to readily target the membrane while others impact different cellular processes. When expressed at endogenous levels, these toxins may interact with specific cellular proteins. This, however, has not been examined to the best of our knowledge.

Effects of toxin protein on bacterial morphology. The AapA1 toxin in *Helicobacter pylori* triggers a massive and rapid morphological transformation of this spiral-shaped bacterium into round coccoid cells (59). Additionally, HokB has been implicated in ghost cell formation, and Fst results in filamentous cells with multiple invaginations affecting cell division with no obvious pores or loss of intracellular material (60, 63). ZorO overproduction decreases membrane potential and cellular ATP levels, however, it does not cause immediate cell lysis based on microscopy (Fig. 2.8) and absence of cell debris (laboratory observation). A marked reduction in the colony forming units though was seen as soon as 15 minutes after induction (22). These differences in cell morphology caused by the type I toxin proteins may be due to differing overproduction levels. However, it likely also implies differences in the nature of the toxin properties, *i.e.*, the direct target of the toxin. For example, Fst overproduction leads to near

immediate condensation of DNA (64). This would greatly impact DNA replication and cellular division, which would be reflective in the observed morphology (65). If ZorO does self-oligomerize in the membrane, the resultant channel formed would be narrow (see above), likely allowing only ion permeation, and would not leak organic molecules or cause morphological alterations. This in turn would explain both the lack of gross morphological changes and visible cellular debris upon ZorO overproduction.

The zor-orz locus: a type I TA system with specificity against aminoglycoside antibiotics.

A role of the type I toxin proteins TisB and HokB has been established in bacterial persistence as a result of membrane depolarization and ATP reduction (1, 17, 23, 49). Persister cells are a subpopulation within a bacterial culture that can survive a high dose of antibiotics but are not genetically resistant (*i.e.* they cannot grow in the presence of the tested antibiotic). Increases in the persister population when cells were treated with ofloxacin (targets DNA gyrase) and tobramycin (aminoglycoside, targets ribosome) have been observed with HokB (1). TisB production can also increase the number of persister cells when treated with ciprofloxacin (targets DNA gyrase), ampicillin (targets cell wall), and streptomycin (protein synthesis inhibitor) (1, 23, 49). This suggests a broader range of protection from their production. We tested for the level of persister cell in EDL933 Δ *zor-orz* locus against ciprofloxacin and ampicillin and did not see significant difference compared to the parental strain (data not shown). However, as shown in this study, increasing the copy number of the *zor-orz* locus (while avoiding its toxicity) improved growth in presence of aminoglycosides but not other antibiotics examined. We also observed an increase in MIC against kanamycin and gentamycin (Table 2.3). Overproduction of some *de novo* synthesized small membrane proteins has been shown to increase MIC against aminoglycosides (66); however, to our knowledge this is the first report of a type I TA system conferring an increase in MIC.

Why is there such specificity for aminoglycosides? A model for ZorO function, as noted earlier, is the possible formation of small oligomers (either dimers or multiple dimers) that lead to ion flux. Similar to that of TisB (67), ZorO is hypothesized to form an

antiparallel dimer within the membrane. The “non-specific” metabolic effect of ZorO does not broadly protect from antibiotic classes other than aminoglycosides while both TisB and ZorO lead to membrane depolarization and ATP depletion (Fig. 2.6) (16, 19). Aminoglycoside uptake occurs in three stages where the latter two are dependent on membrane potential and ATP (68). Therefore, membrane depolarization and decreased ATP levels can reduce the uptake of aminoglycosides. However, our data demonstrated no discernible differences in relative ATP levels for cells harboring empty plasmid versus pBR-*zor-orz* when grown in LB (Fig. 2.13A). Even upon treatment with kanamycin, there were no differences in ATP levels within the first hour of treatment (Fig. 2.13B). This suggests that *zor-orz* has an alternative mechanism in allowing growth in kanamycin. Similarly, additional studies have shown that while small alpha helical peptides that reduce membrane potential can be protective against aminoglycosides, not just any transmembrane helix can, including other type I toxin proteins (66). These data indicate that not all type I toxins behave similarly and that there is likely specificity in their targets and effects on cellular physiology.

The chromosomal copy of *zorO* without its two known translational repressive elements *i.e.* full-length UTR and the OrzO sRNA, however, did not show a difference in growth in LB, LB with kanamycin, or LB with gentamicin as compared to the wild type MG1655 *E. coli* strain in the presence of kanamycin and gentamicin (Fig. 7A, 7B, and 7C). This result was consistent with the EDL933 strains tested (Fig. 7D, 7E and 7F). A similar strain of *E. coli* in which the two translational repressive elements of the toxin *tisB* were removed did result in an increase in the number of persistent cells that survived lethal doses of ampicillin and ciprofloxacin respectively (23). So, why is there a lack of phenotype for ZorO production from the chromosome in absence of the regulation by antitoxin OrzO and 5'UTR? It is possible there may be other yet to be identified regulatory elements that prevents ZorO induced effects and function at chromosomal level under the conditions used in this study. Further, it is possible that under endogenous production, ZorO (and other type I toxins) may interact with specific protein partner(s) to modulate activity and a lack of observable phenotype may be

because levels of ZorO are not high enough under the conditions examined to observe differences across the population of cells.

Is ZorO really a toxin? While ectopic overproduction of ZorO results in a quick cell growth stasis, multiple copies of the *zor-orz* locus with its native regulatory elements do not impact growth of cells in LB or minimal media supplemented with glucose or fructose (Fig. 2.10). Similarly, a single copy of *zorO* (with a processed 5'UTR) on the chromosome of either MG1655 or EDL933 also showed no impact on growth in LB (Fig. 7) or minimal media (data not shown). Reports for other type I toxins TisB (23) and TimP (12) also demonstrated a lack of growth effects in enriched medium such as LB when expressed from the chromosome. Questions thus remain as to when these chromosomally encoded toxins are active and if they truly can induce cell stasis or death when expressed at the endogenous level.

Overall, our data indicate that the 5'UTR of *zorO* plays a vital role in regulating the translation and subsequent cellular impact of the small toxin protein ZorO. Overexpression from the processed 5'UTR significantly impacts membrane depolarization and ATP depletion in the cells without causing a major change in bacterial morphology. Cells harboring multiple copies of the *zor-orz* locus, under control of its native regulatory elements, are not broadly protected against stress but do have increased resistance to aminoglycosides. These results, combined with data from other type I toxins, suggest that type I toxin proteins have unique effects on bacterial cells.

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CHAPTER III

Global impacts of *zor-orz* locus in a medium copy plasmid in response to kanamycin in EHEC

Publication note

This chapter is currently not under review, however, it will be submitted in the future.

Bikash Bogati prepared cells and performed RNA extraction. Sequencing was performed at University of Tennessee Genomics Core facility. Data analysis was done by Bikash Bogati with help from Will Brewer and Sanjeev Dahal. Bikash Bogati and Elizabeth M. Fozo wrote the manuscript

Abstract

Toxin-antitoxin systems are gene pairs in which the transcript, translated protein, or the effect of the translated protein from the toxin gene is neutralized by the product of its cognate antitoxin gene. Of the six types of toxin-antitoxin systems, type I systems encode a small RNA antitoxin that forms complementary base pairs with the toxin encoding mRNA and prevents the translation of the toxin and/or stimulates degradation of the toxin mRNA. The role of chromosomally encoded type I toxin-antitoxin systems has been debated, especially as most studies rely on overexpression of the toxin. The *zor-orz* locus identified in *Escherichia coli* O157:H7 EDL933 is a type I toxin-antitoxin system that consists of tandem duplicated gene pairs- *zorO-orzO* and *zorP-orzP*. Deletion of the *zor-orz* locus in EDL933 does not result in a discernable phenotype; however, amplification of this locus by cloning it onto a medium-copy plasmid (pBR-*zor-orz*) significantly improved cell growth in presence of aminoglycoside antibiotics. No such benefit was observed with other classes of antibiotics tested. This raises the question as to why there is specificity of *zor-orz* locus in resistance against aminoglycoside antibiotics and what is the mechanism behind this improved growth. Herein, we investigated the differences in the global transcriptome of EHEC harboring pBR-*zor-orz* versus empty plasmid after a 15 minute treatment with kanamycin. Multiple copies of the *zor-orz* locus resulted in a reduction in transcripts for flagellar associated genes, whereas genes associated with anaerobic respiration, nitrate reduction, oxidative stress, and sugar transporters were upregulated. These results are useful to further dissect the mechanism of the resistance conferred by the *zor-orz* locus against aminoglycoside antibiotics.

I. Introduction

A toxin-antitoxin (TA) system is a gene pair in which one gene encodes a potentially toxic protein and the other encodes a small RNA (sRNA) or a protein that neutralizes the toxin encoding mRNA, the translated toxin protein or the effects of the toxin (1,2). These gene pairs are distributed throughout plasmids, phages, and bacterial and archaeal genomes (3). Based on the nature of the antitoxin *i.e.*, either a sRNA or a protein, and the mechanism it utilizes to counteract the toxin, TA systems can be classified into six major types (type I – type VI; (4)). In a type I system, the antitoxin sRNA forms complementary base pairs with the toxin encoding mRNA and prevents the translation of the toxin protein (5,6).

Several type I TA systems have been identified in the chromosomes of both Gram-positive and Gram-negative bacteria and are hypothesized to function during specific stress responses (7–9). The deletion of the toxin gene, simultaneous deletion of the toxin and antitoxin genes, or deletion of the antitoxin gene does not always produce a measurable phenotype in laboratory conditions, making their study challenging (10–12). Most of the TA system studies, therefore, utilize an ectopic overexpression system in which the toxin gene is under control of an inducible promoter. However, at the chromosomal level, bacterial cells rely on native regulatory elements, both transcriptional and posttranscriptional, to ensure expression of the toxin gene under the specific, desired condition. Therefore, overexpression systems using inducible promoters on plasmids may not always correspond to the true expression, and hence, function of a TA system in a bacterial cell.

A few studies have addressed the global impacts of type I toxins on cellular processes. The Ldr (long direct repeat) toxin has four homologs (A-D) in the chromosome of *Escherichia coli* MG1655 (13). The overproduction of LdrD affects cell growth, results in loss of cell viability and causes nucleoid condensation (13). Within two minutes of *ldrD* overexpression, microarray analysis revealed an upregulation of the genes involved in purine metabolism (13). A separate comparative study was performed following overexpression of *tisB*, *shoB*, *ibsC* and *ldrD* for 20 minutes in *E. coli* (14). This analysis identified a common set of genes that were upregulated upon overexpression

of all toxins: these included several stress response genes, including *soxS*, which is involved in oxidative stress, and several membrane protein-encoding genes such as those involved in maltose transport. The study also revealed that both TisB and IbsC induced the cell envelope stress response genes *degP* and *spy*, whereas the phage shock genes (*pspABCDE* operon and *pspG*) were significantly induced only by IbsC overexpression (14). The upregulation of the ribose transport and mannose permease genes was unique to *shoB* overexpression. A subtoxic overexpression (that did not affect cellular growth) of the type I toxin *hokB* in *Erwinia amylovora* induced genes associated with ATP synthase, phage shock protein A (*pspA*) and other stress response associated genes (15).

While overexpression analyses have been critical for understanding the toxicity of type I toxins, studies examining their impacts when produced under their normal, chromosomal regulatory systems is critical for elucidating biological function. Since it is challenging to determine the exact conditions in which the toxin overcomes sRNA neutralization and/or is translated, mutation of the antitoxin promoter or deletion of the antitoxin gene is therefore used to study the impacts of the toxin gene. The deletion of the *timR* antitoxin gene in *Salmonella Typhimurium* allowed for detection of the translated toxin protein TimP from the chromosome, however, there was no significant differences in the growth pattern of this strain (12). Upon further examination, although small, a significant increase in activation of the cell envelope stress gene *cpxP* was observed using a P_{cpxP} -*gfp* transcriptional fusion (12). After analyzing the global effects of IbsC overexpression, Fozo et al. confirmed the induction of *pspABCDE* transcripts in an *E. coli* strain in which the promoter region of antitoxin (SibC) was deleted, resulting in increased expression of its cognate toxin *ibsC* (14). Thus, in some cases, bacteria may respond similarly to “mild” overexpression of a toxin gene from the chromosome without observed effects on growth. The homolog of *txpA/ratA* type I toxin, *sprG1/sprF1* has been studied for the differences in the proteome after deletion of this TA system (Δ *sprG1/sprF1*) and after complementation solely with the antitoxin gene. The Δ *sprG1/sprF1* strain had one protein downregulated (Cdr) and one upregulated (Sbi) compared to the wild type *S. aureus* N315 during log phase growth (16). Six

differentially expressed proteins were identified during stationary growth phase (16). In the $\Delta sprG1/sprF1$ deletion strain, a decrease in proteins in extracellular space was observed as compared to the wild type strain with intact *sprG1/sprF1* (16).

The *zor-orz* locus is a type I TA system identified in the chromosome of Enterohemorrhagic *E. coli* O157:H7 EDL933 strain (EDL933 herein) that consists of two homologous gene pairs, *zorO-orzO* and *zorP-orzP* (17). While deletion of these genes in EDL933 does not produce a significant phenotype (in tested laboratory conditions), a plasmid containing the entire *zor-orz* locus was able to significantly improve the growth of *E. coli* in the presence of several aminoglycoside antibiotics (18). This was the first known report of a type I TA system increasing minimum inhibitory concentration against specific antibiotics. To further determine what cellular processes are affected during this resistance to kanamycin by the multiple copies of the *zor-orz* locus, we compared the transcriptomes of the EDL933- $\Delta zor-orz$ cells harboring either pBR322 (vector control) or pBR-*zor-orz* following kanamycin treatment during exponential growth phase. We observed that in cells with pBR-*zor-orz*, multiple genes associated with flagellar motility were down-regulated whereas the biofilm formation regulator (*bssR*) was up-regulated. Significant up-regulation of the genes associated with fumarate respiration and nitrogen metabolism was observed. Overall, these results suggest that the bacterial cells harboring pBR-*zor-orz* are limiting their energy consuming processes such as motility and utilizing alternative paths of respiration.

II. Materials and Methods

Strains, plasmids, and culture conditions

The strains and plasmids used in this study were constructed previously (18). *E. coli* O157:H7 EDL933 deleted for the *zor-orz* locus (EDL933- $\Delta zor-orz$) was transformed with either pBR322 or pBR-*zor-orz*. Ampicillin (100 μ g/ml) was used in all conditions for plasmid maintenance. Overnight cultures were diluted to an OD₆₀₀ of 0.01 in LB and grown at 37°C with shaking (250 rpm). At mid-log growth phase (OD₆₀₀ ~0.3), the cells were exposed to a final concentration of 10 μ g/ml kanamycin. After 15 minutes of

kanamycin treatment, 15 ml aliquots were transferred to 50 ml conical tubes with ice blocks and centrifuged at 3500xg for 10 minutes at 4°C. Cell pellets were stored at -80°C.

RNA Isolation and sequencing

RNA isolation was performed as previously described (17). Briefly, cell pellets were resuspended in 500 µl of solution A (10 mM EDTA, 20 mM sodium acetate, 0.5% SDS, pH 5.5) and then transferred to a 1.5 ml microfuge tube containing 500 µl of acid phenol:chloroform pre-warmed to 65 °C. The mixture was gently vortexed and incubated at 65 °C for 10 minutes, centrifuged at 13000xg for 10 minutes. The supernatant was transferred to a tube containing 500 µl acid phenol:chloroform, pre-heated to 65°C followed by centrifugation. The supernatant was then transferred to a clean microfuge tube and extracted twice with an equal volume phenol:chloroform. This was followed by a chloroform extraction and RNA was precipitated via ethanol precipitation. Pellets were allowed to air dry and resuspended in RNase-free water. The integrity of the extracted RNA was examined via agarose gel electrophoresis and by determining Absorbance 260/280 ratio. Samples were treated with Turbo DNase (Ambion) following manufacturer's instructions. The final concentration and RNA quality was analyzed in a 2100 Bioanalyzer (Agilent). RNA samples were sent to the UT Genomics Core where ribosomal RNA was depleted using Ribominus Transcriptome Isolation Kit for Yeast and Bacteria (Invitrogen). The depleted product was then processed with the TruSeq stranded mRNA sample kit (Illumina) at the fragment, prime, and finish step. The final product was again checked for quality and quantity on the Bioanalyzer, and then loaded at 4 pM with 2% PhiX spike on a v3, 150 flow cell, reading 75 bases paired-end on an Illumina MiSeq at the UT Genomics Core.

Bioinformatic analyses

A general workflow of the bioinformatic analysis is shown in the schematic figure (Fig. 3.1). FastQC was used to check the quality of the raw sequence reads. Sequences shorter than 55 bp were eliminated. Based on the quality score and the per base

sequence content (distribution of A,C,G,T in the sequence reads on each bases), we trimmed 5 bp from the beginning and 3 bp from the end of each read. The trimmed reads were then run through a final quality check with FastQC.

The reads were then aligned to the reference genome *E. coli* O157:H7 str. EDL933 (assembly [GCA_000732965.1](#)) using Spliced Transcripts Alignment to a Reference (STAR). This aligning method uses strategy to account for spliced alignments and therefore, addresses many of the RNA-seq data mapping challenges. After the alignment, gene expression was assembled from the aligned reads by using HTSeq after sorting the alignment file (BAM file) to quantify the number of sequence reads mapped to each gene.

Using the number of mapped reads from assembled reads, differential expression analysis was performed using the library “DESeq2” in RStudio version 1.2.5033. Genes with low counts (<10) were filtered out as they are analogous to the genes which are not expressed. The DESeq function was used for estimating the size factors and dispersions, fitting the negative binomial generalized linear model, and performing Wald tests for differential gene analysis (19). Principle component analysis was used to observe if the replicates clustered. Differentially expressed genes that were statistically significant were used to generate the plots. Differential expression gene network analysis was done by using Cytoscape (v.3.8.2).

