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TITLE: Unlocking the Potential of Bacterial ParE Toxins: Developing a Blueprint for Co-Opting Molecular Time Bombs That Impact Bacterial Cell Survival

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14. ABSTRACT
This project aims to "Unlock[ing] the potential of bacterial ParE toxins: developing a blueprint for co-opting molecular time bombs that impact bacterial cell survival". The central problem addressed by the aims is of treating bacterial infections with an outcome of making existing antibiotics work better, and in understanding a fundamental bacterial mechanism that may help bacteria become resistant. When successful, this will provide an innovative new way to control bacterial growth, including antibacterial resistant strains.

During the funding period we established a potent toxicity of ParE toxins to their native bacterial hosts. Some variation in the absolute toxicity was noted, and appears to reside with the toxin itself rather than the host bacteria. One toxin, as previously noted, is attenuated and does not mediate decreased growth. We found that the extent of toxicity correlates with an increase in the frequency of mutations except for in P. aeruginosa, which appears to have potent repair pathways. However, the increased mutation frequency does not correlate to loss of sensitivity to antibiotics. Surprisingly we note a collateral sensitivity such that ParE expression increases the impact of some antibacterial classes, including anti-gyrase antibiotics. Technical complication limited progress on Aim 2, which was designed to demonstrate antitoxin removal and thus liberation of ParE toxins within cells. Continued optimization of novel detection methods is being explored beyond the current proposal.

15. SUBJECT TERMS
Toxin-antitoxin systems, antibacterial applications, toxicity, mutations

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1. Introduction

The objective of this project is to provide proof-of-concept for a new way to control bacterial growth, including genetically resistant as well as phenotypically persistent bacteria. This approach will manipulate chromosomally-encoded bacterial “time bombs” called toxin-antitoxin (TA) systems. As emerging infections and increases in resistance make the need for antibacterials more pressing, it is also increasingly evident that our homeostatic balance and health also depend on bacteria. This revelation then further challenges antibacterial approaches to minimize impact on beneficial “good” bacteria. **Incorporation of narrow-spectrum antibacterial treatment approaches are highly desirable to minimize disruption of the host microbiome.** Antibacterial discovery has long relied on directed serendipity via screening of natural products and libraries to identify inhibitors and their corresponding bacterial targets. Currently the most fruitful approaches are dominated by derivatization of existing antibacterials; these activities are absolutely required for short-term defenses against infection. However, **longer-term approaches that rely on new and unique strategies are badly needed, especially as emerging resistance is outpacing antibacterial development.**

2. Keywords

Antibacterial strategy, toxin-antitoxin system, gyrase inhibitor, mutation frequency, collateral antibacterial sensitivity, protein degradation

3. Accomplishments

Major goals of the project

Hypothesis: That the presence of ParE toxins within a bacterial cell imparts (1) an increased mutagenic potential that at a native concentrations contributes to emerging antibiotic resistance, and that (2) increasing ParE toxin activity can significantly weaken the bacterial cell’s ability to survive, and this effect will be additive or synergistic with existing antibiotic regimens. To assess this hypothesis the following specific studies are in progress:

Specific Aims:
(1) Determine the spectrum of ParE activity in native hosts by measuring viability, accumulation of mutations, and antibiotic susceptibility as a function of induced ParE toxin expression.
(2) Increase ParE availability *in vivo* as proof-of-concept of a therapeutic approach by engineering each targeted species’ ParD antitoxin degradation model system in an *E. coli* host.

The outcomes of this project will be (1) identifying a fundamental mechanism potentially contributing to rise of resistance, providing a window for potential intervention, and (2) demonstrating proof-of-concept of co-opting this mechanism into a novel treatment that by definition will be specific for a given bacterial species. This idea is directly responsive to the “Area of Encouragement” identified as “Antimicrobial Resistance”, for the “Development of novel and/or innovative interventions to prevent the spread of or treat infections from multi-drug-resistant organisms, focused on hardware-associated infections and biofilms.”

