AWARD NUMBER: W81XWH-20-1-0121

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

PRINCIPAL INVESTIGATOR: Christina R. Bourne, Ph.D.

CONTRACTING ORGANIZATION: University of Oklahoma, Norman, OK

REPORT DATE: March 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
March 2023

2. REPORT TYPE
Annual

3. DATES COVERED
15Feb2022 - 14Feb2023

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

5a. CONTRACT NUMBER
W81XWH-20-1-0121

5b. GRANT NUMBER
PR192335

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)
Christina R. Bourne

E-Mail: cbourne@ou.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Oklahoma Office of Research Administration,
1000 Asp Ave Rm 105
Norman, OK 73019-4039

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 this reporting period we have constructed reagents needed to determine which of the ParE proteins in two pathogenic bacteria have the greatest impact on bacterial cell survival. These assays revealed that of two different ParE toxins in each bacteria tested to date, only one of them is potently toxic and likely to

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRDC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	12	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

	Page
Introduction………………………………………………………….………..…..	4
Body………………………………………………………………………………..	4
Key Research Accomplishments………………………………………..……..	5
Reportable Outcomes……………………………………………………………	9
Conclusion…………………………………………………………………………	10
References………………………………………………………………………..	10
Appendices…………………………………………………………………………	10

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.**

Body

TA systems are a non-secreted component of a bacterial cell’s intrinsic physiologic response. These are protein pairs used to tailor bacterial physiology towards either death (a “time bomb”) or survival, depending on the cellular target of the toxin, in effect acting as resiliency factors. **We propose to co-opt TA systems for health purposes, but this is currently unfeasible because of a lack of fundamental knowledge on how to leverage TA systems as tools.** The current study is focused on ParE toxin subtypes, as we propose these are uniquely useful for an antibacterial approach: in their ability to mediate detrimental DNA degradation to the expressing bacterial cells, and their widespread presence in different Gram-negative bacteria of concern. **Our long-term aim is to co-opt these ParE toxins to directly cause death to only the specific type of bacteria in which they are found, an advantageous narrow-spectrum approach. These types of ParE toxins are present in bacteria of significant concern to human health and are the focus of our investigations: *P. aeruginosa, V. cholera, M. tuberculosis*, and *Burkholderia* sp.** Of specific interest to the funder, these pathogens have a directly negative impact on military personnel in field environments and when dealing with wounds, including biofilm formation, that can occur in non-optimal treatment conditions.

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.”

The short-term impact will demonstrate for the first time a usable approach for co-opting TA systems, and will provide insight into a potential fundamental mechanism of genetic resistance through error-prone repair after low dose toxin-induced DNA damage. ***This study will provide the proof-of-concept badly needed to allow further development into an applied product.*** The long-term potential therapeutic applications will offer very high specificity to a single pathogen, and versatility in providing a means to potentiate current treatments including those with developing resistance.

Keywords

Toxin-antitoxin systems, antibacterial applications, toxicity, mutations

Research Accomplishments

Specific Aim 1: Assessing viability and antibiotic susceptibility as a function of induced ParE toxin expression, and ParE-induced mutations *via* fluctuation analysis.

For this Specific Aim, all Milestones have been achieved.

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.

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).

Subtask 1 is complete.

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

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

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.

Subtask 3: Fluctuation assays to determine if ParE toxins increase native mutagenic capacity

Subtask 3 is complete (see Fig. 1).