Network analyses of Differentially Expressed Genes

Cytoscape (v. 3.8.2) was utilized for network analysis of the differentially expressed genes (DEGs) following the protocol described (<https://cytoscape.org/cytoscape-tutorials/protocols/differentially-expressed-genes/#/ex1-down-string>). The DEGs were applied to the entire STRING (v. 1.7.0) network of *E. coli* O157:H7 str. EDL933 using default settings except *Maximum Additional Interactions* (MAI) being changed to 5 for upregulated and 10 for downregulated genes, after numerous trials using other MAI options. The largest subnetwork was then integrated with the processed gene expression data such that the nodes were filled with colors by continuous mapping using log₂fold change

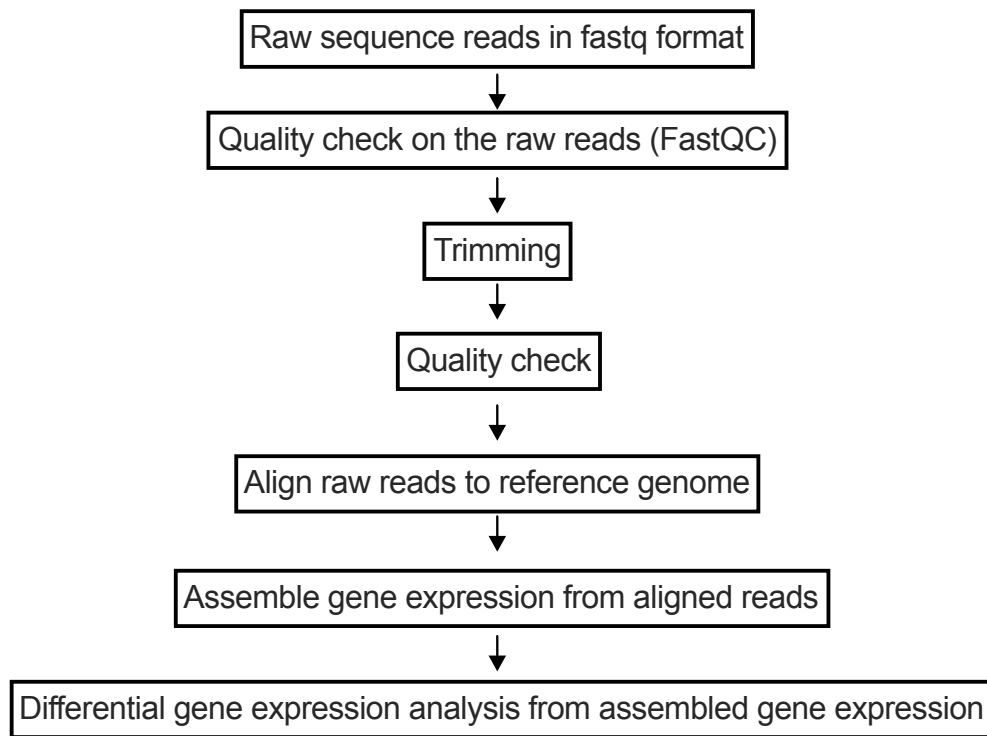


Figure 3.1. Schematic of the workflow for the RNA sequence read alignment and analysis.

information. Following this, the *Functional Annotation Enrichment* tool was used to identify the enriched pathways. Only KEGG, GO and InterPro pathways were chosen, and *Remove Redundant Pathway* option was checked for visualization purpose.

Motility assay

E. coli MG1655 (motile strain) transformed with the indicated plasmids was grown in 5 ml LB with 100 µg/ml ampicillin, 3 µl of the overnight culture was placed at the center of LB plates (0.3% agar with 100 µg/ml ampicillin) and zone of migration was measured at indicated time points.

III. Results

The deletion of *zor-orz* locus does not significantly impact growth.

Our previous findings demonstrated that increasing the copy number of the *zor-orz* locus, by cloning the entire region onto the multicopy plasmid pBR322, allowed for improved growth in media containing the aminoglycoside kanamycin (18). However, we wanted to better determine if loss of the locus would impact normal cellular growth, regardless of the presence of antibiotics. Under normal laboratory conditions, both the parental strain and Δ *zor-orz* strain grew similarly in rich media (LB; Fig. 3.2A)) or in minimal media with glucose as a sole carbon source (Fig. 3.2B). As ZorO translation is repressed not only by the antitoxin OrzO but also by its own 5' UTR, we examined growth of a strain deleted for both repressive elements (Δ 28-*zorO*). Again, we noted no significant differences in growth in either rich media (Fig. 3.2A) or in minimal media supplemented with a 0.2% glucose as a carbon source (Fig. 3.2B).

In silico analysis of the *zorO* promoter and flanking region indicates a potential cAMP receptor protein (CRP) binding site that is located upstream (67 nts) of *zorO* transcription site (data not shown). Expression of *zorO* was decreased in Δ *crp* EDL933 as compared to the wild-type strain (unpublished data). Given that CRP is critical for transitioning carbon sources and the role of nutrient shifts in increasing the number of antibiotic tolerant cells (20,21), we examined growth in M9 minimal media with 0.5mM

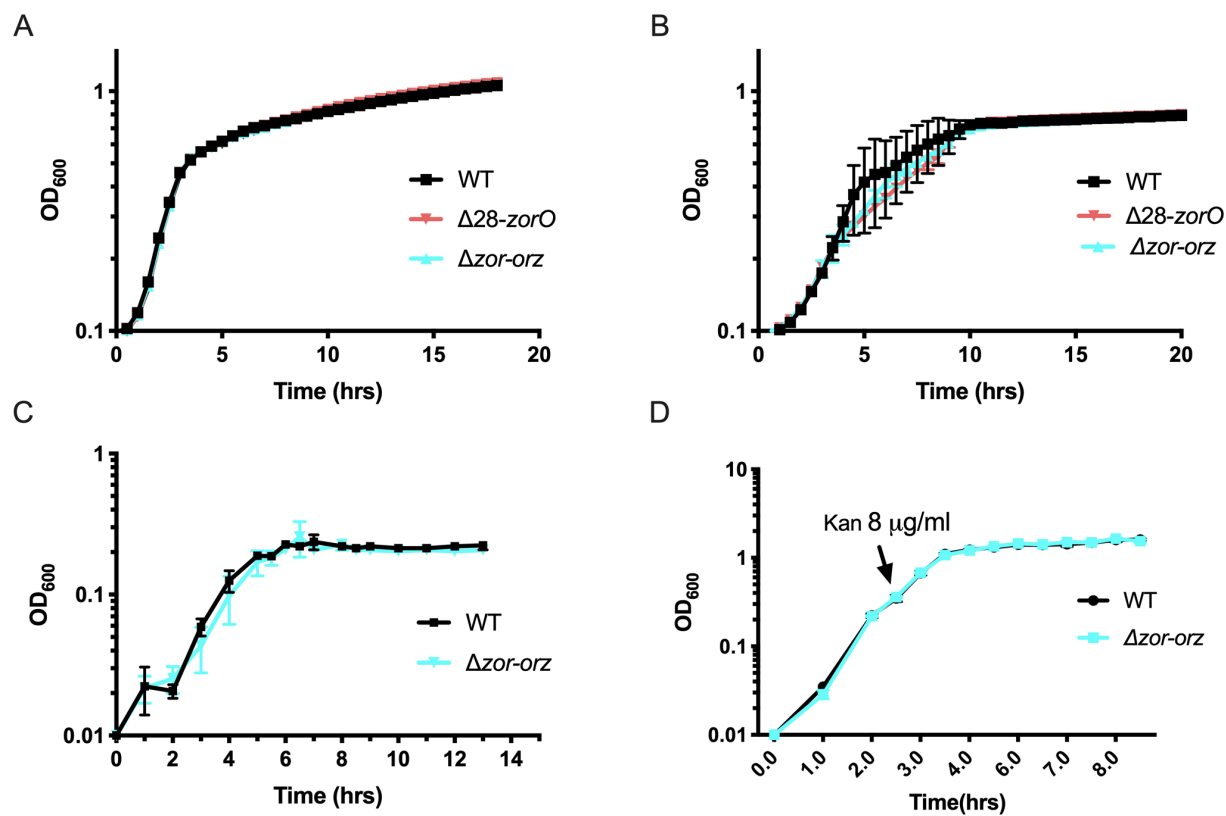


Figure 3.2. Growth curve of the EDL933 strains in LB (A), in M9 minimal media supplemented with 0.2% glucose (B), in M9 minimal media supplemented with 0.5 mM glucose and 6 mM fumarate (C), and in LB with addition of 8 µg/ml kanamycin during mid-log growth phase (D).

glucose and 6mM fumerate. During growth in this medium, glucose should be preferentially used first, and then cells should transition to fumarate as a carbon source. However, while there was a brief stasis at OD₆₀₀ ~ 0.03 signifying possible shift in carbon source utilization, there was no growth difference between the wild type and the Δ *zor-orz* strain (Fig. 3.2C).

Our previous study indicated that multiple copies of *zor-orz* allowed for better growth in the presence of kanamycin (18). Consequently, we tested whether the Δ *zor-orz* strain was more sensitive to kanamycin than the parental. However, addition of kanamycin (8 µg/ml) during exponential growth in LB medium did not show a significant difference between the growth of the parental strain and Δ *zor-orz* strain (Fig. 3.2D). These results indicate that the deletion of the *zor-orz* locus or the regulatory elements preventing ZorO translation (OrzO and 5' UTR) in EDL933 is not sufficient to impart the differences in the growth as measured by optical density.

Multiple copies of *zor-orz* locus: role in growth after kanamycin challenge

Previously, we noted that Δ *zor-orz* harboring pBR or pBR-*zor-orz* grow similarly in rich media (LB) and minimal media but cells harboring pBR-*zor-orz* had improved growth in presence of several aminoglycoside antibiotics and an increased minimum inhibitory concentration for kanamycin and gentamycin (18). When cells were challenged with kanamycin (10 µg/ml) in exponential phase, cells with pBR-*zor-orz* did reach a higher final optical density than that of empty vector (Fig. 3.3). To further elucidate the mechanism by which the *zor-orz* locus enables improved growth in the presence of kanamycin, we challenged the cells with 10 µg/ml kanamycin for 15 minutes and extracted RNA for transcriptomic analyses.

RNA sequencing output and quality control

The overall sequence reads, FastQC results and the number of mapped reads to the reference genome is listed in Table 3.1. The mean total sequence output for all the samples was 3,376,865 reads (SE ± 273,118). The total sequence reads for cells

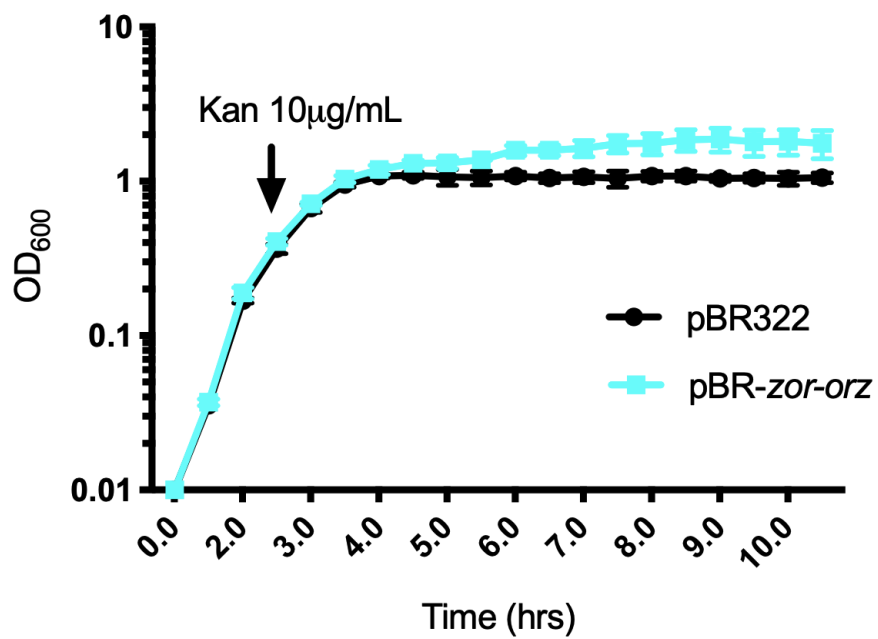


Figure 3.3. Growth curve of EDL933- Δ *zor-orz* strain carrying indicated plasmids in LB medium with addition of 10 μ g/ml kanamycin during mid-log growth phase.

Table 3.1. Total sequencing reads, read quality, and mapping of the RNA sequencing reads to the reference genome

	EDL933-Δzor-orz pBR			Mean	$\pm$ SE
	A	B	C		
Total reads pre-trim	2,908,227	2,923,686	2,647,079	2,826,330	89,736
Total reads post-trim	2,576,086	2,565,634	2,337,867	2,493,195	77,722
Percent GC	53	53	53		
Sequence length	55-75	55-75	55-75		
Quality score	33-37	33-37	33-37		
Total mapped reads	2,252,913	2,231,338	2,043,072		
Unique mapped	499,394	484,048	461,459		
Percent unique mapped	22.17	21.69	22.59		
Percent multiple loci mapped	76.52	76.96	76.2		
	EDL933-Δzor-orz pBR-zor-orz			Mean	$\pm$ SE
	A	B	C		
Total reads pre-trim	3,456,573	4,024,160	4,301,466	3,927,299	248,651
Total reads post-trim	3,011,708	3,566,814	3,805,631	3,461,384	235,170
Percent GC	53	53	53		
Sequence length	55-75	55-75	55-75		
Quality score	33-37	33-37	33-37		
Total mapped reads	2,622,839	3,129,246	3,328,311		
Unique mapped	424,110	499,347	567,106		
Percent unique mapped	16.17	15.96	17.04		
Percent multiple loci mapped	83.06	83.19	82		

harboring pBR was less than that for those harboring pBR-*zor-orz* (2,826,330 vs 3,927,399 respectively, Table 3.1). FastQC was used to assess the quality of the raw sequence reads. After initial FastQC, the reads were trimmed and re-run for quality check. The FastQC results for each of the paired sequence reads for our dataset showed GC content of 53%. The sequence length ranged from 55-75 nts and the phred score was 33-37. On average, 79.6% of the sequences were mapped to multiple loci (ribosomal RNA).

The sequence reads were then mapped to the *E. coli* O157:H7 str. EDL933 (assembly GCA_000732965.1) reference genome using STAR, analyzed for gene expression (using HTSeq) and differential gene expression (using DESeq2).

Overall comparison of transcriptome

The transcriptome of EDL933- Δ *zor-orz* after kanamycin treatment was well differentiated based on the plasmid it harbored, *i.e.* either pBR or pBR-*zor-orz* (Fig. 3.4A). Mean reads of 8 reference genes that are reported to be stably expressed in *E. coli* (22,23) showed similar expression levels (Fig. 3.4B). Expression of a total of 3,398 genes was identified out of 5,579 total genes annotated in the reference genome. Of these, 473 genes showed a significant differential expression ($p < 0.05$, Fig. 3.5).

Differentially expressed genes

The proportional over-representation of the transcripts in the cells harboring the pBR-*zor-orz* plasmids is indicated as upregulated genes and the under-represented transcripts are indicated as the downregulated genes. Of the 234 upregulated genes in the cells harboring pBR-*zor-orz* plasmids those with at least 1.5-log₂fold change are shown in Table 3.1. Most of the significantly upregulated genes belong to those involved in nitrate assimilation, anaerobic respiration, and amino acid biosynthesis (lysine and isoleucine, Table 3.1). The network analysis of the differentially expressed genes followed by the functional annotation enrichment with KEGG, GO, and InterPro pathways shows connections between the overrepresented genes and enrichment of nitrogen metabolism and molybdopterin oxidoreductase pathway (Fig. 3.6). Similar

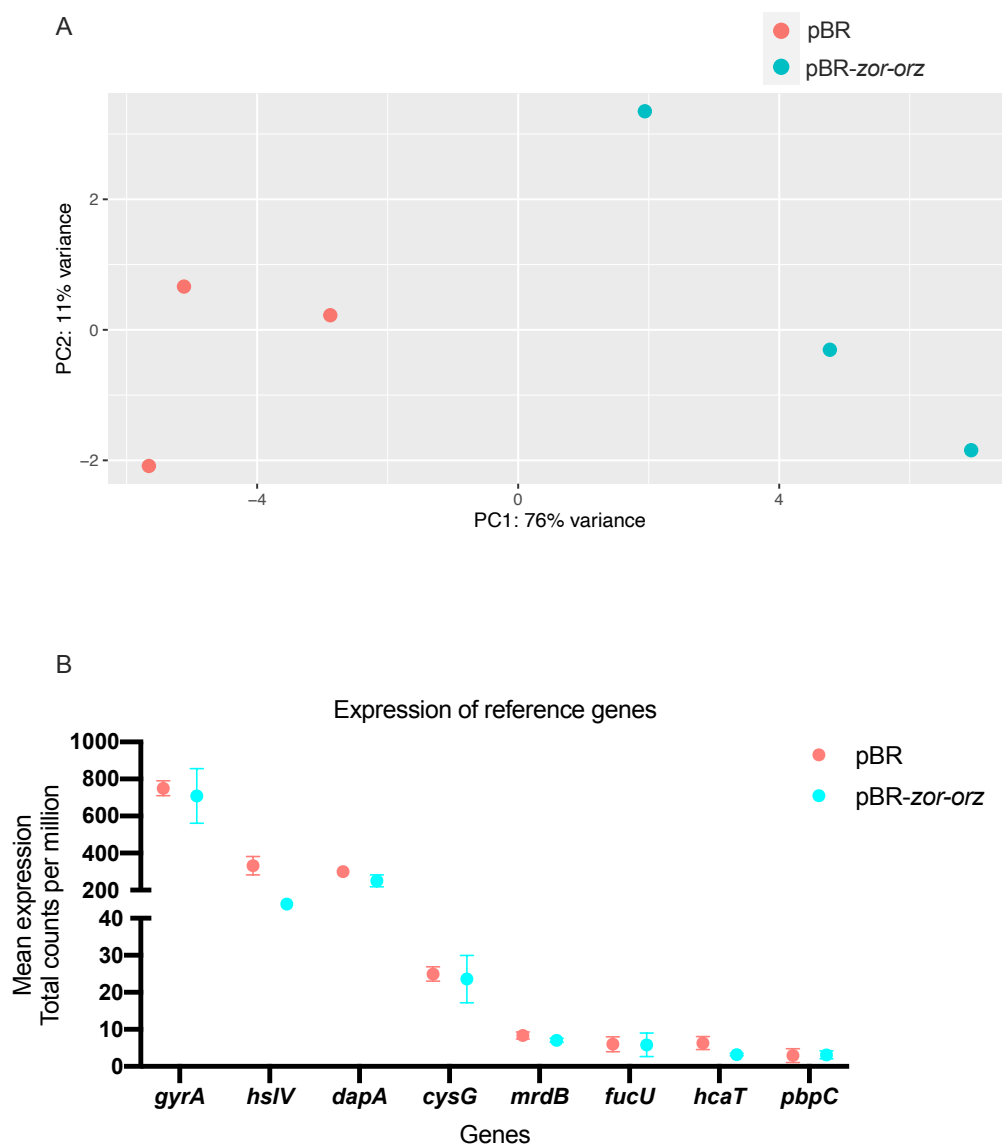


Figure 3.4. Principle component analysis plot of the transcriptome of the EDL933 cells harboring either pBR or pBR-*zor-orz* after treatment with 10 μ g/ml kanamycin for 15 min (A). Mean expression of the reference genes for the cells harboring pBR or pBR-*zor-orz* (B)

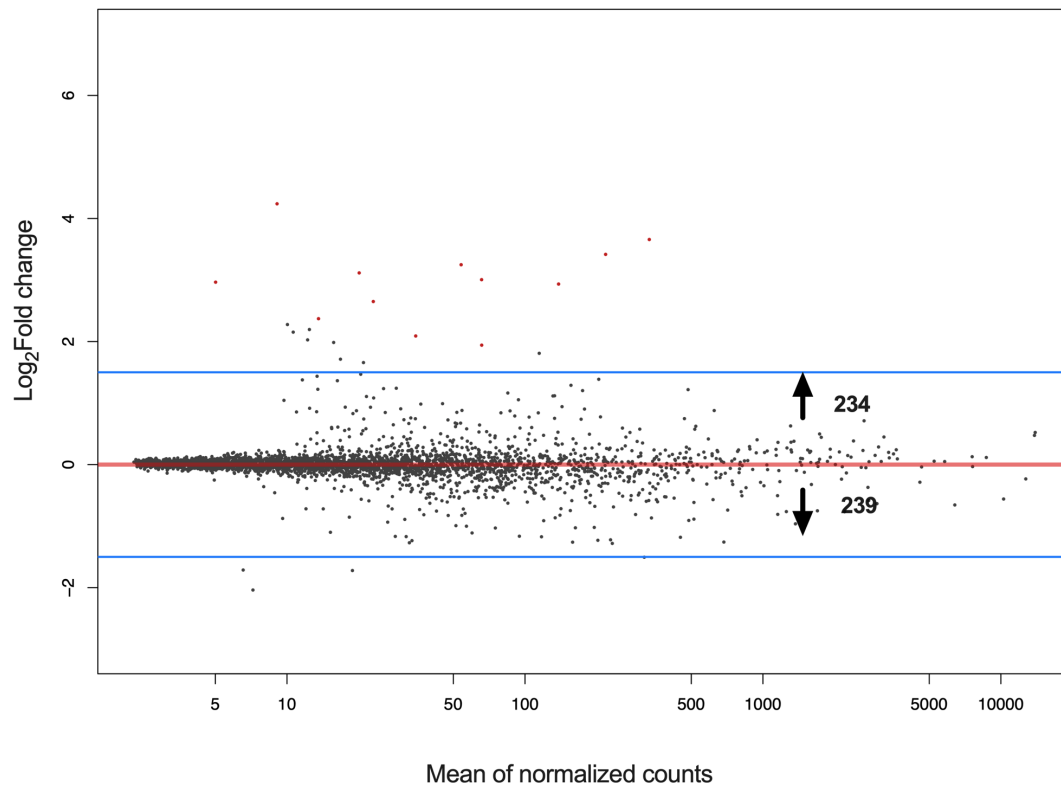


Figure 3.5. Differentially expressed genes represented as a scatter plot. Log₂fold change between EDL933- Δ *zor-orz* cells carrying pBR and pBR-*zor-orz* after kanamycin treatment compared to the mean of normalized counts. Statistically significant genes (p value <0.05) included 234 overrepresented (up-regulated) and 239 underrepresented (down-regulated) genes. Blue lines indicate 1.5-log₂fold changes.