Milestones	Final Status
Aim 1	
1. Determine dose-dependence of individual ParE toxicity	*Completed*
2. Determine impact of ParE expression on mutation accumulation	*Completed*
3. Determine impact of ParE expression on antibiotic susceptibility	*Completed*
Aim 2	
1. SspB induced degradation experimental test system build complete	*Completed*
2. Determine the extent of individual ParD antitoxin degradation in response to SspB induction	*Inconclusive*
3. Determine the impact of degrading individual ParD antitoxins on viability and morphology	*Not achieved*

Accomplishments under these goals

Summary: We have established that toxicity mediated by ParE proteins is directly correlated with mutation frequencies. This relationship varies modestly with individual ParE proteins, deduced by testing the same construct in native host bacteria and in *E. coli* MG1655. The exception to this is the PaParE1 toxin, which we had previously identified as attenuated with toxicity enhanced in *recA*- *E. coli* strains. We have now additional evidence that the outcome of ParE-mediated toxicity is strongly influenced by the pathways in the bacterial strain to mediate DNA repair. Some bacteria, such as *P. aeruginosa*, escape from gyrase-mediated toxicity and limit mutagenic events, even when fluoroquinolone antibiotics are used instead of the ParE toxins. In contrast, *B. cenocepacia* readily accumulates mutations and is very sensitive to ParE toxicity.

Importantly, we have established that the ParE-mediated increase in mutation frequency does not drive antimicrobial resistance (AMR). We had hypothesized that ParE proteins were an intrinsic mechanism to generate stochastic mutations such that selection upon treatment could produce AMR strains. Our current studies demonstrate this is not the case except for protection from other gyrase-targeting antibiotics. In some cases, even relatively non-toxic levels of ParE toxin weakened bacterial cells to potentiate antibiotic action. These findings are anticipated to be submitted for publication by the end of the project.

These outcomes strongly support our long-term application of manipulating ParDE systems to control bacterial growth.

Aim 2 was designed to provide insight into the ease of removing the neutralizing ParD antitoxin from the ParE toxin within a cell. This would establish feasibility of the long-term goal to manipulate the pairing of these two proteins while maintaining ParE-mediated impacts. Unanticipated technical issues arose that limited quantitation of antitoxin levels. Attempts were made to alter our methods; however, only qualitative differences could be visualized, they were not sufficient to draw conclusions. We are continuing to pursue this aim because it is important to our research program, and we will continue to seek funding for this to speed progress.

Methods, Data, and Interpretation

Aim 1 Subtask 1: Cloning ParE toxin genes, ParD antitoxin genes, and ParDE operons into appropriate vectors, establishing bacterial stocks and propagation SOPs, and transformation of constructs. Subtask 1 is complete.

This study has focused on seven unique ParDE TA systems from four bacterial pathogens: *Pseudomonas aeruginosa (*Pa), *Burkholderia cenocepacia* (Bc), *Vibrio cholerae (*Vc), and *Mycobacterium tuberculosis* (Mt).

Table 1. ParDE TA system constructs generated for Aim 1.

Source Bacteria	**Native Operon(s)**	**Successful Cloning** (also with tag)
P. aeruginosa (Pa)	PaDE1, PaDE2	pHerd20T: DE1, (His-)E1, (His-)D1, pHerd20T: DE2, (His-)E2, D2
B. cenocepacia (Bc)	BcDE1	pSCrha2: DE, (Strep-)E, (Strep-)D
M. tuberculosis (Mt)	MtDE1, MtDE2	pMindBAD: DE1, E1, D1, pMindBAD: DE2, (Strep-)E2, D2
V. cholera (Vc)	VcDE1, VcDE2	pBAD33: DE1, (Strep-)E1, (Strep-)D1 pBAD33: (Strep-)DE2, (Strep-)E2, D2

The PaE2 toxin was too potent to obtain transformants in the *P. aeruginosa* PA14 strain, which does not natively encode this TA system and so lacks a chromosomal copy of the antitoxin. We were unable to demonstrate expression in the *M. smegmatis* model system and have relied on *E. coli* MG1655 as a

surrogate for the two MtParDE systems.

Aim 1 Subtask 2: Viability assays to assess toxicity versus induction strength. Subtask 2 is complete (see Table 2 for summary).

Table 2. Summary of results for the viability assays of ParE toxins

Construct	**Bacterial strain tested**	**Max. ∆CFU/mL**	**@ time, %induction**
pSCrhaB2::BcParE	*B. cenocepacia* LMG 16656	4.8-log reduction	15 hrs, 0.002% rha
	E. coli MG1655	3.5-log reduction	8 hrs, 0.2% rha
pBAD33::VcParE1	*V. cholerae* El Tor 16961	4.0-log reduction	8 hrs, 0.2% ara
pBAD33::VcParE2	*V. cholerae* El Tor 16961	5.4-log reduction	8 hrs, 0.2% ara
pHERD::PaParE1	*E. coli* MG1655	0.2-log reduction	8 hrs, 2% ara
	P. aeruginosa PAO1	0.05-log gain	8 hrs, 2% ara
	P. aeruginosa PA14	0.9-log reduction	8 hrs, 2% ara
pHERD::PaParE1	*P. aeruginosa* PAO1	4-log reduction	4 hrs, 0.2% ara
	E. coli MG1655	4-log reduction	4 hrs, 0.2% ara
pMINDBad::MtParE1	*E. coli* MG1655	3-log reduction	4 hrs, 0.2% ara
pMINDBad::MtParE2	*E. coli* MG1655	7-log reduction	4 hrs, 0.2% ara

No toxicity was exerted by expression of any ParD antitoxin, or of any ParDE operon construct. The addition of N-terminal Strep or His tags did not alter toxicity of any tested ParE toxin.