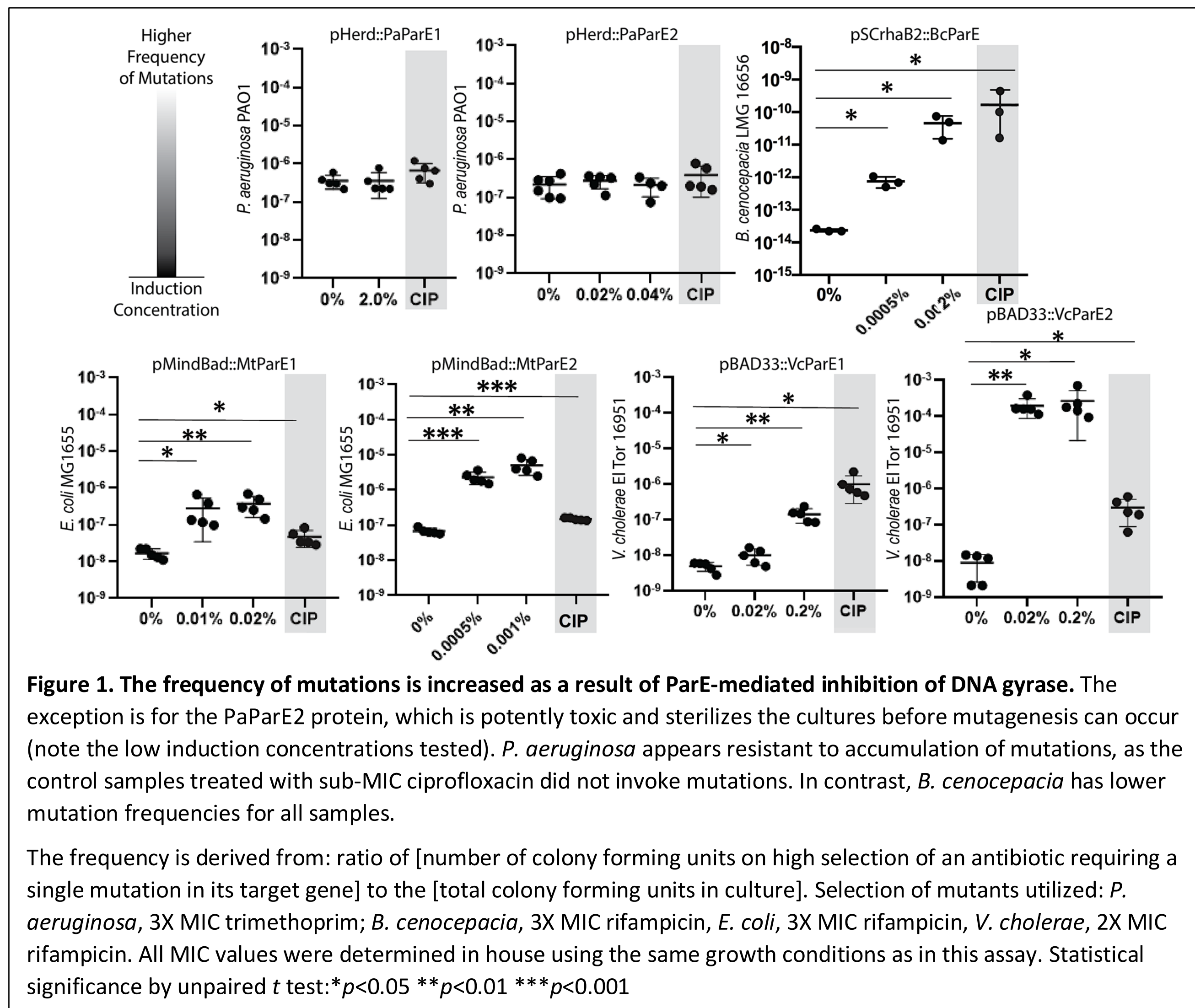

Figure 1. The frequency of mutations is increased as a result of ParE-mediated inhibition of DNA gyrase. The exception is for the PaParE2 protein, which is potently toxic and sterilizes the cultures before mutagenesis can occur (note the low induction concentrations tested). *P. aeruginosa* appears resistant 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

Subtask 4: Antibiotic susceptibility assays as a function of ParE toxin expression

Subtask 4 is almost complete (Tables 3-5), with one remaining set of antibacterial tests.

Remaining tasks:
MtE1 expressed in *E. coli* strain MG1655
MtE2 expressed in *E. coli* strain MG1655

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*

Conclusions from Specific Aim 1:

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.

The remaining tests for the MtParE samples will focus on antibiotics specific for *E. coli*, rather than the planned testing of *Mycobacterial*-specific potential treatments. All other tests are complete and have identified essentially no change in antibiotic susceptibility as a function of ParE toxin expression for both the non-toxic PaParE1 and the remaining potently toxic ParE proteins. *This is encouraging for our approach of manipulating these TA systems to selectively halt the growth of bacterial cells.*

This 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.

Specific Aim 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. Three subtasks were identified for Aim 2, resulting in three Milestone Achievements.

Subtasks 1: Build constructs for the inducible-degradation system

This subtask remains in progress due to setbacks in robust expression of the antitoxin.

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 has been successful, is 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, *left*). We have also added a Strep-tag to the N-terminus of the antitoxin, and a His-tag to the N-terminus of the 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. Throughout these cloning strategies we have used the PaDE1 system to avoid complications of toxicity. All constructs have been verified by nanopore whole plasmid sequencing (Plasmidsaurus).