Table 3.2. Description of significantly overrepresented (upregulated) genes ($p < 0.01$) in cells harboring pBR-zor-orz with at least 1.5-log₂fold change

Gene	Log ₂ Fold Change	p-value	Functional category	Protein Description
<i>ansB</i>	3.20	4E-07	Asparagine metabolism	L-asparaginase 2
<i>bssR</i>	2.29	5E-05	Biofilm	biofilm formation regulator BssR
<i>ccp</i>	2.03	1E-04	Oxidative stress	cytochrome c peroxidase
<i>dcuC</i>	5.65	1E-03	Fumarate transporter	anaerobic C4-dicarboxylate transporter DcuC
<i>dmsA</i>	3.33	2E-08	Anaerobic respiration	dimethylsulfoxide reductase subunit A
<i>fdnG</i>	2.24	4E-08	Anaerobic respiration	formate dehydrogenase-N subunit alpha
<i>feoA</i>	1.69	6E-03	Iron transport	ferrous iron transporter A
<i>frdA</i>	1.68	1E-03	Anaerobic respiration	fumarate reductase (quinol) flavoprotein subunit
<i>fruB</i>	2.01	3E-03	Carbohydrate transport	fused PTS fructose transporter subunit IIA/HPr protein
<i>glpA</i>	3.38	8E-06	Anaerobic respiration	anaerobic glycerol-3-phosphate dehydrogenase subunit A
<i>ilvC</i>	2.00	2E-06	Isoleucine biosynthesis	ketol-acid reductoisomerase
<i>IgoR</i>	1.63	2E-03	Transcriptional regulator	GntR family transcriptional regulator
<i>LsrR</i>	1.54	9E-03	Transcriptional regulator	transcriptional regulator LsrR
<i>lysA</i>	1.80	7E-04	Lysine biosynthesis	diaminopimelate decarboxylase
<i>napF</i>	2.54	6E-05	Oxidative stress	ferredoxin-type protein NapF
<i>narG</i>	3.73	4E-07	Nitrogen metabolism	nitrate reductase subunit alpha
<i>narH</i>	1.78	3E-04	Nitrogen metabolism	nitrate reductase subunit beta

Table 3.1. Continued

Gene	Log ₂ Fold Change	p-value	Functional category	Protein Description
<i>narK</i>	3.51	1E-07	Nitrogen metabolism	nitrate transporter NarK
<i>nirB</i>	4.03	1E-06	Nitrogen metabolism	NADPH-nitrite reductase large subunit
<i>nrfA</i>	2.85	6E-08	Nitrogen metabolism	ammonia-forming nitrite reductase cytochrome C552 subunit
<i>sad</i>	2.03	2E-11	Nitrogen metabolism	succinate-semialdehyde dehydrogenase
<i>ttdR</i>	2.62	3E-04	Transcriptional regulator	DNA-binding transcriptional activator TtdR
<i>ubiU</i>	2.72	2E-04	Metal ion binding	U32 family peptidase
<i>uhpT</i>	1.75	3E-03	Carbohydrate transport	hexose-6-phosphate:phosphate antiporter
<i>ycbJ</i>	2.37	9E-05	Biofilm and oxidative stress	YcbJ family phosphotransferase
<i>ychH</i>	1.69	6E-04	Biofilm and oxidative stress	stress-induced protein YchH
<i>yddB</i>	3.81	2E-03	Iron transport	TonB-dependent receptor plug domain-containing protein
<i>ygeV</i>	1.88	2E-05	Transcriptional regulator	sigma-54-dependent Fis family transcriptional regulator
<i>yjil</i>	2.69	2E-05	Carbohydrate transport	Yjil family glycine radical enzyme

analyses for the underrepresented genes show enrichment of protein folding, heat response and flagellar assembly pathways (Fig. 3.7).

Of the 239 underrepresented (downregulated) genes, 13 had at least 1.5- $\log_2$ fold and most of these were associated with the flagella (Fig. 3.8A). Other significantly downregulated genes included those encoding heat shock proteins (Fig. 3.8B). The other downregulated genes include *espR4* that encodes a type 3 secretion system effector-like protein and LdtC (L,D-transpeptidase) that functions in peptidoglycan crosslinking. Several heat shock proteins such as GroEL, GroES and DnaK have been reported to be induced by kanamycin (24). The $\log_2$ fold change for these and other heat shock proteins, however, is lower in the pBR-*zor-orz* harboring cells (Fig. 3.8B).

As flagellar associated genes were downregulated in cells harboring pBR-*zor-orz*, we tested if the presence of pBR-*zor-orz* can impact motility of *E. coli* MG1655. We saw a trend in decrease in the motility of the cells harboring pBR-*zor-orz* in early time points and it was statistically significant at 9 hours ($p = 0.026$; Fig. 3.9). Since cells with pBR-*zor-orz* grew better in presence of kanamycin compared to the cells with pBR (18), motility testing of these cells with kanamycin was not comparable.

IV. Discussion

Though several aspects of type I toxin systems have been examined, a paucity of research has addressed the global impact for these toxins. We determined that neither deletion of the chromosomal copy of the *zor-orz* locus nor the amplification of locus copy number resulted in altered growth of *E. coli* EDL933 in the conditions tested (LB, M9 supplemented with glucose, fructose or glucose and fumarate, Fig. 3.2 (18)). However, cells harboring pBR-*zor-orz* grew significantly better in the presence of, or subsequent to being challenged with aminoglycoside antibiotics such as kanamycin (Fig. 3.3, (18)). Therefore, to better understand the global processes that contribute to this improved growth, we challenged cells, grown to exponential phase, with kanamycin and examined the primary transcriptional response to the antibiotic.

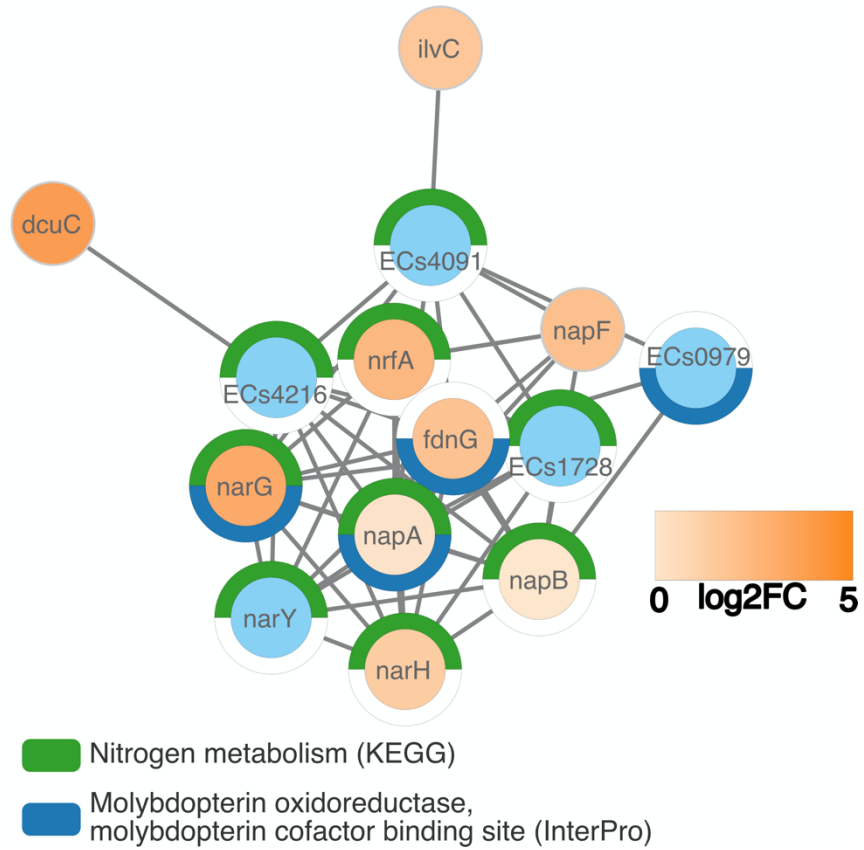


Figure 3.6. Network analysis of upregulated genes using Cytoscape (v.3.8.2) with maximum additional interactions of 10. The split donut around the node indicates the enrichment pathways identified.

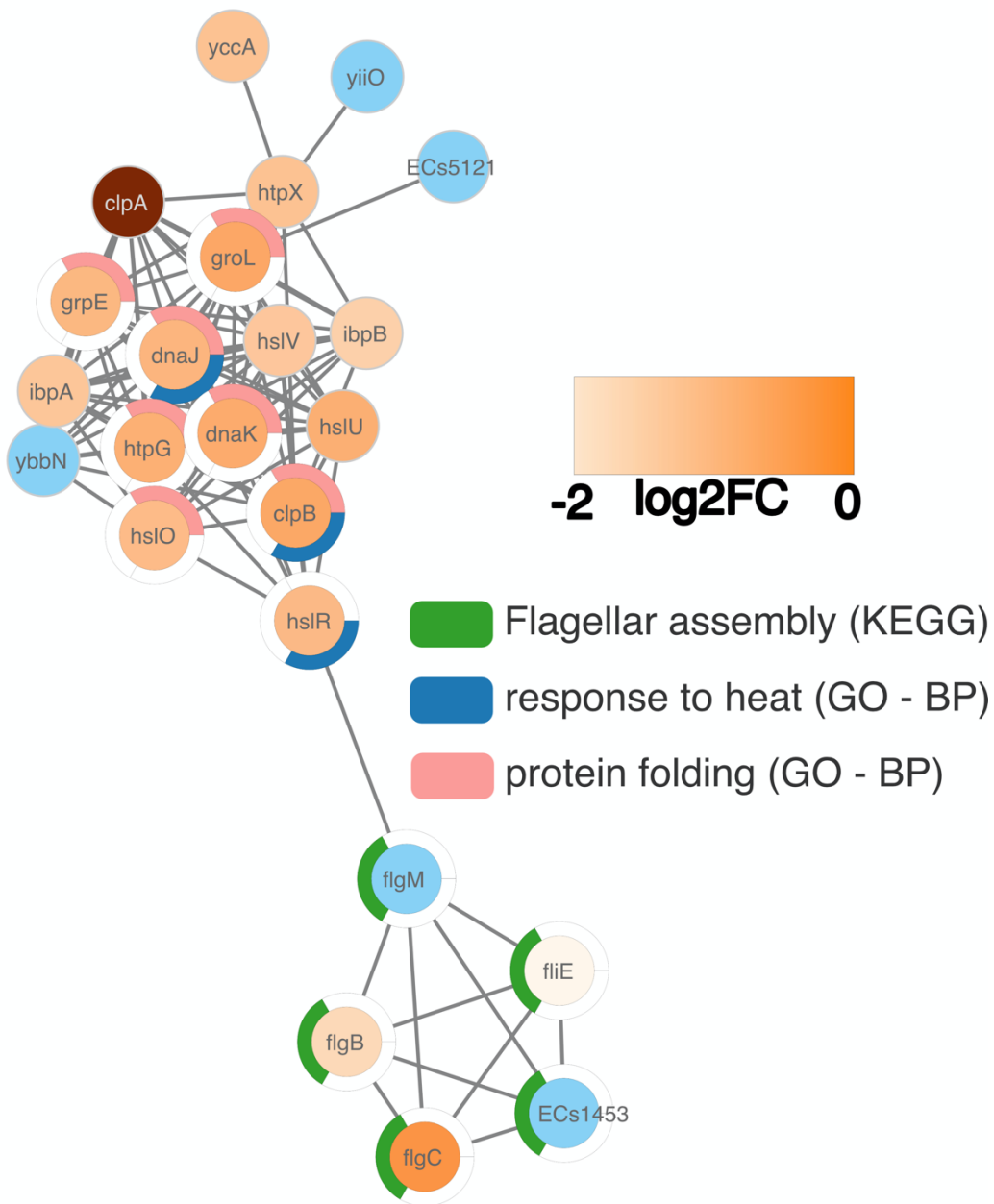
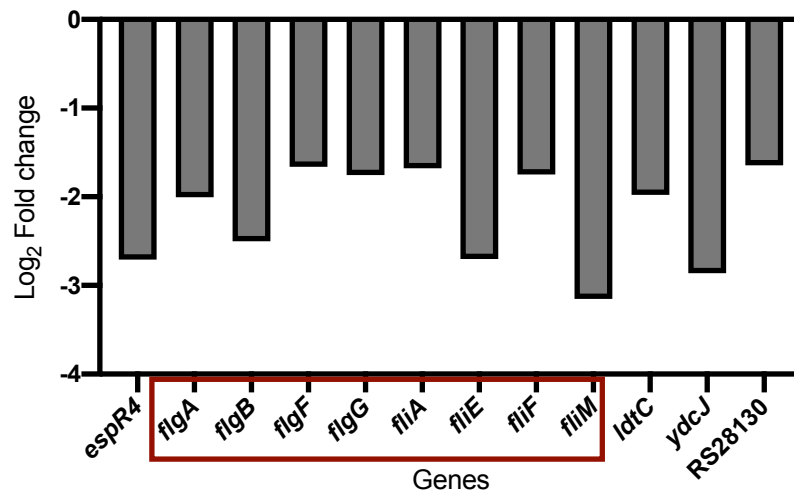


Figure 3.7. Network analysis of downregulated genes using Cytoscape (v.3.8.2) with maximum additional interactions of 5. The split donut around the node indicates the enrichment pathways identified.

A



B

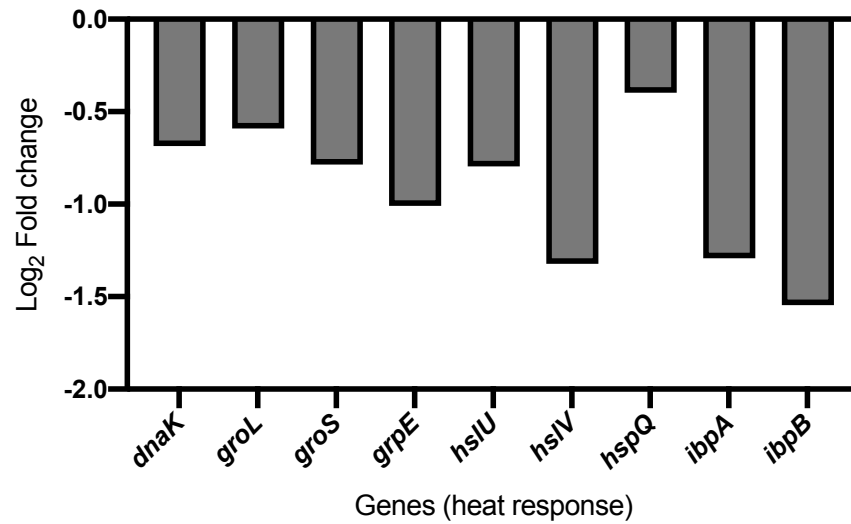


Figure 3.8. Genes that are significantly underrepresented (downregulated ($p < 0.01$)) in cells harboring pBR-*zor-orz*. At least 1.5- $\log_2$ fold (A), Flagella associated genes indicated in red box. Genes related to heat shock response that are significantly downregulated (B).

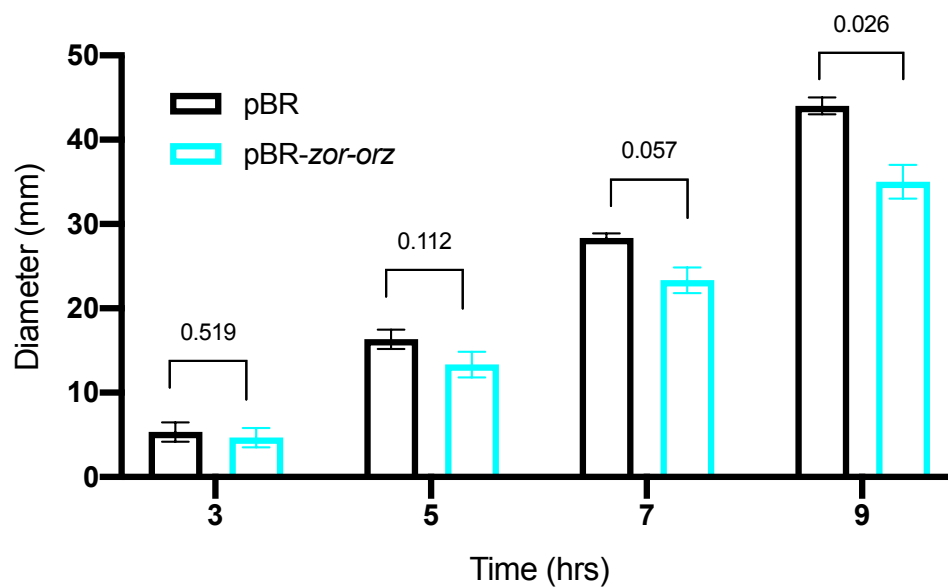


Figure 3.9. Motility of *E. coli* MG1655 with pBR or pBR-zor-orz. Diameter of the colony on 0.3% LB medium (with 100 μ g/ml ampicillin to maintain plasmids) was measured at indicated time points. $n = 3$, shown are mean $\pm$ SD. The p values were calculated using a multiple unpaired t test with Welch correction.