Aim 1 Subtask 3: Fluctuation assays to determine if ParE toxins increase native mutagenic capacity Subtask 3 is complete (see Fig. 1). The method for these investigations required considerable optimization to balance expression, toxicity, and time (see legend for details of procedure).

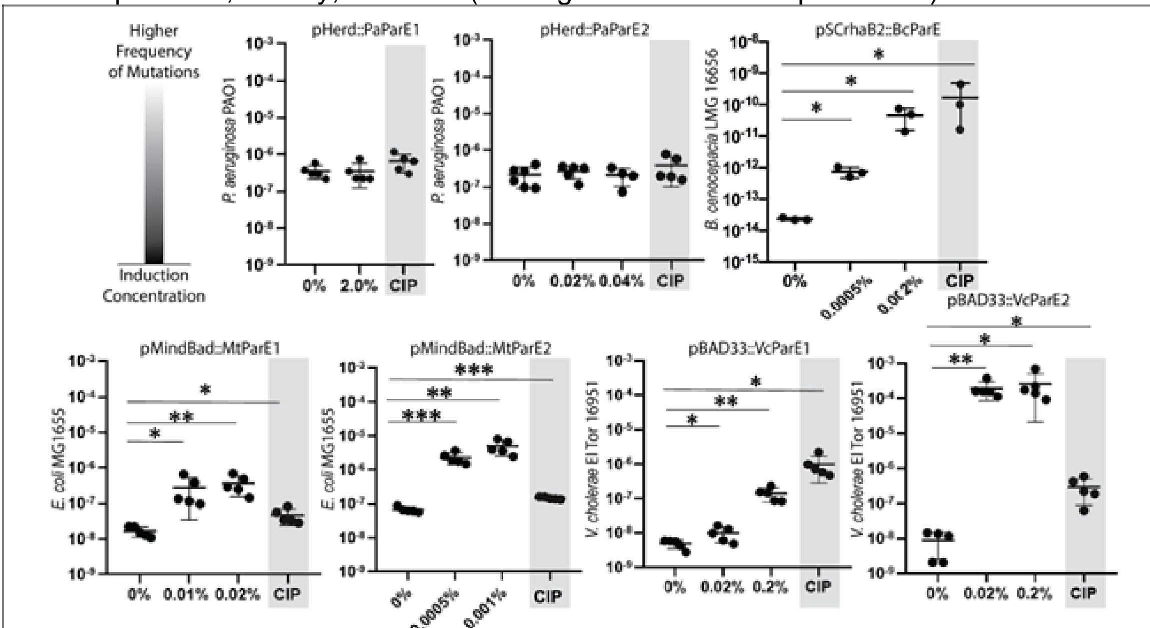

Figure 1. The frequency of mutations is increased as a result of ParE-mediated inhibition of DNA gyrase. The exception is for the PaParE2 protein, which is potently toxic and sterilizes the cultures before mutagenesis can occur (note the low induction concentrations tested). *P. aeruginosa* appears resistant to accumulation of mutations, as the control samples treated with sub-MIC ciprofloxacin did not invoke mutations. In contrast, *B. cenocepacia* has lower mutation frequencies for all samples.

The frequency is derived from: ratio of [number of colony forming units on high selection of an antibiotic requiring a single mutation in its target gene] to the [total colony forming units in culture]. Selection of mutants utilized: *P. aeruginosa*, 3X MIC trimethoprim; *B. cenocepacia*, 3X MIC rifampicin, *E. coli*, 3X MIC rifampicin, *V. cholerae*, 2X MIC rifampicin. All MIC values were determined in house using the same growth conditions as in this assay. Statistical significance by unpaired *t* test:*$p<0.05$ **$p<0.01$ ***$p<0.001$

Aim 1 Subtask 4: Antibiotic susceptibility assays as a function of ParE toxin expression
Subtask 4 is complete (see Tables 3-6).