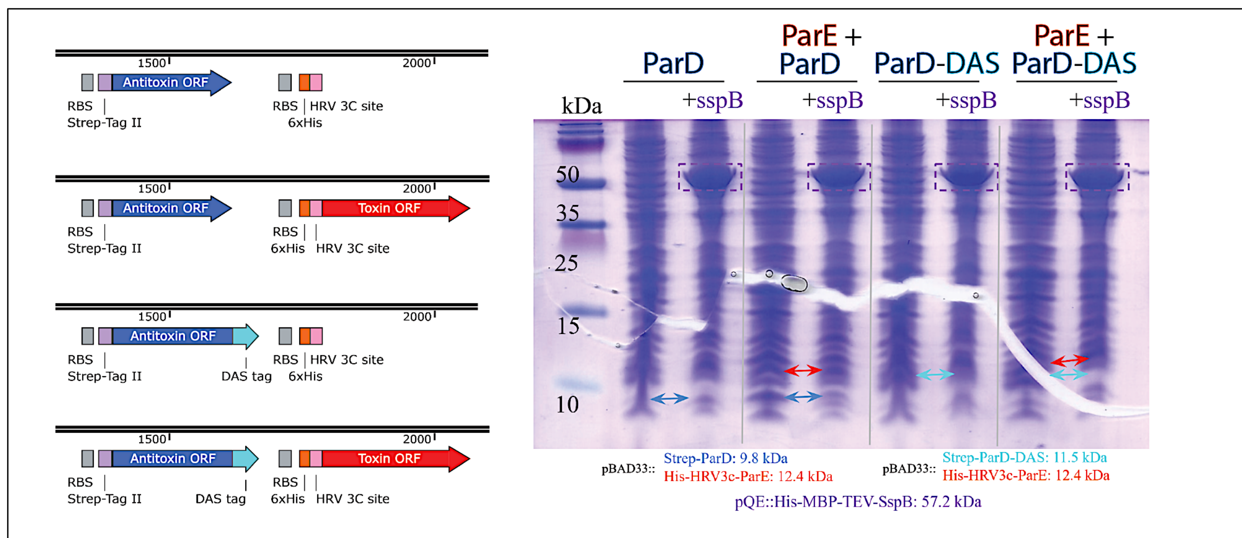

Figure 2. Construct design for inducible degradation of ParD antitoxins. *(Left)* Schematic of open reading frames (ORFs) for antitoxin (blue) and toxin (red), including tags for detection by Western blotting. Of the four total constructs, two have a degradation tag ("DAS", cyan) appended to the antitoxin ORF. ***(Right)*** Results from pilot expression assay; expected molecular weights are indicated below the Coomassie-stained gel, and the gel is annotated with color-coded arrows. Note that induction of SspB is hypothesized to increase antitoxin degradation in the absence of ParE toxin.

Pilot expression studies (Fig. 2, *right*) demonstrate robust expression of SspB from the pQE vector, while ParD antitoxin and ParE toxin are harder to distinguish (annotated colored arrows, Fig. 1). It appears that antitoxin is

expressed and this is reduced when the SspB protease adaptor is induced. Further, this appears roughly the same extent of loss even when ParE toxin is present, suggesting the protease system is powerful enough to separate these tightly interacting proteins. Western blotting detects the His-tag appended to both SspB and the ParE toxin, while the Strep-tagged ParD antitoxin was not detected. We are continuing to optimize Strep detection by Western blotting and may need to incorporate an enrichment step to facilitate robust detection.

As the Aim is seeking to measure ParD antitoxin, which is apparently expressed to low levels, we sought to increase its signal. Given the strong expression of the SspB protein, we have swapped constructs between these vectors. We have successfully subcloned the ParD antitoxin constructs, with and without the ParE toxin constructs, as diagramed in Fig. 1 into the pQE plasmid. The SspB insert was also moved and now resides in the pBAD33 plasmid. Pilot expression studies demonstrate robust expression of SspB and ParE toxin, while ParD antitoxin detection remains low and is still being optimized for 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.