The significant downregulation of flagella-associated genes along with the upregulation of anaerobic respiration indicated that the cells could be minimizing ATP requiring processes (motility), while simultaneously using alternate ATP generating mechanisms. Overexpression of ZorO abundantly indicates cellular ATP level depletion within 15 and 30 minutes of overexpression (18,25) however, differences in ATP levels were not observed between cells harboring the control plasmid or pBR-*zor-orz* after 1 hour of 4 µg/ml kanamycin treatment compared to the empty vector in EDL933- Δ *zor-orz* strain (18). This suggests that the impact on ATP during overexpression might be occurring at the chromosomal level in a less pronounced manner which could not be resolved by the previously used luciferase-based ATP assay. Similarly, while we observed a trend of decreased motility of *E. coli* cells with pBR-*zor-orz* in LB medium with ampicillin at early time points (Fig. 3.9), the diameter of motility was statistically significant at 9h ($p = 0.026$). These results suggest the presence of subtle effects on cellular physiology, with preference for the downregulation of flagellar genes.

The upregulation of nitrogen metabolism genes *narG*, *narH*, and *nark* indicated the occurrence of the reduction of nitrate to nitrite, together with further reduction to ammonia by *nirB*. This ammonia can be then used for the biosynthesis of arginine, glutamine, and glutamate. Similarly, other amino acid biosynthesis genes such as *lysA* (lysine biosynthesis) and *ilvC* (Isoleucine biosynthesis) were also upregulated suggesting that the translation process is not affected in cells with pBR-*zor-orz*.

Aminoglycoside antibiotics cause the misfolding of newly synthesized proteins. As a result, bacteria use heat shock proteins and chaperones as an early defense mechanism to protect cells from these antibiotics (26). However, our observations indicated that the transcripts for heat shock proteins and chaperones were underrepresented in cells harboring pBR-*zor-orz*, suggesting a decreased incidence of misfolded proteins compared to the empty vector cells. This demonstrated that the *zor-orz* locus functions to either prevent the entry of kanamycin into the cell or rapidly exports it before it can impact the process of translation. To distinguish this, we attempted to quantify the extracellular kanamycin (in spent media) using mass spectrometry after kanamycin treatment of cells with pBR or pBR-*zor-orz*. However, we

were unable to detect any significant difference, likely because the kanamycin concentration was very low ($< 0.01\text{nM}$) in these samples.

Altogether, our data indicate that the presence of pBR-*zor-orz* can offer cellular protection from kanamycin-induced protein misfolding and helps cells to grow in the presence of very minimal concentrations of antibiotics. Amplification of the *zor-orz* locus has helped identify the function of cellular growth improvements in aminoglycoside antibiotics (18). Although, the use of native regulatory elements of *zor-orz* in the plasmid is more similar to native physiological induction conditions, compared to the use of the overexpression system, it is important to determine the mechanistic impacts of the *zor-orz* locus at the chromosomal expression level. Based on transcriptomic comparison, future experiments can focus on investigating the effects of nitrogen limitation, oxygen, and temperature on the growth of bacterial strains that possess different variations or a deletion of the *zor-orz* locus.

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CHAPTER IV

Role of charged amino acid residues in ZorO mediated toxicity and bacterial growth in presence of kanamycin

Publication note

This chapter is currently not under review; however, it will be submitted in the future.

Mutant constructions, western blot analysis, flow cytometry analysis, ATP assays and growth curves were performed by Bikash Bogati. Oana Stoiculescu and Thomas Nipper made the point mutants of the *zorO* to study the role of charged amino acid residues and performed preliminary experiments on the effects of the point mutants of ZorO on cell growth inhibition. The pBR based constructs and chromosomal mutants of *zorO* were made by Bikash Bogati. Bikash Bogati and Elizabeth M. Fozo wrote the manuscript.

Abstract

Many type I toxin-antitoxin systems encode a small, hydrophobic protein with several charged residues distributed across the sequence of the toxin protein. While some of the membrane targeting type I toxins have been shown to form pores in the membrane and affect membrane potential and cellular ATP levels, the role of the specific charged amino acid residues has been studied for only a few toxins. Herein, we use ectopic overexpression after mutating the codons encoding charged amino acids within *zorO*. These codon mutations were conducted in order to study their impacts on growth inhibition, membrane depolarization, ATP depletion, and the localization of this small protein. We found that the charged residues are critical for ZorO mediated toxicity and that altering a single charged amino acid can completely abolish the toxic effects of ZorO. In addition to localization to the membrane, as seen with wild-type ZorO, cells overexpressing the non-toxic mutants accumulated the protein within the membrane and cytoplasm. Previously, we noted that amplification of the *zor-orz* locus (consisting of both the *zorO-orzO* and *zorP-orzP* toxin-antitoxin pairs) resulted in increased resistance to kanamycin. Within, we also verified that the improved growth in kanamycin could be imparted with multiple copies of the *zorO-orzO* gene pair. When the arginine at the 23rd position was replaced with leucine, which is nontoxic via overexpression analysis, multiple copies of *zorOR23L-orzO* continued to confer growth advantage in the presence of kanamycin. The chromosomal copy of the *zorO-orzO* locus in *E. coli* MG1655, however did not improve growth in the presence of kanamycin. Surprisingly, removal of the antitoxin *orzO* and/or the first 28 nucleotides of *zorO* from the *zorO-orzO* gene pair also did not show growth difference in LB or LB with kanamycin. This indicated that increasing the translation of ZorO by deleting translational repressive elements does not necessarily improve *zorO-orzO* mediated growth in the presence of aminoglycosides. While charged residues in ZorO are important for its toxicity, the non-toxic mutant still improves growth in the presence of kanamycin, thus suggesting a more complex nature of the *zorO-orzO* gene pair function than previously suggested.

I. Introduction

First identified in plasmids, type I toxin-antitoxin (TA) systems are also found in bacterial chromosomes. The toxin gene encodes for a small protein (usually less than 60 amino acids) and the antitoxin gene encodes for a small RNA (sRNA). While overexpression of the toxin often results in growth inhibition and cell lysis, co-expression of the sRNA neutralizes the toxicity. This is achieved by sRNA complementary base pairing with the toxin-encoding mRNA and subsequent prevention of toxin translation (1,2). Despite their small size, overexpression studies indicate that not all type I toxins impact cells via the same mechanism.

Some type I toxins have cellular targets such as RNA (SymE) and DNA (RalR), whereas several others have either been experimentally validated to localize to the bacterial inner membrane or have a predicted alpha helix structure (3–5). Pore formation after membrane localization has been established for the *hokB/sokB* and *tisB/istR1* TA systems (6,7). The 49 amino acid HokB forms a dimer via disulfide interactions between the cysteine residues at the 46th position of two HokB proteins (8). This not only affects membrane potential but also results in leakage of ATP from the cell (6). Ectopic overexpression of HokB in *E. coli* functions in persistence against the antibiotics tobramycin and ofloxacin, likely due to a reduction in membrane potential and ATP levels (9). Using a combination of *in vitro* and *in vivo* tools, the 29 amino acid TisB toxin has been shown to form a dimer-of-dimers giving rise to a tetrameric charged zipper structure permitting ion conductance (7,10). This results in membrane depolarization and affects cellular ATP levels, leading the cell into a transient dormancy (11). The transcription of *tisB* occurs in DNA damaging conditions as it is normally repressed by a SOS response regulator (LexA); thus, it is speculated that TisB production during SOS is needed to allow the cell time to repair DNA damage before replicating DNA (12, 13).

Apart from type I toxins, overexpression of *de novo* synthesized small hydrophobic proteins has been shown to cause membrane depolarization and affect aminoglycoside uptake resulting in an increase in the minimum inhibitory concentration (MIC) for those antibiotics in *E. coli* (14). Despite their effect on membrane

depolarization and cellular growth (15,16), neither IbsC or LdrD overexpression resulted in an increased MIC against aminoglycosides (14).

While many type I toxins are predicted to localize to the cytoplasmic membrane, there are variations in the predicted orientations within the membrane and the length of the predicted transmembrane domain (5). Many membrane-associated type I toxins have a global positive charge (5), which can play an important role in binding to the negatively charged bacterial membrane and/or with interactions to other proteins. For some toxins, both positively and negatively charged amino acid residues are distributed within the toxin sequence. For the toxin TisB, the charged amino acids have been observed to lead to the formation of self-dimers and tetramers that form small pores in the membrane (7). Some other toxins will associate with the membrane upon high overexpression, but their primary effects are likely not the membrane. For example, severe overexpression of Fst results in the toxin localizing to the membrane, but the primary effect is nucleic acid condensation (20,21). It is therefore important to distinguish whether a toxin functions by acting on the bacterial membrane or if the membrane associated phenotypes are the result of saturating the membrane with a small, hydrophobic protein.

ZorO is the toxin component of a bacterial type I TA system, *zorO-orzO*, first identified in the chromosome of *Escherichia coli* O157:H7 EDL933 (17,18). It is located in a region known as the *zor-orz* locus, which encodes, in tandem, a second highly homologous type I TA gene pair *zorP-orzP* (17,18). The small protein ZorO localizes to the bacterial inner membrane (25). ZorO translation is regulated by a sRNA, OrzO, via complementary base pairing with the *zorO* mRNA, and by secondary structure formed within its own long 5' untranslated region (UTR) within its mRNA (17,26). Ectopic overexpression of *zorO* using plasmids results in growth stasis, membrane depolarization, and ATP depletion in *E. coli* MG1655 (which does not possess *zorO*) without a significant impact on bacterial gross morphology (19). Although toxic when overproduced, we observed that cells with multiple copies of the entire *zor-orz* locus (including the toxins, antitoxins and their native promoter elements) were able to grow significantly better in aminoglycosides (19).

To determine the amino acid residues involved in both toxic and beneficial effects of ZorO production, we overexpressed mutants of ZorO and identified key amino acids that conferred toxicity. We found that changes in several positively charged or negatively charged amino acids either decreased or completely abolished ZorO toxicity. We also observed the impact of these point mutations on ZorO localization. Furthermore, we studied how these mutations alter the growth of the cells in aminoglycoside antibiotics utilizing a medium copy plasmid and chromosomal strains integrated with *zorO-orzO* variants.

II. Materials and Methods

Bacterial strains and plasmids All bacterial strains and plasmids used in this study are listed in Table 4.1. The sequences of all oligonucleotides are listed in Table 4.2.

Growth conditions. Overproduction studies. Cultures for overproduction of ZorO and its derivatives were grown as described previously (17). Briefly, *E. coli* UTK007, a derivative of MG1655 with constitutive *araE*, was transformed fresh (not older than 10 days) with the indicated plasmid derivatives of pAZ3 using electroporation (17). The resulting transformants were grown overnight in 5 mL of LB medium (with 25 µg/ml chloramphenicol final concentration) at 37°C with shaking and diluted to an OD₆₀₀ of 0.01. At an OD₆₀₀ of ~ 0.3, arabinose was added to a final concentration of 13.32 mM (0.2%) or 3.33 µM (0.00005%) as indicated in the text. *E. coli* UTK007 carrying empty vector (pAZ3) or uninduced (no arabinose) were used as controls. OD₆₀₀ was measured and cells were harvested after 30 mins of overexpression. Averages ± standard deviations for a minimum of three replicates are shown.

Microplate growth curves. Overnight cultures were grown in LB medium. Ampicillin (100 µg/ml final concentration) was added when testing cells transformed with pBR322 (27) or pBR-*zor-orz* (19). Overnight cultures were diluted to an OD₆₀₀ of 0.2 in 1 mL growth medium. A 96 well microplate was prepared by adding 190µl of the culture media (LB) containing the antibiotics as indicated. Then 10 µl of 0.2 OD₆₀₀ culture (diluted in sterile

medium from overnight culture) was added to obtain a 200 μ l total volume of a 0.01 OD₆₀₀ culture. Absorbance was recorded on a Gen5™ Microplate reader (BioTek Instruments, Inc.) every 30 min after a 15 sec of shaking for 24 hrs at 37°C. Shown are averages $\pm$ standard deviations for a minimum of three replicates.

Plasmid construction. Specific point mutants were created by site directed mutagenesis of the pAZ3-FLAG-zorO plasmid as previously described (17) using the indicated oligonucleotides (Table 4.2). Briefly, plasmids amplified with indicated oligonucleotides were DpnI digested, purified, and transformed into *E. coli* TOP10 cells. Plasmid DNA was extracted and confirmed via sequencing.

The pBR322 derived plasmids were constructed via Gibson assembly. The inserts were amplified using the indicated oligonucleotides whose sequences are available in Table 4.2. The insert for pBR-zorO-orzO was amplified from the *E. coli* O157:H7 DNA: for Δ 28-FLAG-zorO, it was amplified from EDL933- Δ 28-FLAG-zorO. The R23L and AAG mutant of the pBR- Δ 28-F-zorO plasmids were constructed by performing site directed mutagenesis of the pBR- Δ 28-F-zorO plasmid.

Membrane depolarization assay. Membrane depolarization assays were performed as described previously (19). Membrane potential sensitive dye Bis-(1,3-diethylbarbituric acid) trimethine oxonol (DiBAC₄-3; Invitrogen TM) was used to determine the effect of ZorO production on membrane depolarization. A 25 mg/mL stock solution was prepared in dimethyl sulfoxide. At indicated time points, 50-100 μ l of culture was taken, flooded with 4ml 1X phosphate buffer saline (PBS) and centrifuged for 10 min at 4°C. Cells were resuspended in 1 ml PBS and stained with DiBAC₄-3 (10 μ g/ml final concentration) for 20 min in the dark. After washing with 1x PBS twice, cells were re-suspended in 0.5 ml of 1x PBS and analyzed by flow cytometry in an LSR II flow cytometer (Becton

Table 4.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source	Primers
<u>Bacterial strains</u>			
<i>E. coli</i> MG1655	<i>E. coli</i> F ⁻ λ <i>ilvG</i> <i>rfb</i> -50 <i>rph</i> -1	Lab stock	
<i>E. coli</i> Top10	Cloning vector	Lab stock	
UTK007	DJ480 PCP- <i>araE</i>	Lab stock	
UTK102	UTK007- <i>lepB</i> -SPA	(19)	
MG- <i>zorO</i>	MG1655 recombineered with P _{zorO} -full length UTR- <i>zorO</i>	This study	EF1869, EF1865A
MG- Δ 28-FLAG- <i>zorO</i>	MG1655 recombineered with P _{zorO} - Δ 28UTR-FLAG- <i>zorO</i>	This study	EF1865A, EF1869B
MG- <i>zorO-orzO</i>	MG1655 recombineered with <i>zorO-orzO</i>	This study	EF1864A, EF1865A
<u>Plasmids</u>			
pAZ3	Cm(R)	(17)	
pAZ3-FLAG- <i>zorO</i>	Cm(R)	(19)	
pAZ3- <i>zorO</i> -D2N	Cm(R)	This study	EF1576 EF1577
pAZ3- <i>zorO</i> -D2K	Cm(R)	This study	EF1574, EF 1575
pAZ3- <i>zorO</i> -K7Q	Cm(R)	This study	EF1580, EF1581
pAZ3- <i>zorO</i> -K7E	Cm(R)	This study	EF1578, EF1579
pAZ3- <i>zorO</i> -E16Q	Cm(R)	This study	EF1184, EF1185
pAZ3- <i>zorO</i> -E16R	Cm(R)	This study	EF1215, EF1216

Table 4.1. Continued

Strain or plasmid	Relevant characteristics	Source	Primers
pAZ3- <i>zorO</i> -R23L	Cm(R)	This study	EF1186, EF1187
pAZ3- <i>zorO</i> -R23E	Cm(R)	This study	EF1217, EF1218
pAZ3- <i>zorO</i> -D26N	Cm(R)	This study	EF1311, EF1312
pAZ3- <i>zorO</i> -D26K	Cm(R)	This study	EF1309, EF1310
pAZ3- <i>zorO</i> -K29Q	Cm(R)	This study	EF1315, EF1316
pAZ3- <i>zorO</i> -K29E	Cm(R)	This study	EF1313, EF1314
pBR322	Amp(R)	(17)	
pBR- <i>zor-orz</i>	Amp(R)	(19)	
pBR- <i>zorO-orzO</i>	Amp(R)	This study	EF1835, EF1836, EF1837, EF1838
pBR- <i>zorO</i> R23L- <i>orzO</i>	Amp(R)	This study	EF1186, EF1187
pBR-plac- <i>orzO</i>	Amp(R)	(17)	
pBR- <i>zorO</i>	Amp(R)	(19)	
pBR- Δ 28-FLAG- <i>zorO</i>	Amp(R)	This study	EF1836, EF1839, EF1837, EF1840

Table 4.2. Oligonucleotides used in this study

Name	Oligonucleotide sequence, 5'→3'
EF1217	GAGTTATTAGTGGCTCTGTTAGAGTTGATTGATTTGTTGAAGTAAC
EF1218	GTTACTTCAACAAATCAATCAACTCTAACAGAGCCACTAATAACTC
EF1309	GTGGCTCTGTTACGGTTGATTAAGTTGTTGAAGTAACGGGGCAAATTG
EF1310	CAATTTGCCCCGTTACTTCAACAACTTAATCAACCGTAACAGAGCCAC
EF1311	GTGGCTCTGTTACGGTTGATTAATTTGTTGAAGTAACGGGGCAAATTG
EF1312	CAATTTGCCCCGTTACTTCAACAAATTAATCAACCGTAACAGAGCCAC
EF1313	CTGTTACGGTTGATTGATTTGTTGGAGTAACGGGGCAAATTGAAACGCATC
EF1314	GATGCGTTTCAATTTGCCCCGTTACTCCAACAAATCAATCAACCGTAACAG
EF1315	CTGTTACGGTTGATTGATTTGTTGCAGTAACGGGGCAAATTGAAACGCATC
EF1316	GATGCGTTTCAATTTGCCCCGTTACTGCAACAAATCAATCAACCGTAACAG
EF1574	GATTATAAAGATGATGATGATAAAAAGACGCTGACACAAAAGTTAACCGTG
EF1575	CACGGTAACTTTTGTGTCAGCGTCTTTTATCATCATCATCTTTATAATC
EF1576	GATTATAAAGATGATGATGATAAAAACACGCTGACACAAAAGTTAACCGTG
EF1577	CACGGTAACTTTTGTGTCAGCGTGTTTTTATCATCATCATCTTTATAATC
EF1578	GATGATAAAGACACGCTGACACAAGAGTTAACCGTGCTCATTGCCGTACTG
EF1579	CAGTACGGCAATGAGCACGGTAACTCTTGTGTCAGCGTGTCTTTATCATC
EF1580	GATGATAAAGACACGCTGACACAACAGTTAACCGTGCTCATTGCCGTACTG
EF1581	CAGTACGGCAATGAGCACGGTAACTGTTGTGTCAGCGTGTCTTTATCATC
EF1835	CATCGTCATCCTCGGCACCGTCCATCGATTTTATATTCAACGC

Table 4.2 (continued)

Name	Oligonucleotide sequence, 5'→3'
EF1836	GTCCCTGATGGTCGTCATCTACGGATCCCCCTTAGATGCG
EF1837	CGCATCTAAGGGGGGATCCGTAGATGACGACCATCAGGGAC
EF1838	GCGTTGAATATAAAATCGATGGACGGTGCCGAGGATGACGATG
EF1839	GCGCTCATCGTCATCCTCGGCACCGTCCGAATAAATTATAACCATATCC
EF1840	GGATATGGTTATAATTTATTCTGGACGGTGCCGAGGATGACGATGAGCGC
EF1864A	CTCTAAATAATTCAAATTGGTCCGATCCGGCGCAACGTCCCCATCGATTTTA TATTCAACGCACC
EF1865A	CATCCCTGAAATGTATTAATGAAATAAACGATATTTTACGCGCGTGTAGGCT GGAGCTGCTTCG
EF1865A	CATCCCTGAAATGTATTAATGAAATAAACGATATTTTACGCGCGTGTAGGCT GGAGCTGCTTCG
EF1869	CTCTAAATAATTCAAATTGGTCCGATCCGGCGCAACGTCCCCATCGTCATC CTCGGCACCGGATCC
EF1869B	CTCTAAATAATTCAAATTGGTCCGATCCGGCGCAACGTCCCCATCGTCATC CTCGGCACCGGATCC

Dickinson) with a 488nm laser. Samples were run at ~3000 events per second and fluorescence was collected in the fluorescein isothiocyanate channel. Data was analyzed using the FlowJo software package (FlowJo LLC, Ashland, OR, USA) such that the cells showing at least 10^3 abu or more fluorescence were gated and mean fluorescence intensity was obtained. All tests were done with a minimum of three biological replicates. Shown are averages $\pm$ standard deviations for a minimum of three replicates.