Table 3. MIC values (µg/mL) for cultures expressing ParE toxins

P. aeruginosa PAO1		PIP	TOB	LEV	MPN
pHerd::PaParE1	*0% ara*	12	4		
	1% ara	12	4		
pHerd::PaParE2	*0% ara*	12	4	0.38	0.125
	0.1% ara	4	4	0.19	0.016*

**denotes a 2-fold or greater change*

Table 4. MIC values (µg/mL) for cultures expressing ParE toxins

B. cenocepacia LMG 16656		MPN	RIF	PIP	PIP/TAZO	LEV
pSCrhaB2::BcParE	*0% rha*	4-6	24	>256	>256	6
	0.0005% rha	6-8	32	>256	128	6
	0.002% rha	6	32	>256	96*	4-6

**denotes a 2-fold or greater change*

Table 5. MIC values (µg/mL) for cultures expressing ParE toxins

V. cholerae El Tor N16961		AZM	DOX	LEV
pBAD33::VcParE1	*0% ara*	2	0.25	0.008
	0.02% ara	2	0.25	0.016
	0.2% ara	2	0.38	0.023*
pBAD33::VcParE2	*0% ara*	2	0.38	0.008
	0.02% ara	1	0.094*	0.004
	0.2% ara	1	0.094*	0.008

**denotes a 2-fold or greater change*

Table 6. MIC values (µg/mL) for cultures expressing ParE toxins

E. coli MG1655		TMP	MPN
pMindBad::MtParE1	*0% ara*	0.047	<0.016
	0.02% ara	0.023*	<0.016
pMindBad::MtParE2	*0% ara*	0.094	<0.016
	0.01% ara	0.047*	<0.016

**denotes a 2-fold or greater change*

Aim 2: The premise of this design is to append a degradation signal peptide (called “DAS”) to a protein of interest, and selectively express a protease adaptor (SspB) that will recognize this signal and deliver the protein of interest to the bacterial ClpP protease system. We sought to test if the ParD antitoxin could be removed from the ParE toxin within the bacterial cells by appending the signal peptide to the ParD antitoxin. As noted above, however, antitoxin detection proved difficult and resulted in only qualitative results.

Aim 2 Subtasks 1: Build constructs for the inducible-degradation system

Our experimental design is based closely on previous studies. We selected the inducible expression vector pQE encoding the SspB adaptor protein to deliver tagged cargo to ClpP for degradation in the cell. We then utilized a pBAD33 backbone for cloning of the TA system components, as this vector has a unique origin of replication, selection marker, and inducible promoter from the pQE vector.

	Antibiotic resistance marker	*ori*	Promoter induction signal
pBAD33	Chloramphenicol	p15A	arabinose
pQE	Ampicillin	pBR322	lactose or IPTG
E. coli* W3110 Δ*sspB	Kanamycin		

pBAD33 backbone derived from pBAD33.lon, a gift from Robert Sauer (Addgene plasmid # 22145)
pQE-80L MBP-SspB Nano was a gift from Brian Kuhlman (Addgene plasmid # 60409)
E. coli W3110 Δ*sspB* strain was a kind gift from Peter Chien, U Mass Amherst.

Our original approach was to modify the pBAD33 plasmid to carry two copies of the arabinose-inducible promoter (P_{ara}) to ensure equal production of the toxin and antitoxin proteins, as the typical arrangement in the TA operon produces excess antitoxin. However, we were not successful in building this construct despite considerable effort.

Our next approach, which was successful, was to drive expression of both antitoxin and toxin from the same promoter but to add an additional ribosome binding site before the second open reading frame (Fig. 2). We have also added a Strep-tag to the N-terminus of the ParD antitoxin, and a His-tag to the N-terminus of the ParE toxin ORF, to allow detection of each by Western blotting. Additionally, we have appended to the C-terminus of the antitoxin a tag for recognition by the SspB adaptor (“DAS”). Throughout these cloning strategies we have used the PaParDE1 system to avoid complications of toxicity. All constructs were verified by nanopore whole plasmid sequencing (Plasmidsaurus).