In order to move this aim forward in the remaining time we are evaluating (a) if expression of ParD antitoxins is less stable in this *E. coli* strain independent of any induced degradation, indicating we may need to rely on another strain for our experiments, and (b) if other ParDE systems will be more robustly expressed, particularly as the survival of the cells will rely on effective neutralization of the more toxic ParE proteins. We note that, using a Gibson assembly approach, building additional constructs is relatively straightforward. However, it is unlikely that we will have time remaining to test the six ParDE systems used in Aim 1. *We will prioritize at least one ParDE system from Vc and Mt, in addition to the Bc system.*

Subtasks 2: Determining extent of ParD antitoxin degradation with and without the cognate ParE toxin present

> *This subtask is pending completion of subtask 1 and is anticipated to take up to two weeks once constructs are in hand.*

Subtasks 3: Assessing the gain of phenotypes expected for gyrase inhibition as a function of ParD loss

> *This subtask is pending completion of subtask 1 and is anticipated to take up to two weeks once constructs are in hand.*

Milestones Achieved:	***Proposed***	***Actual***
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	0.25	*Remaining MtParE samples to be tested in E. coli MG1655* Completed by April 1, 2023
Aim 2		
1. SspB induced degradation experimental test system build complete	2	*In progress* Completion of selected sample subset by May 31, 2023
2. Determine the extent of individual ParD antitoxin degradation in response to SspB induction	2	*Pending* Completion of selected sample subset by May 31, 2023
3. Determine the impact of degrading individual ParD antitoxins on viability and morphology	2	*Pending* Completion of selected sample subset by May 31, 2023

Overall Conclusions

Despite initial delays and subsequent Covid-19 impacts, we have completed Aim 1, and completed pilot studies for Aim 2.

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.

Overall Outcomes

The successful completion of this project will (1) increase understanding of a fundamental mechanism of ParE toxin-mediated inhibition of DNA gyrase and subsequent impacts on bacterial cell physiology, and (2) will demonstrate the selective *in vivo* degradation of ParD from the ParDE complex. ***This study will provide the proof-of-concept badly needed to allow further development 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.***

Impact

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.*

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.

Training

Data reported for this project were collected by graduate students; each had to learn specific skills to carry out the required studies. Of the four graduate students employed on this project, all learned molecular biology techniques needed to produce constructs, three were trained in BSL2 bacteriology, and two will incorporate these studies as chapters in their dissertations. I have recently undertaken training of two undergraduate students who are working directly with me to establish methodology for Aim 2.

Changes and Problems

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. This caused modest delays but the remaining personnel have successfully completed that portion of the study.

During the current 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.

Reportable Outcomes / Products

Training: 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.

Products: 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. To support other funding, we have used some of these clones as a starting point for production of these proteins for use in structural studies to define the molecular mechanism of gyrase inhibition.

Findings: 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 (May, 2023).

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

Our remaining studies from Aim 2 will provide insight into the ease of removing the neutralizing ParD antitoxin from the ParE toxin within a cell. The long-term goal will be to manipulate the pairing of these two proteins while maintaining ParE-mediated impacts.

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 uptake of the inducing sugar, arabinose. Further studies are confirming this explanation, and we anticipate submission of a manuscript in the coming four months describing this “path of least resistance” to “resistance”.

Participants and 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. To date, this has included graduate students (Chih-Han Tu, Shengfeng Ruan, Michelle Holt, and Kevin Snead), undergraduate students (Randon Kanchanakomtorn and Nour Bou Alwan), and myself (Christina R. Bourne, PI).

Current and Recently Completed Funding

US Department of Defense W81XWH-20-1-0121 2/15/20–5/31/23
"Unlocking the Potential of Bacterial ParE Toxins: Developing a Blueprint for Co-Opting Molecular Time Bombs that Impact Bacterial Cell Survival"
Role: Principal Investigator

NIH National Institute of General Medical Science (1P30GM145423-01) 10/22 – 05/24
Pilot project: "Defining host-pathogen interactions of Chlamydia trachomatis inclusion membrane proteins using a structure-function approach"
Role: Co-I (Lead: Erika Lutter, OSU PI: Ann West, OU)

NIH National Institute of General Medical Science (1P30GM145423-01) 09/22 – 05/24
Pilot project: "Structure/function of oxysterol-binding protein to guide therapeutic development"
Role: Co-I (Lead: Anthony Burgett, OUHSC PI: Ann West, OU)

OU Faculty Investment Program 01/23 – 01/24
"Advancing structure-function studies of DNA gyrase"
Role: Principal Investigator

Special Reporting Requirements

Not applicable.

References

Not applicable.

Appendices

Conference presentations during this reporting period:

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.**, "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.**, "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.