ATP measurements. ATP levels were measured as described previously (19), using the BacTiter Glo ATP Assay System Bioluminescence Detection Kit for ATP Measurement (Promega) according to the manufacturer's instructions. The observed relative luciferase unit (RLU) values were normalized to OD₆₀₀ at the time of cell harvest. A minimum of three biological replicates were performed per strain/condition. Shown are averages $\pm$ standard deviations for a minimum of three replicates.

Western blot and dot-blot analyses. Western blot and dot-blot analyses was done as described previously (19). Total protein was extracted from a 50 ml culture (after overexpression for 30 minutes) via bead beating. Protein concentration was measured using a Bradford Protein Assay. Protein samples (10 μ g) were separated on an SDS-PAGE gel, transferred to the immobilon-FL membrane, and probed with a rat derived α -FLAG tag primary antibody (BioLegend) and an α -IgG secondary antibody (LI-COR Biosciences) that fluoresces at 680 nm. LepB was tagged with a SPA tag (UTK102) to serve as a loading control.

Separation of the insoluble fraction (membrane) from the soluble fraction was done via ultracentrifugation (100,000 g, 4°C, 45 min) to yield supernatant (cytoplasmic proteins) and a pellet (membrane proteins). The pellet was washed with cold PBS and resuspended in 100 μ l PBS.

Dot blots were performed by spot inoculating a nitrocellulose membrane with 5 μ l of sample containing 10 μ g or 20 μ g of protein and processing the membrane as above (probed with α -FLAG primary antibody α -IgG secondary antibody) (28).

III. Results

ZorO: a small hydrophobic protein

The *zorO* gene encodes for a small protein (29 amino acids in length) that is predicted to form an alpha helix (Phyre 2.0; Fig. 4.1A; (29)) and localizes to the bacterial inner membrane (19,25). Several computational tools predict that ZorO has a transmembrane helix (Fig. 4.1B). The orientation of this small protein in the membrane, *i.e.* the location of the N-terminus or the C-terminus towards the cytoplasm or periplasm, varies between these prediction tools (Fig. 4.1B) and is yet to be determined experimentally for ZorO. The grand average hydropathy value (GRAVY), calculated by assigning each amino acid residue with a hydropathy value and dividing it by the length of the protein sequence, is 1.4 for the ZorO sequence (Fig. 4.1C; (30)) indicative of its hydrophobic nature.

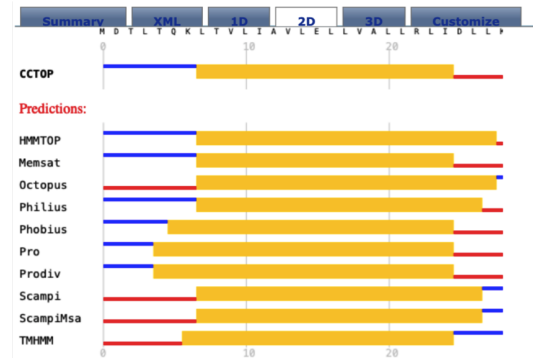
ZorO has three positively charged and three negatively charged residues uniformly distributed within the sequence (red and blue colored residues respectively in helical wheel projection in Fig. 4.1D). The addition of a FLAG tag (DYKDDDDK) reduces the hydrophobicity index (0.38 for FLAG-ZorO) as the FLAG tag is rich with charged amino acid residues. However, the membrane localization of ZorO is not affected by the FLAG tag (19). To examine the role of the specific charged residues within the ZorO sequence, we mutated the respective codons to encode either an oppositely charged amino acid or a neutrally charged amino acid. As expected, these mutations did not alter the hydrophobicity index of the ZorO sequence except when a positively charged residue (arginine) was mutated to hydrophobic leucine (R23L, Table 4.3).

A

MDTLTQKLTVLI AVLLELLVALLRLIDLLK

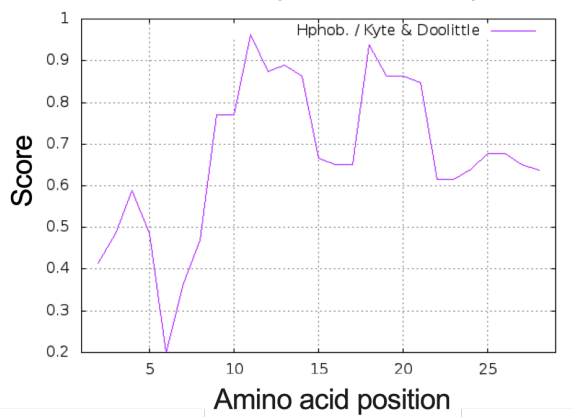


B



C

ProtScale output for ZorO sequence



D

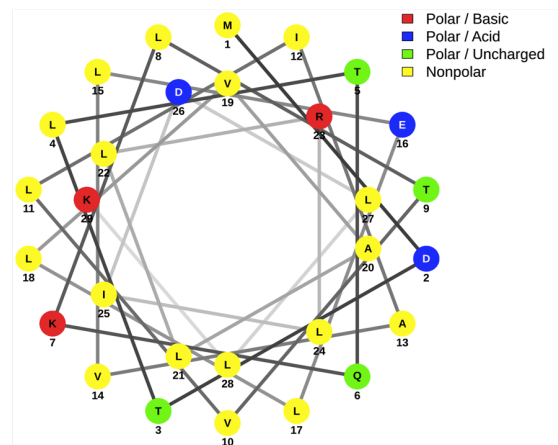


Figure 4.1. Structure and hydropathy prediction of ZorO. Alpha-helical structure of ZorO predicted by Phyre 2.0 (A). Predicted transmembrane domain and orientation (B); Blue line at N-terminus indicates N-terminus towards the periplasm, yellow bar represents the predicted transmembrane domain. The ProtScale value based on hydrophobicity scale of ZorO sequence (C). Helical wheel projection of ZorO sequence (D).

Table 4.3. Point mutants of the charged amino acid residues of ZorO and their respective GRAVY index

Amino acid	Position	Mutated to	Symbol	GRAVY index
Wild type			ZorO	1.40
Aspartate	2	Asparagine	D2N	1.39
		Lysine	D2K	1.38
Lysine	7	Glutamine	K7Q	1.41
		Glutamate	K7E	1.40
Glutamate	16	Arginine	E16R	1.36
		Glutamine	E16Q	1.40
Arginine	23	Leucine	R23L	1.68
		Glutamate	R23E	1.43
Aspartate	26	Asparagine	D26N	1.40
		Lysine	D26K	1.38
Lysine	29	Glutamine	K29Q	1.41
		Glutamate	K29E	1.41

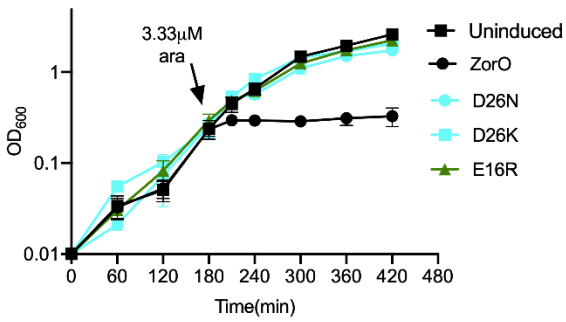
Role of charged amino acid in ZorO induced growth inhibition

Despite the lack of major differences in GRAVY, we elected to examine the contribution of charged residues in ZorO activity by making the point mutations (Table 4.3) and monitoring the effects after overexpression. The resulting mutant *zorO* sequences, which also contained the sequence for an N-terminus FLAG tag, were overexpressed from the arabinose inducible promoter (P_{BAD}) on a high-copy plasmid by adding 3.33 μ M of arabinose during mid-log growth phase (OD600 ~0.3) and assessing for growth inhibition. To maximize ZorO translation, the 5' UTR was eliminated, leaving only the ribosome binding site (22 nt before the start codon) and mutating the sequence to prevent stem formation with the ribosome binding site (RBS; (26)).

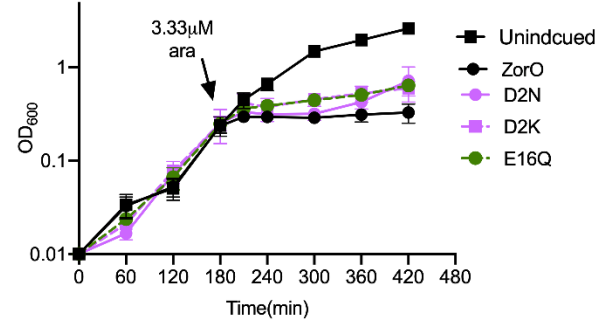
Of the negatively charged amino acid residues glutamate and aspartate (E16, D2 and D26), mutating E16 to glutamine (E16Q) still maintained growth inhibition although it was not as severe when compared to wild type ZorO, whereas, mutating E16 to the oppositely charged arginine (E16R) abolished ZorO toxicity (Fig. 4.2A). When an aspartate at the C-terminus (D26) was mutated to either lysine or asparagine, this too eliminated the effects of ZorO (Fig. 4.2A). This indicated that this residue was critical for inducing growth stasis, whereas the mutation of the other aspartate residue (D2) to lysine or asparagine (D2K, D2N) mirrored the growth stasis induced by wild-type ZorO (Fig. 4.2B).

Similar analyses with the positively charged amino acids arginine and lysine (R23, K7, K29) were also performed. The conversion of the lysine residues at the 7th and 29th position to the neutrally charged amino acid glutamine showed growth inhibition similar to the wild type ZorO (Fig 4.2C). However, cells grew well if either lysine residue was mutated to a negatively charged glutamate (Fig. 4.2D). When arginine was converted to leucine which has a neutral charge (R23L), cells grew normally (Fig. 4.2D), and we note that the GRAVY score for this was the most distinct from wild type ZorO than the other mutant proteins examined. Surprisingly, altering the arginine at the 23rd position to negatively charged glutamate (R23E) maintained toxicity (Fig. 4.2C). Overall, based on these data, altering the charged residues (other than D2)

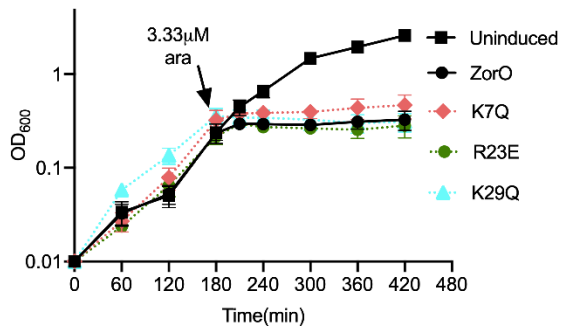
A



B



C



D

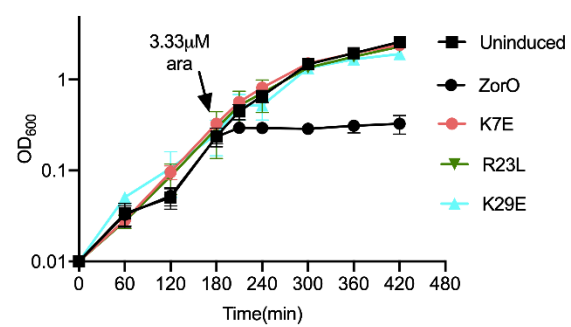


Figure 4.2. Role of charged amino acid residues in ZorO mediated growth inhibition. Negatively charged amino acid mutations that result in a non-toxic ZorO (A) or maintain ZorO toxicity (B). Positively charged amino acid mutations that maintains ZorO toxicity (C) or makes ZorO non-toxic (D). LB medium with 3.33 μM arabinose induction at mid-log phase. $n=3$, shown are mean $\pm$ standard deviation (SD). The uninduced cells and the ZorO overexpressing cells serve as controls.

have impacts on their ability to inhibit growth after ZorO overproduction. This confirms that charged residues play a key role in ZorO toxicity.

Impact of charged residues in ZorO localization

Previous overproduction studies indicated that ZorO predominantly localizes to the bacterial inner membrane (25), which likely contributes to its effects on membrane polarization and ATP levels. The observed lack of toxicity of the above point mutants could be due to inappropriate localization and/or reduced expression and/or stability of the ZorO variants. We thus examined the levels of the ZorO variants via western analysis and their localization after induction by 13.3 mM (0.2%) arabinose and separation of the soluble (cytoplasmic) and insoluble (membrane) fractions (Fig. 4.3).

The non-toxic ZorO mutants were present in large quantities in comparison to wild-type ZorO and accumulated both in the insoluble and soluble fraction (Fig. 4.3A and B). Despite a large fraction of ZorO localizing to the membrane with these mutants, there were no observed effects on growth inhibition (Fig. 4.2). The R23L mutant was of particular interest as a large amount of ZorO protein in this mutant accumulated in the cytoplasmic fraction (47%) (Fig. 4.3B). Although the remaining 53% of the protein localized in the membrane, the mutant had no impact on cellular growth stasis (Fig. 4.2D). All toxic mutant derivatives of ZorO (D2N, D2K, K7Q, E16Q, R23E and K29Q) were detected only in the membrane fraction and not in the soluble fraction, which corresponded to similar localization behavior seen in wild type ZorO (Fig. 4.3C).

Impact of charged amino acid residues on ZorO induced membrane depolarization and ATP depletion

Apart from growth inhibition, ZorO overexpression results in membrane depolarization and ATP depletion. We, therefore, examined both 30 minutes after overexpression of each of the charged residues mutants, regardless of toxicity or not, and compared to that of the wild type ZorO. Consistent with the results observed for the growth patterns, we found that cells overproducing a non-toxic ZorO mutant (K7E,

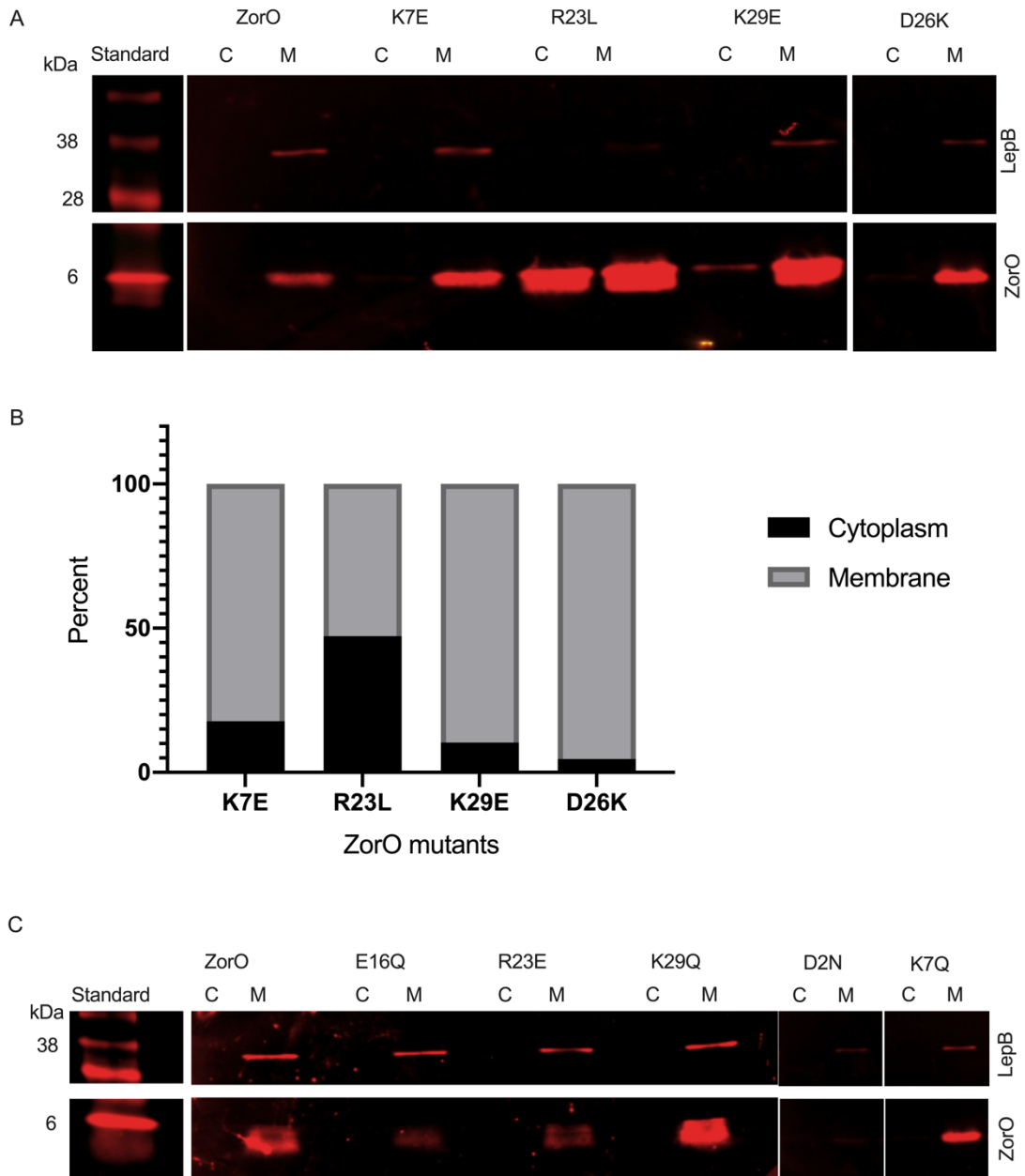


Figure 4.3. Western blot analysis of ZorO mutants. The non-toxic mutants of ZorO (A). Quantification using ImageJ of the non-toxic mutants in the cytoplasm and the membrane fraction (B). Localization of ZorO mutants that are toxic (C). The band at ~38kD standard denotes to the LepB (membrane protein) that serves as control for separation of the soluble and insoluble protein and loading control.