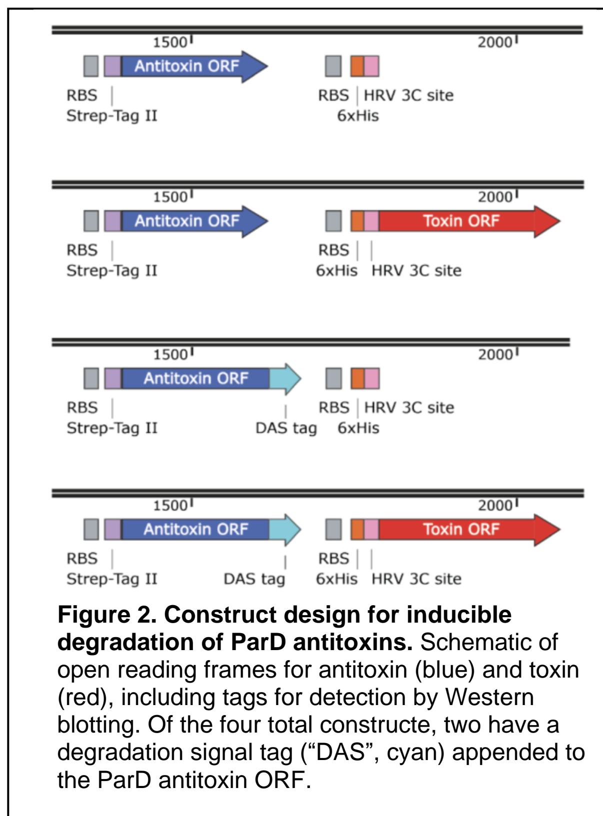

Figure 2. Construct design for inducible degradation of ParD antitoxins. Schematic of open reading frames for antitoxin (blue) and toxin (red), including tags for detection by Western blotting. Of the four total constructe, two have a degradation signal tag (“DAS”, cyan) appended to the ParD antitoxin ORF.

Pilot expression studies demonstrated robust expression of SspB from the pQE vector, while ParD antitoxin and ParE toxin were difficult to distinguish. It appeared that antitoxin is expressed and this is reduced when the SspB protease adaptor is induced. However, Western blotting probing for Strep-tagged ParD antitoxin could not detect this difference.

As the Aim is dependent on measuring ParD antitoxin amounts, we sought to increase its signal. Given the strong expression of the SspB protein, we swapped the inserted ORFs between these vectors. Pilot expression studies again demonstrate robust expression of SspB and ParE toxin, while ParD antitoxin detection remains too low for reliable detection.

This is unexpected, as the ParD and ParE proteins are driven by the same promoter, and further, the ParE is downstream in the gene order, yet it is robustly expressed.

These studies are carried out in a unique *E coli* strain that has been mutated to remove the native SspB protein. We repeated the expression studies in both cloning and protein expression strains of *E. coli*, yet have the same limited detection. We have also evaluated alternative detection methods, as Western blotting has proved difficult likely due to the enriched positive charge of the ParD antitoxin proteins (pI values of ~ 9, on average) combined with their small size (~ 9-12 kDa). An in-house collaboration with Dr. Si Wu attempted to measure antitoxin amounts using a top-down mass spectrometry approach; however, the level of expression was too low. We reasoned that an enrichment method, while presenting additional limitations and requiring reproducible capture of all tagged protein, may be helpful. Attempts to enrich antitoxin using beads with affinity to the Strep tag followed by electrophoresis are promising with respect to the measurable signal, but reveal many contaminants and potentially incomplete depletion from the lysate. We are continuing to optimize this approach.

Of note, we have been motivated to develop a new generalized method for detection of these small and low abundance proteins (both toxins and antitoxins) that could address the technical issues from this aim. This method is listed under "Products", below.

Aim 2 Subtasks 2: Determining extent of ParD antitoxin degradation with and without the cognate ParE toxin present
Inconclusive. A robust detection method for antitoxin levels is required.

Aim 2 Subtasks 3: Assessing the gain of phenotypes expected for gyrase inhibition as a function of ParD loss
Not achieved.

Conclusions

Our hypothesis is that lower amounts of ParE toxin, such as at bacteriostatic levels, may increase the number of cells surviving and thus containing repaired DNA. These repair pathways are intrinsically error-prone, and thus may contribute to an undesired increased resistance. Subtask 3, however, highlights a lack of increased mutagenic capacity at non-toxic (bacteriostatic) ParE induction levels. This would be ideal for our approach; however, we wanted to assess the potential for antibiotic tolerance to impact our translational goal. When combined with results from subtask 2 (viability assays), it appears that overall increased levels of mutations correlate with higher toxicity, but rather than promoting mutations for survival they instead contribute to loss of viable cells. This collateral sensitivity may prove useful and is an ideal outcome for the application of our translational approach, where ParE-toxin levels could be manipulated to control bacterial growth without imparting resistance to antibiotics. Additionally, this gives us insight into origins of antibacterial resistance, which are unlikely to arise from stochastic mutations such as those mediated by gyrase inhibition by ParE toxins.