R23L, D26K and K29E) maintained membrane polarization better than cells overproducing wildtype ZorO (Fig. 4.4A). Overproduction of the toxic mutants resulted in membrane depolarization examined by DiBAC4(3) staining followed by flow cytometry analysis (Fig 4.4A).

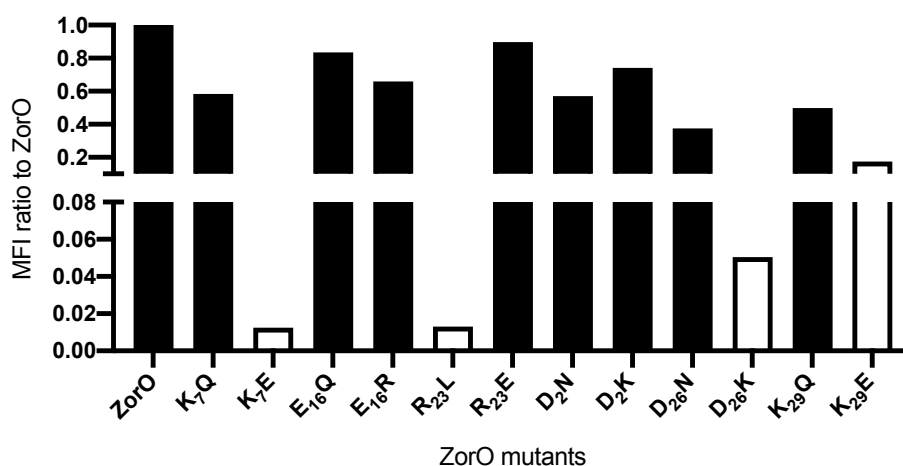
We determined the ratio of relative ATP level of cells overexpressing ZorO mutants for 30 minutes (T30) after 3.33 μ M arabinose induction to that before induction (T0). There was no ATP depletion observed in cells overproducing the non-toxic ZorO variants while those overproducing mutant toxic proteins had ATP loss (Fig. 4.4B). These data indicates that the specific charged residues are essential for the ZorO mediated effects on membrane depolarization and ATP depletion.

Role of *zorO-orzO* gene pair in bacterial growth in presence of aminoglycosides

We previously observed that amplifying the *zor-orz* locus by cloning it onto a medium-copy plasmid (pBR322; referred to as pBR-*zor-orz*) with its native regulatory elements improves the growth of *E. coli* in the presence of kanamycin and gentamycin (19). To test if the *zorO-orzO* gene pair is sufficient to improve bacterial growth we cloned the *zorO-orzO* gene pair onto pBR322 (pBR-*zorO-orzO*). Cells harboring pBR-*zorO-orzO* were able to grow similarly to cells with pBR-*zor-orz*, and significantly better compared to cells containing the empty vector in the presence, of kanamycin, gentamycin, streptomycin, and neomycin (Fig. 4.5). No difference in growth was observed for erythromycin (50 μ g/ml). These results demonstrate that the *zor-orz* locus improves bacterial growth in the presence of several aminoglycosides and that the *zorO-orzO* pair alone is sufficient to achieve this function.

Several type I toxins have been linked to persistence, a mechanism by which a subpopulation of cells can survive lethal dose of antibiotics, mainly by cell growth arrest and making the antibiotic target unavailable. In case of the *zor-orz* locus, we observed resistance against aminoglycoside antibiotics (with an increase in MIC) rather than persistence (19). We were curious how the non-toxic mutant ZorO R23L impacted growth in kanamycin to conclude if the beneficial effect of *zorO* could be separated from its

A



B

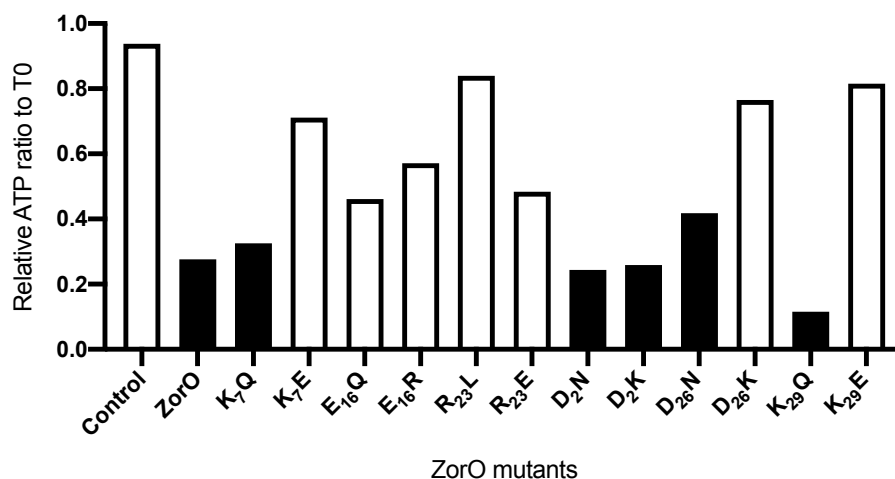
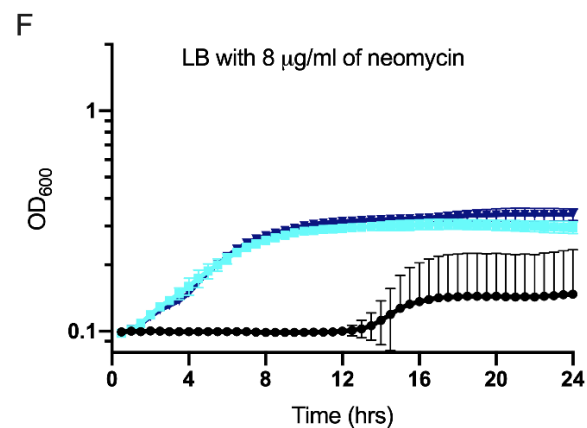
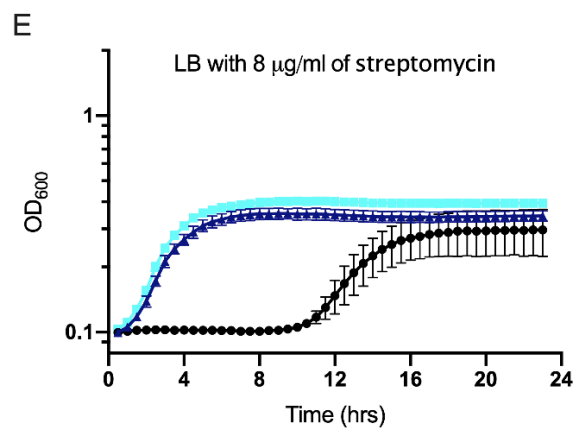
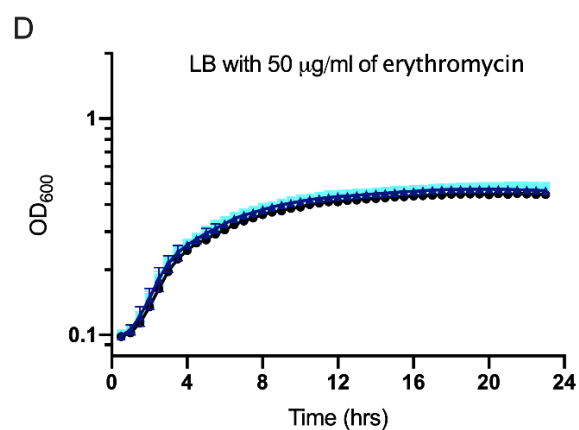
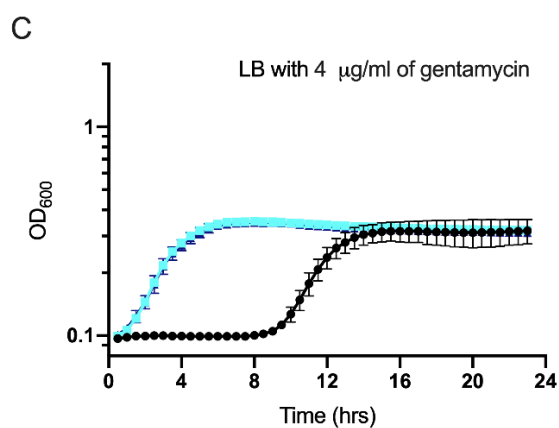
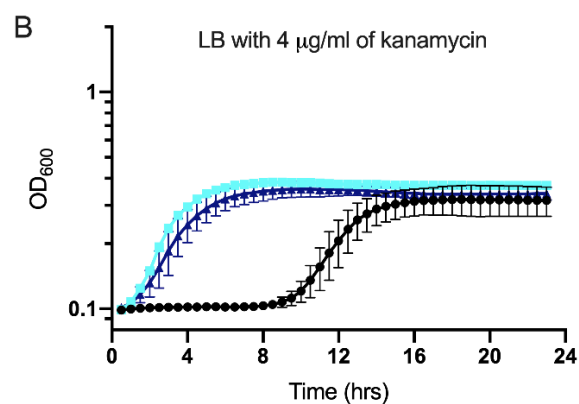
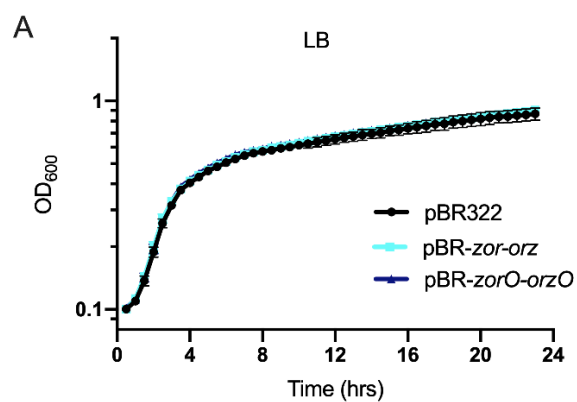


Figure 4.4. Role of charged amino acid residues in ZorO mediated membrane depolarization and ATP depletion. Ratio of mean fluorescence intensity compared to that of the wild type ZorO of the cells after overexpression of indicated ZorO mutant and DiBAC4(3) staining (A); the non-toxic mutants (white bars) have less membrane depolarized cells. Ratio of mean relative luciferase unit corresponding to cellular ATP levels normalized to OD₆₀₀ in the cells after 30 minutes (T30) overexpression of indicated ZorO mutant to the ATP level at the time of induction (T0) (B); ATP levels in non-toxic mutants are indicated with white bars.

Figure 4.5. The *zorO-orzO* gene pair is sufficient to improve growth of *E. coli* in the presence of several aminoglycoside antibiotics. Growth in LB (A), LB with 4 µg/ml kanamycin (B), 4 µg/ml gentamycin (C), 50 µg/ml erythromycin (D), 8 µg/ml streptomycin (E), and 8 µg/ml of neomycin (F) in *E. coli* UTK 007 strain with indicated plasmids. $n \geq 3$, shown are mean $\pm$ standard deviation (SD).



inherent toxicity (Fig. 4.2 and 4.3). To test this, we performed site directed mutagenesis of pBR-*zorO-orzO* to create pBR-*zorO-R23L-orzO*. *E. coli* harboring this plasmid grew similarly to the others in LB media (Fig. 4.6A). Surprisingly, pBR-*zorO-R23L-orzO* was also able to improve the growth of *E. coli* in the presence of kanamycin (4 µg/ml) in LB medium (Fig. 4.6B). This was unexpected especially as the overexpression study showed that the R23L mutant does not result in a significant growth inhibition, membrane depolarization and ATP depletion, all of which have been associated with kanamycin resistance.

Bacterial growth after removal of the two regulatory elements: antitoxin OrzO and first 28 nts of *zorO*

We observed that cells have improved growth in the presence of kanamycin when harboring multiple copies of the *zor-orz* locus or the *zorO-orzO* gene pair (Fig. 4.5, (19)). Translation of the *zorO* mRNA is regulated by the antitoxin OrzO and the long 5' UTR (17,26). The processed form of *zorO* (missing first 28 nts at 5'UTR, Δ28) is translated far greater than when expressed from full-length 5' UTR (3,4). Therefore, we hypothesized that the cells harboring multiple copies of *zorO* either with its full-length or processed 5' UTR would have even better growth in the of kanamycin. To this end plasmids were constructed with a full-length 5'UTR *zorO* (pBR-*zorO*) and a processed 5'UTR (pBR-Δ28-FLAG-*zorO*) with an N-terminus FLAG tag. Further, to test if R23L shows any differences in the growth pattern, we also constructed a pBR-Δ28-FLAG-*zorO-R23L*.

The growth of cells harboring these plasmids was similar in LB media (Fig. 4.7A). Unlike the significant improvement in growth observed with the *zor-orz* locus or the *zorO-orzO* gene pair in presence of aminoglycoside antibiotics (Fig. 4.5), *E. coli* harboring pBR-*zorO*, pBR-Δ28-FLAG-*zorO* or pBR-Δ28-FLAG-*zorO-R23L* did not result in improved growth pattern in LB with kanamycin (4 µg/ml) (Fig. 4.7B).

To test if the *zorO* is translated when present with its own promoter, we used the pBR-Δ28-FLAG-*zor* vectors and extracted the protein after growing cells until mid-log phase in LB. Although we were not able to detect ZorO via western blot we were successful in detecting it using a dot-blot assay (Fig. 4.8).

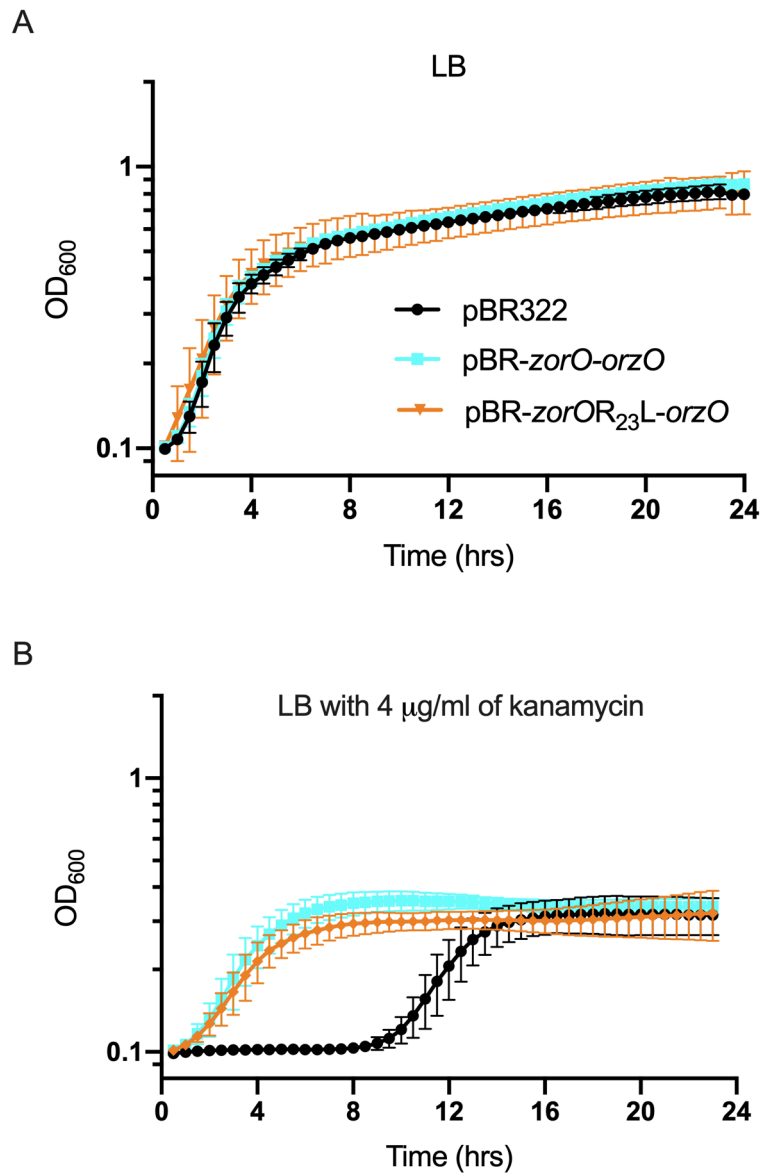
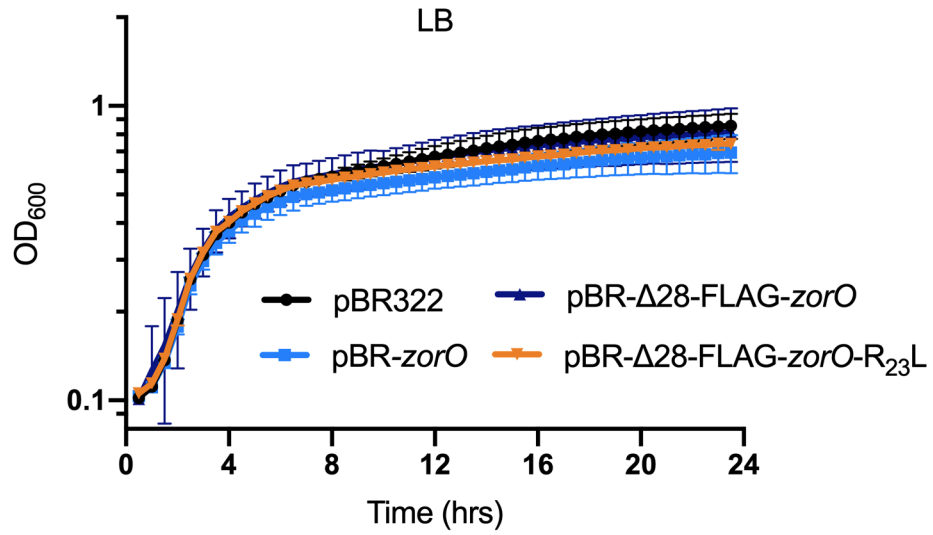


Figure 4.6. Growth curve of the *E. coli* UTK007 cells with indicated plasmids in LB (A) and LB with 4 μ g/ml kanamycin (B). $n \geq 3$, shown are mean $\pm$ standard deviation (SD).

A



B

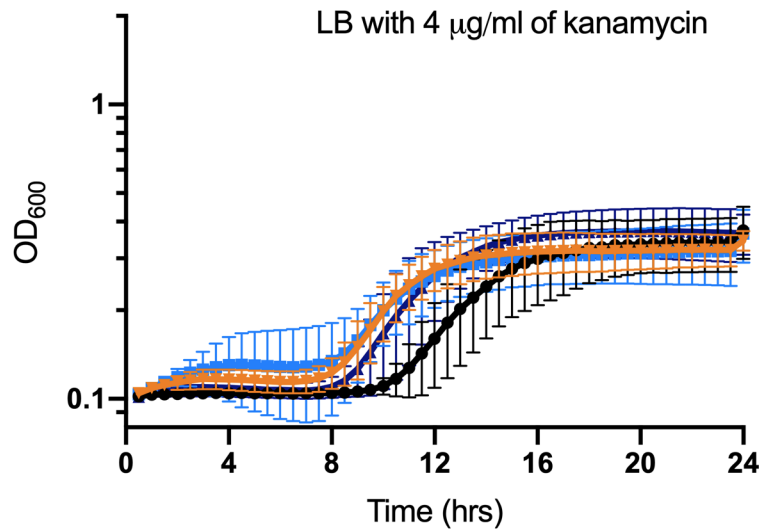


Figure 4.7. Growth curve of the *E. coli* UTK007 cells with indicated plasmids in LB (A) and LB with 4 μ g/ml kanamycin (B). $n \geq 3$, shown are mean $\pm$ standard deviation (SD).