What opportunities for training and professional development has the project provided?

The support of graduate students as part of their training is appreciated and very valuable to their development as well as progress of the project. In addition, this year two undergraduates are working directly with the PI to establish protocols for Aim 2.

How were the results disseminated to communities of interest?

A manuscript encompassing all achieved results is in preparation and submission for publication is anticipated before the end of the calendar year. Results from this project have been presented at conferences and are listed below (6. Products).

What do you plan to do during the next reporting period to accomplish the goals?
Not applicable.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

We have identified that six of the seven tested ParE toxin proteins reduce bacterial host cell viability in a dose-dependent manner. Lower concentrations of ParE induction lead to bacteriostatic effects, while up to a 5-log reduction can be achieved in actively growing cultures. *This is promising for our application of manipulation of these systems to control bacterial growth.*

Further studies indicate that a loss of viability is due to DNA damage, evident by increased rates of mutation, but that these are not causing overt changes in the MIC values for different classes of antibiotics. *This supports our application of potentiating antibiotic treatments by manipulating these specific TA systems.*

The remaining objectives from Aim 2 are designed to test if ParE toxins can be liberated from a complex with their cognate antitoxins. A poor detection limit for the antitoxin precluded definitive measurement,

which limited this line of investigation. Methods development studies beyond the scope of this award are already underway to improve antitoxin quantitation. *The next logical step for these studies are proposing viable approaches to manipulate toxin levels, including induced degradation of antitoxin and/or small molecule inhibitors of these interactions, and these will be proposed in on-going funding applications.*

What was the impact on other disciplines?

This project is directly responsive to the pressing need for alternative antibacterial strategies. The demonstration that ParE toxins can be co-opted will be transformative in multiple fields, including microbial physiology, therapeutic development, and the wider TA community. The outcomes have the potential to offer (1) very high specificity to a single pathogen, (2) versatility in providing a means to re-sensitize "tolerant" metabolic states to current treatments in a potentiating approach, (3) will provide insight into a potential fundamental mechanism of genetic resistance through error-prone repair, now recognized as likely only after such high doses of toxin-induced DNA damage that the cells are not viable, and (4) targeting of the antitoxin is predicted to be less prone to resistance because of the need to maintain a productive pairing between cognate toxins and antitoxins.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?

Valuable reagents have been generated, and in the process essential training of graduate students has taken place. Graduate students have presented outcomes from these studies at the Great Plains Infectious Disease conferences and at the ASM Missouri Branch regional meetings.

This study provides initial proof-of-concept demonstrating that, in general, most ParE toxins are feasible to target as they lead to reduced bacterial cell viability without increasing antibiotic tolerance or resistance. Our objective is to further development our idea into an applied product that co-opts this mechanism into a novel treatment with strong potential to potentiate existing antibiotics, and that by definition will be a narrow-spectrum approach thus sparing the normal microbiota.

5. Changes/Problems

Changes in approach and reasons for change.
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them.

A one year no-cost extension was granted on Oct. 20, 2021. This was requested based on delays early in the project initiation, including a complete laboratory closure (March 24 – May 22, 2020) with limited access until July 1, 2020. Further limitations were experienced with procuring supplies, and a hiring freeze and issues with visa processing preventing hiring of a postdoctoral fellow as was originally planned. Supply chain issues have been a continual issue (for example, pipette tips are routinely backordered for four months). Additionally, during 2022 the graduate student assigned to complete the studies for *B. cenocepacia* left the program early.

Technical issues arose in using *Mycobacteria smegmatis* as a surrogate host for two of the toxins under study. Expression vectors (pMind) were tested but no toxicity prompted examination of expression levels, revealing that no detectable toxins were produced. We altered this vector to add an inducible promoter; however, this did not restore induction in the *Mycobacterial* strain. These vectors were able to readily produce the toxin in *E coli* cultures, and therefore these were used to complete tasks in Aim 1.

Additional technical issues in detecting antitoxin levels directly from bacterial lysate prevented completion of Aim 2. Visualization of the antitoxin protein bands directly on PAGE gels was not reliable due to low expression and many other protein bands. We moved to detection by Western blotting; however, the highly basic nature and small size of the antitoxin protein precluded successful transfer to membranes. We altered the expression format to increase the amount antitoxin, as well as sampled shorter intervals in case

degradation happened quickly, but were unable to overcome the signal to noise issues. During the final time period we attempted to enrich the antitoxin samples for analysis; this approach holds promise but will require further optimization before clear and reproducible signals can be obtained.

During the final reporting period an additional 10 week no-cost extension was requested to give more time to complete Aim 2. This was granted, shifting the completion date from Feb 15, 2023 to May 31, 2023.

Changes that had a significant impact on expenditures.
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to report.

6. Products

Reagents
We have now built many constructs for expression of these toxic systems. This includes modification of the pMind vector to replace the *tet* inducible promoter with that from a pBAD vector, allowing *ara* induction. We have also generated constructs with affinity tags (Strep, His) position in multiple cloning sites, which may be useful to others. These will be made available to other researchers upon request, and may be deposited with the Addgene repository after publication. Transformed strains may also be available upon request upon demonstration of BSL2 ready facilities and necessary shipping documentation.

Books or other non-periodical, one-time publications.
Nothing to report.

New lines of investigation developed from this study
We have initiated additional studies that build directly from Aim 1 subtask 2 to carry out top-down proteomic experiments to correlate qualitative numbers of ParE toxin molecules per noted toxicity. Pilot studies using the MtE1 and MtE2 toxins have demonstrated very low amounts of these ParE proteins; methods are being developed to increase the signal of these within a lysate while preserving the relative abundance information.

An additional observation that has seeded new investigations is the partial survival of cultures at longer time points of ParE exposure. We routinely observe a dramatic reduction of viable bacterial cells in the 4-8 hour window after induction of ParE; however, this effect plateaus and in some cases cultures begin to re-grow at 18-24 hours. Initially we hypothesized that suppressor mutants were arising, likely in the gyrase enzyme, allowing escape from ParE inhibition. This is of high interest as the molecular mechanism of ParE-gyrase interaction remains unknown. However, experimentation has pointed instead to alterations in plasmid copy number. A manuscript will be submitted before the end of the calendar year describing this “path of least resistance” to “resistance”.

Other publications, conference papers, and presentations.

Davis, J., Bourne, C.R. “Characterizing the mutagenic capacity of an attenuated ParE toxin from Pseudomonas aeruginosa” American Society for Microbiology Branch Meeting, March 13th-14th, 2020, Liberty, MO. *Cancelled due to CoVid-19

Davis, J., Bourne, C.R. “Characterizing the mutagenic capacity of an attenuated ParE toxin from Pseudomonas aeruginosa” OU Undergraduate Research Day, May 1, 2020, online.

Bourne, C.R. “Surprises in Gyrase-inhibiting ParDE TA systems” Oral Presentation, Joint Meeting of the Missouri and Missouri Valley Branches of the America Society for Microbiology, March 20th, 2021, virtual.

Holt, M., Ruan, S.R., Bourne, C.R. “Intersection of ParE toxin expression and antibiotic susceptibility in pathogenic bacteria” Poster-Oral hybrid. IDeA Central Region Conference, July 26th, 2021, virtual.

Ruan, S., Holt, M., Tu, C-H, Bourne, C.R. “Phenotypic impacts of ParE toxin expression in the native host bacteria” Oral Presentation, Great Plains Infectious Disease Conference, Nov. 6th, 2021, Columbia MO.

Bourne, C.R. Invited Seminar, TA Collective International Seminar Series, Virtual, March 2022, “A tale of a tail: how to prevent a picomolar interaction in a type-II ParDE TA system”

Ruan, S., Bourne, C., "Exploring Arabinose metabolism Impairment in Cells Overexpressing ParE Toxins," Flash Talk, Missouri Valley Branch of the American Society for Microbiology Annual Meeting, March 2022, Manhattan KS.

Tu, C.-H., Ruan, S., Bourne, C., “TA System ParE-mediated Gyrase Inhibition Invokes Toxicity and Increases Mutagenic Frequency Without Impacting Antibacterial Susceptibility**,”** Poster Presentation, Missouri Valley Branch of the American Society for Microbiology Annual Meeting, March 2022, Manhattan KS.

Ruan, S., Bourne, C., "Impact of ParE Toxin Expression on the Antibiotic Susceptibility of Pseudomonas aeruginosa," Poster Presentation, Missouri Valley Branch of the American Society for Microbiology Annual Meeting, March 2022, Stillwater OK.

Bourne, C.R. Invited Seminar, Oklahoma State University, Stillwater, OK, September 2022, “DNA gyrase inhibition by TA system proteins”

Bourne, C.R. Invited Seminar, Kansas State University, Manhattan, KS, September 2022, “Manipulating TA system interfaces to enable new bacterial control strategies”

Ruan, S., Bourne, C., "Arabinose-Inducible MtParE1 Toxin Expression Drives Changes in Arabinose Metabolism," Poster Presentation, Great Plains Infectious Disease Research Conference, Nov. 2022, Columbia, MO.