Role of *zorO-orzO* gene pair and *zorO* variants at chromosomal level in MG1655 strain

Given our above data, we wanted to determine if kanamycin protection could be induced via production from single chromosomal copy. Thus, we investigated the effects at the chromosomal level by recombineering *zorO-orzO*, *zorO* and $\Delta 28$ -FLAG-*zorO* into the chromosome of *E. coli* MG1655. In LB media alone, the growth of these strains was similar (Fig. 4.9A). Unlike the improved growth in kanamycin that was observed with pBR-*zorO-orzO*, we were unable to see the growth benefit with the chromosomal copy of *zorO-orzO* in MG1655 (Fig. 4.9B). Similarly, MG-*zorO*, MG- $\Delta 28$ -FLAG-*zorO*, and MG- $\Delta 28$ -FLAG-*zorO*-R23L also had similar growth patterns in LB with kanamycin (Fig. 4.9B).

OrzO overexpression does not improve growth in kanamycin

The above results (Fig. 4.9) raised the question if the improved growth in kanamycin was due to the antitoxin OrzO. To test this, we overexpressed OrzO from the lac promoter (pBR-P_{lac}-OrzO) and monitored cell growth in LB or LB with kanamycin (17). Cells overexpressing OrzO had similar growth pattern to those with empty vector in LB medium and did not improve growth in LB with kanamycin (Fig. 4.10A and 4.10B). Interestingly, cell growth was slowed with OrzO overexpression in kanamycin (Fig. 4.10B).

IV. Discussion

The inner membrane localization of ZorO in *E. coli* and its effect on membrane potential and ATP depletion has been established using an overexpression system (19,25). We also showed previously that the 5' UTR regulates the translation and subsequent impacts of ZorO (19). Herein, we demonstrate that the charged amino acids of ZorO are critical for growth inhibition, membrane depolarization and depletion of cellular ATP levels. Specifically, five out of six charged amino acids, when mutated individually, had impacts on ZorO mediated cell growth inhibition showing the significance of the interaction with these residues in ZorO toxicity. Further, improved growth of the bacterial

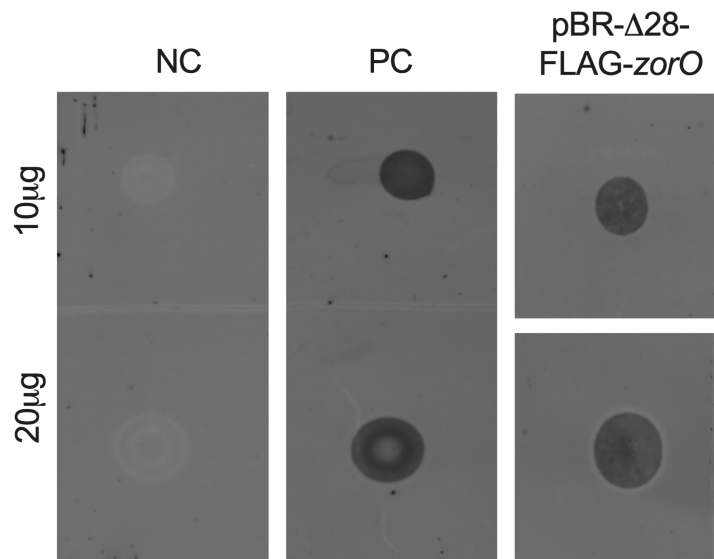


Figure 4.8. Dot blot analyses for the detection of ZorO from *E. coli* UTK007 cells with pBR-Δ28-FLAG-zorO. NC- Negative control with empty vector. PC- Positive control with P_{BAD}-FLAG-zorO.

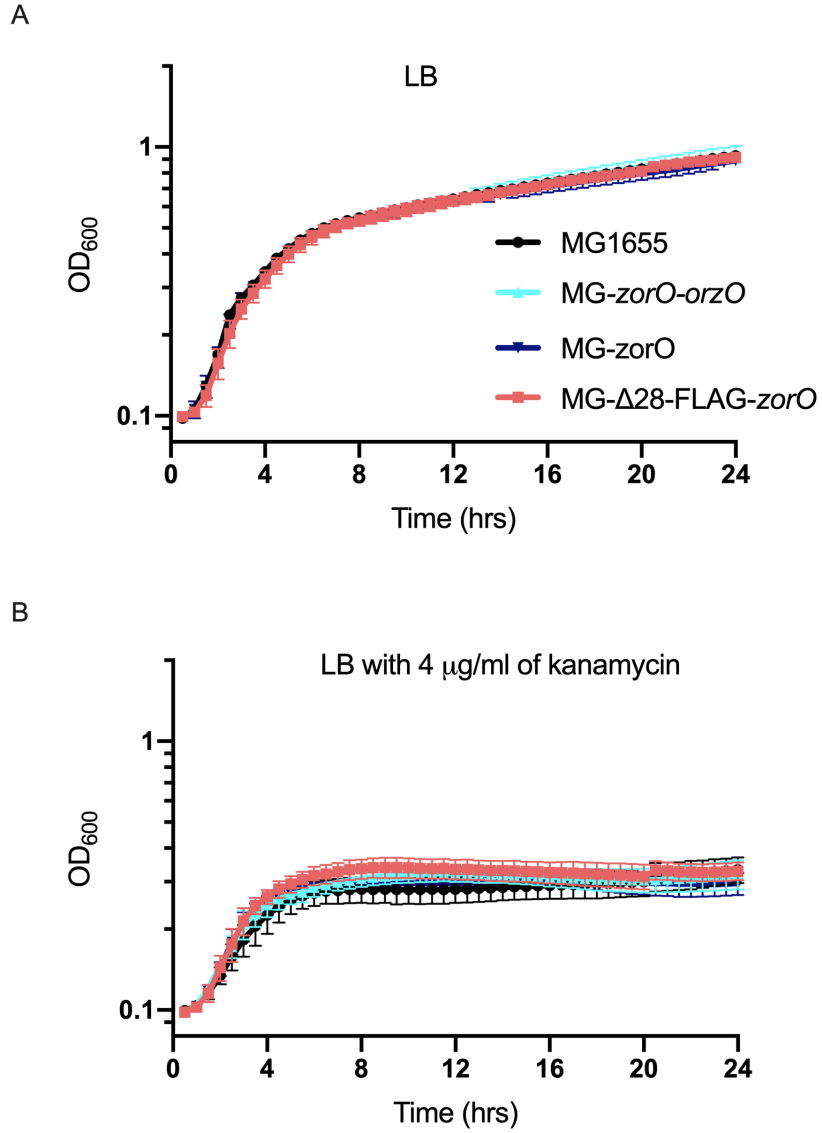


Figure 4.9. Growth curve of *E. coli* MG1655 recombineered with different *zorO* variants in the chromosome (*zorO-orzO*, *zorO* and Δ 28-FLAG-*zorO* LB (A) and LB with 4 μ g/ml kanamycin (B). $n \geq 3$, shown are mean $\pm$ standard deviation (SD).

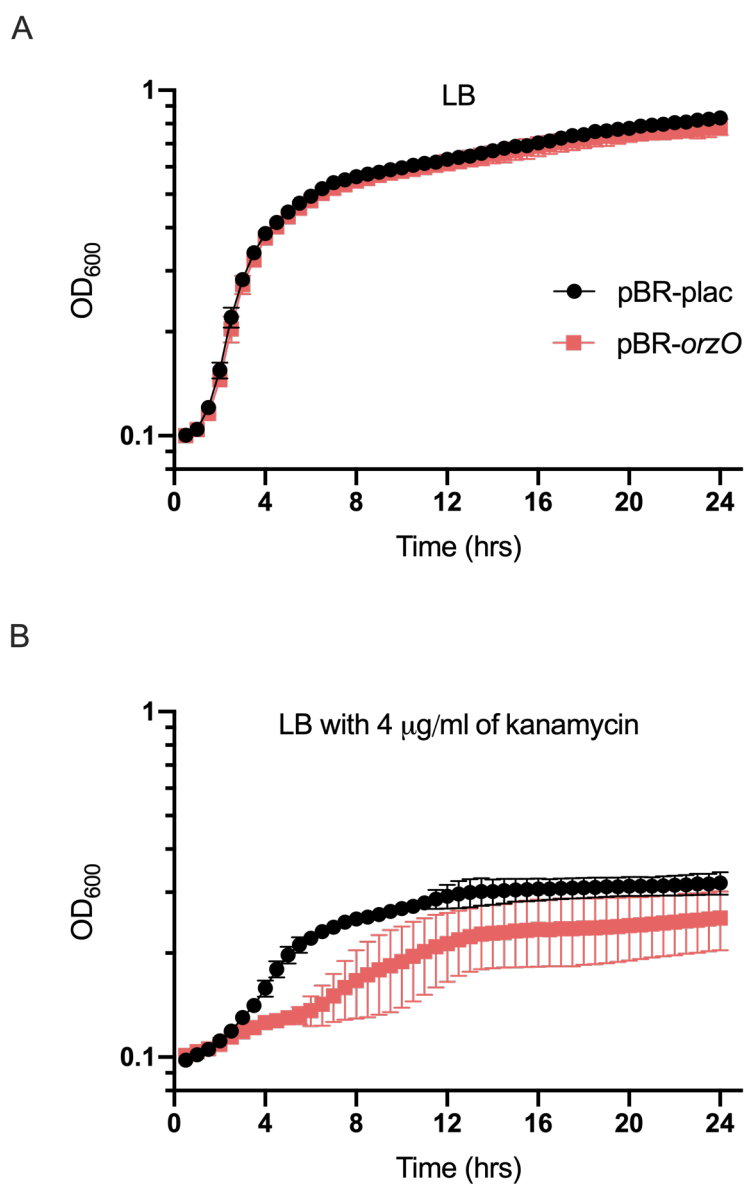


Figure 4.10 Growth curve of *E. coli* UTK007 harboring pBR-plac or pBR-P_{lac}-orzO in LB medium (A) and in LB with 4 µg/ml kanamycin (B). 1 mM IPTG was added at the beginning to induce OrzO production. $n \geq 3$, shown are mean $\pm$ standard deviation (SD).

cells in antibiotics conferred by harboring multiple copies of the *zor-orz* locus (pBR-*zor-orz*) with the native regulatory elements is maintained by pBR-*zorO-orzO*, pBR-*zorO*, pBR- Δ 28-FLAG-*zorO* and a non-toxic mutant of ZorO (Δ 28-FLAG-*zorO*-R23L). As the non-toxic mutant pBR-*zorO*-R23L-*orzO* was able to allow for improved growth in kanamycin, membrane depolarization and ATP depletion are not required for this benefit.

Charged residues in ZorO are important for ZorO mediated toxicity

The inner membrane targeting small toxin protein ZorO is hydrophobic and contains six charged amino acid residues (20% of its sequence) distributed within the sequence. Most prediction tools show a transmembrane ((TM)) domain within the sequence, although the length of the predicted domain varies based on the tool used (Fig. 4.1B). Only two of the six charged residues, glutamate at 16th position and arginine at 23rd position, are predicted to be in TM domain by all the tools used. Upon mutation of these two different residues, we observed a loss in ZorO mediated toxicity (Fig. 4.2). While the mutation of aspartate at the 2nd position did not impact ZorO mediated toxicity, all other amino acid residues that were mutated either made ZorO less toxic or completely rescued the cells from the ZorO toxicity (Fig. 4.2 and 4.3). Most of the prediction tools suggest ZorO is oriented with N-terminus at the periplasmic side (Fig. 4.1B) and the aspartate at the 2nd position is not predicted to be in the TM domain which might be the possible reason that mutation of this residue does not impact ZorO toxicity. This demonstrates that the charged amino acid residues are critical, either to form an oligomer of ZorO, bind to the bacterial membrane, or to bind to another protein and result in subsequent toxic effects.

Examination of the localization of the non-toxic mutants reveals that the interaction of ZorO with the membrane alone is not sufficient for inhibiting cell growth. The non-toxic mutants of ZorO have the protein accumulate in the cytoplasm in addition to membrane localization (Fig. 4.4A). These results suggest that the effects of ZorO on cells upon overproduction are not simply due to membrane saturation by these artificially overproduced membrane-targeting proteins. Further, the accumulation of the

protein in the cytoplasm could be merely the result of the cells being able to continuously grow and translate the protein (as it is not toxic) after arabinose induction.

Role of pBR-*zorO-orzO*, pBR-*zorO* and pBR- Δ 28-FLAG-*zorO* gene pair in bacterial growth in presence of aminoglycoside

Using pBR-*zorO-orzO*, we verified that *zorO-orzO* is sufficient and can improve cellular growth to a similar degree as that of pBR-*zor-orz* against several aminoglycoside antibiotics (Fig. 4.5). We also confirmed that the non-toxic (R23L) mutant of *zorO* (pBR-*zorO*-R23L-*orzO*) equally improved the growth of the cells in presence of kanamycin (Fig. 4.6B), suggesting that toxicity can be decoupled from growth in kanamycin.

Based on these observations we expected removal of the regulatory elements of *zorO* translation, the *OrzO* sRNA and the first 28 nts of the *zorO* 5'UTR would increase the growth in presence of kanamycin. However, cells harboring pBR-*zorO* or pBR- Δ 28-FLAG-*zorO* did not show improved growth in kanamycin (Fig. 4.7B). Since these constructs did not have the antitoxin *OrzO*, we hypothesized if the improved growth in kanamycin was due to the sRNA antitoxin; thus, we overexpressed *OrzO* using pBR-*P_{lac}-orzO* and noted no improved growth (Fig. 4.10A and 4.10B). Indeed, it appeared that overexpression of *OrzO* resulted in reduced ability to grow in kanamycin versus the negative control. Since pBR-*P_{lac}-orzO* is overproducing *OrzO* (17) unlike the other pBR plasmids we used previously to study the impact on growth in presence of kanamycin, further work is ongoing to analyze differences when *OrzO* alone is expressed from its own promoter or a constitutive promoter.

These results are intriguing as we observe a clear growth advantage of cells that harbor multiple copies of the *zorO-orzO* gene pair and even the R23L mutant. However, the absence of such effects with pBR-*zorO*, pBR- Δ 28-FLAG-*zorO* (Fig. 4.7B) or overexpression of *OrzO* (Fig. 4.10B) indicates that *zorO* mRNA could be responsible for such improved growth in kanamycin. Cells overexpressing *OrzO* had a mild negative effect on growth (Fig. 4.10B) which might be the result of accumulation of *OrzO* sRNA in absence of *zorO* mRNA. If *zorO* mRNA is responsible for the improved growth in

kanamycin, then it would explain why the non-toxic R23L mutant is also able to improve growth. Perhaps translation occurs too quickly in our other constructs lacking repressive elements such that the mRNA cannot accumulate and function. Additional work is ongoing to distinguish the underlying mechanism behind these differences in the growth in kanamycin. These results also suggests that the interaction of OrzO sRNA either to form a double stranded RNA with *zorO* or to modify *zorO* mRNA is required to provide the *zorO-orzO* mediated function of improved growth in kanamycin.

ZorO translation *in vivo* without ectopic overproduction

The transcription of the genes in *zor-orz* locus has been shown from the chromosome in the EDL933 strain (18). However, in case of the type I TA system where the regulation occurs post-transcriptionally at multiple levels (2,31), mere presence of transcripts might not correspond to the translation. *In vivo* ZorO translation has been demonstrated only by utilizing an ectopic overexpression system (19,25). Herein, we showed that ZorO translation can occur *in vivo* derived from its own promoter after the two regulatory elements- antitoxin OrzO and the first 28 nt of the *zorO* 5'UTR- are removed (Fig. 4.8). Only a few studies have reported the detection of the translated toxin protein from the chromosome (without overexpression) in a strain that lacks the antitoxin or cannot transcribe the antitoxin (4,32). We were unable to detect the translated ZorO protein from the chromosome, even after removing the translation-preventing elements which suggest that the translation occurs at very low levels. By amplifying the signal, we successfully detected ZorO via dot blot in cells harboring pBR- Δ 28-FLAG-*zorO* (Fig. 4.8). This demonstrates that using such vectors can be useful to verify *in vivo* translation of type I toxins. They can also be useful to identify the conditions in which type I toxin translation is increased eliminating the detrimental effects seen during severe ectopic overexpression from inducible promoters.

Chromosomal copy of *zorO-orzO*, *zorO* or Δ 28-F-*zorO* is not sufficient to improve cell growth in presence of kanamycin

There is one copy of the *zorO-orzO* gene pair and a homolog *zorP-orzP* in the chromosome of EDL933 (17,18). To understand the physiological relevance of this gene pair, we constructed *E. coli* MG1655 (that naturally lacks *zor-orz* locus) by recombineering *zorO-orzO*, *zorO* or $\Delta 28$ -FLAG-*zorO* in its chromosome. Upon investigation of the growth pattern of these cells, we did not see an improved growth in presence of kanamycin as compared to wild-type MG1655. We were also not able to detect ZorO from the MG- $\Delta 28$ -FLAG-*zorO* strain via dot-blot even when a large quantity of protein (100 μ g) was loaded. The lack of phenotype upon chromosomal mutation and/or deletion of the type I TA system is common and therefore demands tools alternative to the ectopic overexpression system. The use of medium copy plasmid vectors with native regulatory elements (as done in this study) is one way to approach this. Further use of more sensitive methods such as mass spectrometry with focus on small hydrophobic proteins could resolve the issue with the detection of the protein.

Overall, the results from our study demonstrate a method to identify translation of toxin protein under control its native regulatory elements. Further our results indicate the possibility of type I toxins functioning not only as small proteins but also as RNAs.

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CHAPTER V

Conclusions and future directions

The work in this dissertation demonstrates for the first-time *in vivo* translation of the small toxin protein ZorO, its impacts on cellular processes, insights into the mechanism of toxicity and a possible beneficial function of the *zor-orz* locus in bacteria.

The *zor-orz* locus, first identified in *Escherichia coli* O157:H7 EDL933 (EHEC; referred to as EDL933 herein), is a type I toxin-antitoxin (TA) system (1, 2). Previous studies provided insights into the interaction of the *zorO* mRNA and OrzO small RNA (sRNA) and the results of this interaction (1). In addition, it was shown that the *zorO* transcript has a 5' untranslated region (UTR) longer than the actual coding sequence for the protein. Northern analyses after *zorO* overexpression revealed that a small fraction of these *zorO* transcripts were shorter: primer extension and 5' RACE (3) confirmed that these shorter transcripts had a processed 5' UTR ($\Delta 28$, (4)). *In vitro* translation of these two forms of *zorO* mRNA (full-length and $\Delta 28$) demonstrated that the processed *zorO* mRNA has nearly 10-fold increase in its translation as compared to the full-length 5' UTR (4). This shows a multi-layered, tight regulation of *zorO* translation.