Tu, C.-H., Bourne, C., "Characterization of V. cholerae ParE toxin proteins from ParDE TA systems," Poster Presentation, Great Plains Infectious Disease Research Conference, Nov. 2022, Columbia, MO.

Tu, C.-H., Bourne, C., "Characterization of V. cholerae ParE toxin proteins from ParDE TA systems," Poster Presentation, Missouri Valley Branch of the American Society for Microbiology Annual Meeting, March 2022, Stillwater OK.

Ruan, S., Bourne, C. “Exploring arabinose metabolism impairment in cells overexpressing ParE toxins,” Poster Presentation, Missouri Valley Branch of the American Society for Microbiology Annual Meeting, March 2023, Manhattan KS.

Kanchanakomtom, R., Bou Alwan, N., Bourne, C. “Investigating ParDE Toxin-Antitoxin Systems Expression,” Poster Presentation, Undergraduate Research Day, May 2023, Norman OK.

Bou Alwan, N., Kanchanakomtom, R., Bourne, C. “Analyzing Pseudomonas-ParE Expression Within Toxin-Antitoxin Systems,” Poster Presentation, Undergraduate Research Day, May 2023, Norman OK.

Ruan, S., Bourne, C. “*Escherichia coli* cells avoid death by reducing plasmid copy number after ParE toxin over-expression,” Department of Chemistry and Biochemistry Research Day, June 2023, Norman OK.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
A challenge in the electroporation of *V. cholerae* El Tor strains has resulted in development of important alterations to standard techniques. The fluctuation assay, used to measure mutation frequencies, required novel optimization to normalize for expression of protein toxin rather than addition of an exogeneous drug or compound. Both of these advances in techniques are disclosed in the pending publication.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products
Nothing to report.

7. Participants & Other Collaborating Organizations

The project is carried out solely by members of the Bourne laboratory at the University of Oklahoma Department of Chemistry and Biochemistry.

Name:	*Christina R. Bourne*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-6192-3392*
Nearest person month worked:	*35*
Contribution to Project:	*Dr. Bourne has overseen all experimental methods, design, partial data collection, interpretations, expenditures, reporting, and presentations*
Funding Support:	*9 mo salary provided by University, partial summer salary paid from this award*

Name:	*Shengfeng Ruan*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*18*
Contribution to Project:	*Shengfeng contributed to the design and execution of cloning, and refined methods for CFU counting. He generated the altered pMind vector series, and collected and analyzed all the data for Mt and Pa toxins.*
Funding Support:	*Outside of the current proposal this student was funded in part by Department Teaching Assistantships, core facility Research Assistantships, a state grant (to CB), faculty support funds (to CB), pilot funding on another project, and a College Finishing Fellowship.*

Name:	*Chih-Han Tu*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*11*
Contribution to Project:	*Chih-Han generated some clones for, collected, and analyzed all the data for Vc toxins.*
Funding Support:	*Outside of the current proposal this student was funded in part by Department Teaching Assistantships, a state grant (to CB), faculty support funds (to CB), pilot funding on other projects.*

Name:	*Michelle Holt*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*10*
Contribution to Project:	*Michelle generated clones for, collected, and analyzed most of the data for Bc toxins (prior to her departure in Spring 2022).*
Funding Support:	*Outside of the current proposal this student was funded in part by Department Teaching Assistantships.*

Name:	*Kevin Snead*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Kevin devised the strategy for Aim 2 clones and initiated their construction (prior to his graduation in May 2023).*
Funding Support:	*Outside of the current proposal this student was funded in part by Department Teaching Assistantships and by a state grant (to CB).*

Name:	*Jake Davis*
Project Role:	*Undergraduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Jake contributed to early development of the fluctuation assay used in Aim 1; this was later refined for implementation on this proposal.*
Funding Support:	*Not applicable*

Name:	*Nour Bour-Alwan*
Project Role:	*Undergraduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Nour contributed to evaluating expression for constructs in Aim 1*
Funding Support:	*Not applicable*

Name:	*Randon Kanchanakomtorn*
Project Role:	*Undergraduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Randon contributed to evaluating expression for constructs in Aim 1*
Funding Support:	*Not applicable*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

8. Special Reporting Requirements

None to report.

9. Appendices

Award Expiration and Transition Plan (starts next page)

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.
This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** or **No**
These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

4. What will be **the next step(s)** for this project?

5. How would you classify your **lead candidate product**? *Please choose the best option or add explanation for multiple selections.*
(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy):
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify:

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.