The work in this dissertation further addressed if the differences observed from *in vitro* translation studies occurs *in vivo* and if the severity of ZorO induced toxic effects differs under the two 5'UTRs (3). We combine the conventional tools to study TA systems (ectopic overexpression) with the use of a medium-copy plasmid vector cloned with the *zor-orz* locus and its variants under its native regulatory elements (3). This is a closer representation than inducible overexpression of toxins and may better identify physiological roles of the TA systems in a bacterial cell, as cells are able to maintain these plasmids with no obvious toxic effects. The employment of such a strategy is particularly useful because in many cases deletion of these chromosomal regions does not have obvious growth phenotypes. We show that this amplification of the *zor-orz* locus improved cell growth in the presence of several aminoglycoside antibiotics (3). We also examined the changes in the global transcriptome of EDL933 to identify the cellular processes affected during the growth in the presence of kanamycin conferred by the *zor-orz* locus. To better understand the role and contribution to ZorO-induced toxicity of specific charged residues within ZorO, we performed site directed mutagenesis and studied the impacts on ZorO-mediated effects and ZorO localization after ectopic

overproduction. Finally, we examined if a non-toxic mutant of ZorO impacts cell growth in presence of kanamycin.

ZorO localization, role of 5'UTR in translation and its impacts

While small proteins are challenging to study because of their inherent small size, those targeting the cytoplasmic membrane pose an additional difficulty. A common strategy to identify and characterize proteins is to use specific antibodies to monitor detection and possible protein-protein interactions; however, given the limited epitopes that small proteins (including type I toxins) possess, it can be challenging to successfully generate antibodies against them. As an alternative, scientists can introduce a tag sequence either at N-terminus or C-terminus of the protein, but such tags on small proteins (and sometimes even on large proteins) can impact their function; indeed, adding a green fluorescent protein tag to the C-terminus of ZorO resulted in the protein being non-toxic (data not shown). To add to this complication for our studies with ZorO, its translation is regulated post-transcriptionally by the sRNA OrzO and an inhibitory region within its own 5' UTR (1, 4). Similar obstacles have also hindered the study of other type I toxin proteins. Most of the studies investigating these toxins, therefore, utilize ectopic overexpression systems by cloning the toxin genes under an inducible promoter in a multi-copy plasmid vector. Using such an overexpression system, we identified that ZorO localizes to the bacterial inner membrane *in vivo* and it can be translated from both its full-length and processed ($\Delta 28$) 5'UTR (3). Both the translation and the impacts of ZorO were significantly increased when overexpressed from the $\Delta 28$ UTR (3).

ZorO overproduction results in a rapid increase in membrane depolarization and depletion of cellular ATP levels (3, 5) and the severity of this impact is significantly increased when overexpressed from $\Delta 28$ UTR compared to the full-length UTR (3). We predict that ZorO forms small channels by inserting into the membrane; however, we do not observe ATP leakage or distorted cell morphology upon ZorO overproduction indicating that the predicted channels are small (3). These results are further supported

as we do not see leakage of small fluorescence dye calcein from the lipid vesicles contain synthetic ZorO peptides (3).

Function of the *zor-orz* locus

The chromosomal deletion strains of the *zor-orz* locus in EDL933 (Δ *zor-orz* and Δ 28-FLAG-*zorO* strains) did not show differences in growth compared to the wild type strain in LB or M9 minimal medium supplemented with glucose or fructose (3). While similar observations are reported with other TA gene pair deletion mutants, it raises the question if the *zor-orz* locus has a true biological function. Many of the type I toxins studied in this regard have utilized an overexpression system to identify the role of these proteins in bacterial persistence (6). To eliminate the complication of marked growth inhibition observed upon overproduction of ZorO and to test the effect of the *zor-orz* locus from its native promoter, we cloned the *zor-orz* locus with its regulatory elements into pBR322. *E. coli* MG1655 or EDL933- Δ *zor-orz* harboring pBR-*zor-orz* had a similar growth pattern and no significant differences in membrane depolarization or ATP levels compared to cells harboring empty vector (3). There was, however, significantly improved growth in the presence of several aminoglycoside antibiotics for those harboring pBR-*zor-orz* (3). Cells with pBR-*zor-orz* plasmid had in fact resulted in an increase in MIC against kanamycin and gentamycin (3). Similar results were observed with a plasmid harboring only the *zorO-orzO* locus (pBR-*zorO-orzO*). While increased MIC against aminoglycoside antibiotics has been reported during overexpression of *de novo* synthesized small proteins (7), this is the first study showing the role of a type I TA system to impact MIC, to our knowledge. Persistence, a mechanism by which a sub-population of bacterial cells can survive lethal doses of antibiotics and can replenish the population upon removal of antibiotic pressure has been linked with the type I toxins TisB and HokB (6, 8–10). It was surprising to see that the *zor-orz* locus improved growth of bacterial cells in the presence of lower concentrations of aminoglycoside antibiotics as this has not been reported for other systems (3). Therefore, to identify the key cellular processes that contribute to improved growth in aminoglycosides, we treated mid-log bacterial cultures (EDL933- Δ *zor-orz*)

harboring either pBR322 or pBR-*zor-orz* vector with 10 µg/ml of kanamycin for 15 minutes and extracted RNA to perform transcriptomics.

The transcriptomic data identified 473 differentially expressed genes ($p < 0.05$; 234 upregulated and 239 downregulated) in cells harboring pBR-*zor-orz* compared to those harboring the empty vector. Functional enrichment of the genes with a log₂-fold change of 1.5 or greater showed that genes associated with nitrogen metabolism (*narG*, *narH*, *narK*, *nirB*, *nrfA*) and anaerobic respiration (*dmsA*, *fdnG*, *frdA*, *glpA*) were upregulated. The flagella associated genes (*flgA*, *flgB*, *flgF*, *flgG*, *fliE*, *fliF*, *fliM*) were among the most significantly downregulated genes. While the overproduction of ZorO results in depletion of ATP, the cells with pBR-*zor-orz* treated with kanamycin appear to shut down one of the major ATP consuming processes *i.e.* flagellar motility and upregulate the genes associated with nitrate metabolism and anaerobic respiration for energy. The heat shock protein encoding genes have been reported to be upregulated during kanamycin treatment in *E. coli* to prevent the misfolding of the newly synthesized protein caused by aminoglycoside (11). Contrary to this, the pBR-*zor-orz* harboring cells have many heat response genes down regulated, suggesting lower intracellular kanamycin levels in those cells either by blocking its entry or active export.

ZorO- native translation and role of charged amino acids

Previous work demonstrated *in vitro* ZorO translation but not *in vivo* (4). The work within this dissertation (3) and others (5) showed *in vivo* translation of ZorO after overexpression from an inducible plasmid vector. To better understand the role of ZorO in cellular physiology, we switched from an arabinose inducible overproduction system (P_{BAD}) to the use of the native *zorO* promoter (P_{zorO}). We then used a medium-copy plasmid vector (pBR322) and cloned $\Delta 28$ -FLAG-*zorO* with its own promoter (no overexpression). We were able to detect ZorO using a dot-blot assay from the cells transformed with pBR- P_{zorO} - $\Delta 28$ -FLAG-*zorO* after growing in LB medium. Note that this strain lacks the OrzO antitoxin. This is the first evidence verifying ZorO translation under its own promoter. This direct evidence of *in vivo* ZorO translation can now be used to identify the conditions which result in “high” levels of this protein. To test if we can

detect ZorO via its native promoter at the chromosomal level (single-copy), we constructed a chromosomal mutant in EDL933 strain by replacing the entire *zor-orz* locus with the *zorO* gene that has a processed 5' UTR ($\Delta 28$) and an N-terminus FLAG tag ($\Delta 28$ -FLAG-*zorO*). The efforts to detect ZorO in this strain via western blot or dot-blot analysis were not successful when grown in LB, likely because of the detection limit of the method. A similar strain was generated in non-pathogenic *E. coli* MG1655 (lab strain), but still, we were unable to detect ZorO. This indicates the need of more sensitive advanced techniques such as mass spectrometry (with optimization to target small hydrophobic proteins) to investigate the translation of the type I toxins at the chromosomal level.

To investigate the mechanism of the toxicity mediated by ZorO, we again implemented the use of multi-copy plasmids to increase the copy number of *zorO*. We created several ZorO mutants to identify key amino acid residues that contribute to its toxicity. Since charged amino acids in a protein play important roles in interacting with cellular targets or in the formation of self-oligomers, we focused on mutating the charged amino acids that are distributed within the ZorO sequence. Mutational analyses revealed that in fact specific charged amino acid residues, glutamate (E) at the 16th position and arginine at the 23rd position (R23), are critical for proper localization and ZorO mediated toxic effects on cellular processes. Mutating E16 to an oppositely charged residue, arginine (E16R), or R23 to a neutrally charged amino acid, leucine (R23L), reduced ZorO mediated growth inhibition (Fig.4.2D). The lysine residues at positions 7 and 29 are also critical as observed via diminished toxicity when changed to glutamate. While the mutation of glutamate at the N-terminus (D2) did not impact ZorO toxicity, the one at the C-terminus (D26) mutated to either lysine or glutamine rescues cells from ZorO toxicity (Fig. 4.2A and B). The orientation of ZorO within the membrane could be responsible for toxicity of the D26 variant. Topology predictions tools suggest a mixed orientation for ZorO, some tools suggesting N-terminus in and others suggesting out, but the true orientation has not been experimentally validated. This indicates the role of charged amino acids present both inside and outside of the predicted transmembrane helix might be important for the electrostatic interactions or membrane

insertion. Analysis of ZorO mutant variants via western blot to verify if there were any differences in protein stability and localization showed that the non-toxic mutants had more protein accumulated in their membrane as well as in the cytoplasm unlike wild-type ZorO or the toxic derivatives. However, the membrane localization of the non-toxic variants was not sufficient to affect the cell growth.

Two other membrane associated type I toxins (HokB and TisB) have been shown to form multimers (12, 13). While HokB forms a dimer via disulfide bonds between the cysteine residues of two monomers, TisB forms a dimer-of-dimers forming a tetrameric charged zipper structure (12, 13). While we can see ZorO migrating at a higher molecular weight (especially with the R23L mutant) via SDS-PAGE, indicating a possible oligomeric state, similar to studies examining synthetic ZorO interacting with liposomes, further evidence is needed to verify if ZorO oligomerizes. This is important for better understanding of the mechanism of the ZorO toxicity.

Considering our results from the loss of ZorO toxicity after mutating specific charged residues, we further investigated whether multiple copies of *zorO*-R23L-*orzO* could improve growth in the presence aminoglycosides. We mutated plasmid pBR-*zorO-orzO* to pBR-*zorOR23L-orzO* (maintaining native *zor-orz* promoters and regulatory elements). We then tested its ability to improve growth in the presence of kanamycin. Surprisingly, *E. coli* harboring pBR-*zorO*-R23L-*orzO* had similar improved growth in LB with kanamycin as the cells harboring pBR-*zorO-orzO*. This indicates ZorO might function in two distinct ways: improved growth of the cells in the presence of aminoglycoside antibiotics when produced “non-toxically” and then reduced growth if produced at too high of a level.

Removal of the elements preventing *zorO* translation *i.e.* the antitoxin OrzO and the first 28 nts from *zorO* mRNA in the pBR plasmid did not improve bacterial growth in medium with kanamycin (Fig. 4.7). This observation was unexpected as increasing *zorO* translation (by removing translation repression elements) did not correspond to an increase in the growth advantage of the cells in kanamycin. This also led to the question if OrzO played role in the growth advantage, and we tested this by overexpressing OrzO but we did not see improved growth (Fig. 4.10).

While ZorO has similar characteristics to other type I toxins such as TisB, mainly, localizing to the bacterial inner membrane, and affecting cells by membrane depolarization and ATP depletion, the improved growth in the presence of antibiotics was specific to aminoglycoside in the case of the *zor-orz* locus (3). TisB increases persister cells against multiple class of antibiotics with no reports on increase in minimum inhibitory concentration (9, 10), whereas with ZorO, we observed an increase in resistance against the aminoglycosides. Unlike TisB, we were unable to see differences in membrane depolarization at the chromosomal level even after removing the regulatory elements *i.e.* antitoxin and the inhibitory region of its 5'UTR. Why ZorO and the *zor-orz* TA system behave differently than the *tisB-istR* system despite their similarities is still unknown. Further, as the non-toxic mutant derivative (pBR-*zorO*R23L-*orzO*) was capable of improving the growth in presence of kanamycin suggests that the *zor-orz* system does not follow the conventional mechanism of cell growth arrest observed with TisB.

How is the *zor-orz* locus able to improve the growth of the cells in presence of aminoglycoside antibiotics? While the improved growth effects by non-toxic mutant pBR-*zorO*-R23L-*orzO* indicated involvement of a mechanism, separate from the one by which ZorO is toxic, current work in the lab further indicates that translation of *zorO* might not be essential for the *zor-orz* mediated improved growth in presence kanamycin. A pBR-*zorO*-AAG-*orzO* mutant, in which the start codon of the ZorO is mutated to the AAG, is also able to improve cellular growth in the presence of kanamycin. To support this observation, the plasmid constructs that contained the *zorO* 5' UTR (fused to a fluorescent reporter) on a separate plasmid (pACYC) also showed improved growth in kanamycin (Shore S., unpublished results). This is an interesting finding that opens the door to a different modality for type I toxins: perhaps they may be able to function as an RNA to improve the cell growth in specific condition. Future work should be aimed at dissecting this. In silico analyses can be used to determine if there are specific targets with sequence complementarity that the *zorO* mRNA may bind to and regulate their production. These results then can be employed to see if the possible targets of *zorO* have a differential gene expression level in our transcriptomics data.

The use of a plasmid vector either overexpressing or constitutively expressing the *zorO* 5'UTR can further validate the current results obtained for the improved growth in kanamycin. We can then further investigate if other type I TA gene pairs show similar model of improved growth in a specific growth environment as a function of the transcribed RNA.

The identification of small open reading frames encoding small proteins including those that occur as type I TA system is increasing (14). While the use of advanced techniques such as mass spectrometry-based proteomics and ribosome foot printing combined with transcriptomics and proteomics has experimentally validated the presence of these small open reading frame encoded proteins (15–18), the biochemical and functional characterization of these small proteins remains to be a challenge. Further, the chromosomally encoded type I TA system appear to function in a much diverse way than suggested.

Future directions

Based on our point mutation studies, we observed that the charged residues are critical in ZorO mediated toxicity (Fig. 4.2). The mutation of the aspartate at the 2nd position, however, did not impact ZorO toxicity. This might be the result of how ZorO is associated within the membrane and the location of the C-terminus vs the N-terminus in either the cytoplasm or periplasm. Experiments using liposomes or inner membrane vesicles derived from *E. coli* and subsequent treatment with proteinase K can shed more light on the topology (C_{in}-N_{out} or N_{in}-C_{out}) of ZorO in the membrane (19). If ZorO could efficiently insert into the liposomes, then it would indicate that the membrane insertion of ZorO does not depend on any insertion pathway, whereas if the insertion is only observed in the inner membrane vesicles, then it would indicate that a specific insertion pathway is essential. However, we need to be aware that since ZorO is hydrophobic, spontaneous insertion into the liposomes can occur over time. The role of signal recognition particle (SRP) and migration of small protein encoding mRNAs to the membrane have been observed with other small proteins that are similar size to ZorO (19).

Laboratory observation based on SDS-PAGE analysis of FLAG tagged ZorO and point mutants of ZorO (particularly the R23L mutant) shows additional higher molecular weight band(s). This suggests that ZorO might form an oligomer or interact with other proteins within the cell. Use of a native page gel can be useful to identify if ZorO forms oligomers; protein-protein interaction can be studied using bacterial two hybrid, co-immunoprecipitation, or pull-down assays.

While we anticipated that the removal of the regulatory factors of *zorO*, the OrzO sRNA and the inhibitory region of the 5'UTR of the *zorO* mRNA, would enhance ZorO translation and further improve growth in presence of aminoglycosides, the result indicated otherwise (Fig. 4.7). Overexpression of OrzO alone was also not able to improve the growth in kanamycin, but instead resulted in worse growth than the negative control. This could be the result of OrzO basepairing with RNAs other than *zorO*. While co-expression of *zorO* and *orzO* neutralize each other, overexpression of OrzO alone could now make OrzO available to interact with other RNAs and impact cell growth. Growth analyses with a regulated OrzO expression at different levels can further help understand the role of OrzO. Another possible hypothesis is that both *zorO* and *orzO* are required for the improved cellular growth in the presence of aminoglycosides. Further work will be done to rule out if this is the case and if the cells require a double stranded RNA (after *zorO* and OrzO interaction) or processing of the *zorO* mRNA upon interaction with the OrzO sRNA to improve growth in presence of aminoglycosides. In addition, other targets of the OrzO sRNA or *zorO* mRNA can be assessed using bioinformatic tools and then experimentally verified.

E. coli harboring pBR-*zor-orz* plasmid is able to grow immediately in presence of kanamycin (4 µg/ml) as compared to the empty vector control that starts growing after a long lag (~8-10 hours). The transcriptomics results show downregulation of genes associated with heat shock proteins in cells with pBR-*zor-orz*, indicating the cells are not stressed by the antibiotic. This indicates that the presence of the *zor-orz* locus can function either to decrease the uptake of the antibiotic into the cytoplasm, increase the efflux of the antibiotic from the intracellular space, or increase ribosomes which are the targets of aminoglycoside antibiotics. Comparison of 30S ribosomal subunit levels of

overnight cultures of cells harboring pBR vs pBR-*zor-orz* will be helpful in identifying the basal level of targets available for aminoglycosides in these two groups of the cells. The overnight cultures can also be compared for the presence of any modification of the aminoglycoside target such as 16s rRNA methylation.

Overall, rising above the challenges of studying small proteins encoded in the bacterial chromosome, this dissertation work demonstrates that a type I TA system can function in a way not previously demonstrated. While the overexpression of the toxin gene is detrimental to the cells through membrane depolarization and ATP depletion, the *zor-orz* locus in a medium copy plasmid improved cell growth in the presence of aminoglycoside antibiotics, linking its possible true biological function while expressing via native regulatory elements (and not overexpression). Using several point mutants in an artificial overexpression system, we were able to identify the specific charged residues involved in ZorO mediated toxicity. The non-toxic ZorO mutant derivative R23L was still able to improve growth in the presence of kanamycin indicating that ZorO-induced toxicity and *zor-orz* mediated function during growth in the presence of aminoglycoside antibiotics are not directly linked. This has opened the doors to further study the “unusual” mechanism that the *zor-orz* gene pair utilizes to improve survival and growth in presence of specific antibiotics.

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VITA

Bikash Bogati was born in a bucolic village in Nepal. He joined laboratory science in 2001 because he was told that after three years of training, he would be able to get a job that makes a good living and won't require him to study anymore. Fast forward two decades; he is still in the school system. Raised by a father who was not able to complete high school and a mother who cannot write her name, Bikash (if you are reading this in future), finished his Ph.D. in Microbiology from the University of Tennessee, Knoxville in December 2021.

Bikash started as a bozo in the Fozo lab. He now knows that earning a PhD is just the beginning and he has a lot more to learn from and add to his life. He believes that compounding small, everyday actions is the key to achieving big impacts. Volunteering, hiking in the Smoky Mountains, playing tennis & volleyball, baking and being around friends are a few things that significantly helped him endure the journey of graduate school. Helping people around him and sharing smiles with them makes him happy. Bikash regards himself as a global citizen and aims to work towards alleviating the burden of infectious disease and antibiotic resistance.