AWARD NUMBER: W81XWH-20-1-0121

TITLE: Unlocking the potential of bacterial ParE toxins: Developing a blueprint for co

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CONTRACTING ORGANIZATION: University of Oklahoma Office of Research Admin

REPORT DATE: March 2021

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MARCH 2021	Annual	15 Feb 2020 - 14 FEB 2021

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

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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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
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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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.
Four subtasks were identified to achieve Aim 1, culminating in three Milestone Achievements.

Subtask 1: Cloning ParD and ParDE genes into appropriate vectors, bacterial propagation, and transformation / conjugation of constructs.

The proposed study focused on six unique ParDE TA systems from four bacterial pathogens (*Pseudomonas aeruginosa,* Pa; *Burkholderia cenocepacia*, Bc; *Vibrio cholera,* Vc; *Mycobacterium tuberculosis*, (smegmatis model, Mt)).

An additional ParDE system (PaDE2) has been added for Pa, as it has subsequently been published as active and involved in prophage activation in approx. one-third of clinical isolates.

Similarly, as additional putative ParDE system (BcDE2) has been included for evaluation in Bc. Both TA systems are highly conserved among strains of *cenocepacia*. The initially selected cloning vector, pMLBad, was found to have poorer control over expression versus the pSCrha2 construct; subsequent cloning and testing has utilized only the pSCrha2 vector.

Table 1. TA systems targeted for study, and progress on Subtask 1

Source Bacteria	Native Operon(s)	Successful Cloning (vector: construct)
P. aeruginosa	PaDE1	Yes (pHerd20T: toxin, antitoxin, operon)
	PaDE2*	Yes (pHerd20T: toxin, antitoxin, operon)
B. cenocepacia	BcDE1	Yes (pSCrha2: toxin, antitoxin, operon) Yes (pMLBad: toxin, antitoxin, operon)

	BcDE2*	Yes (pSCrha2: toxin, antitoxin, operon)
M. tuberculosis	MtDE1	Pending (pMyc, pMind, primers designed)
	MtDE2	Pending (pMyc, pMind, primers designed)
V. cholera	VcDE1	Pending (existing clones pending transfer)
	VcDE2	Pending (existing clones pending transfer)

Subtask 2: Viability assays

These have been completed for PaDE1 and PaDE2 in strain PA14 (**Fig. 1**, summarized in **Table 2**); assays are in progress to assess toxicity to strain PAO1. The use of two different strains is important, because native chromosomal antitoxin could provide some protection in our over-expression assay. Strain PA14 does not contain the DE2 system, toxicity of ParE2 to this strain cannot be attenuated.

We have found the ParE2 toxin to be potently toxic to strain PA14 (results with PAO1 are pending but appear similarly toxic). In the image below, this ParE2 toxin is not included as its slower growth essentially result in no colonies until the latest time point, and these smaller colonies are barely visible on plated media.

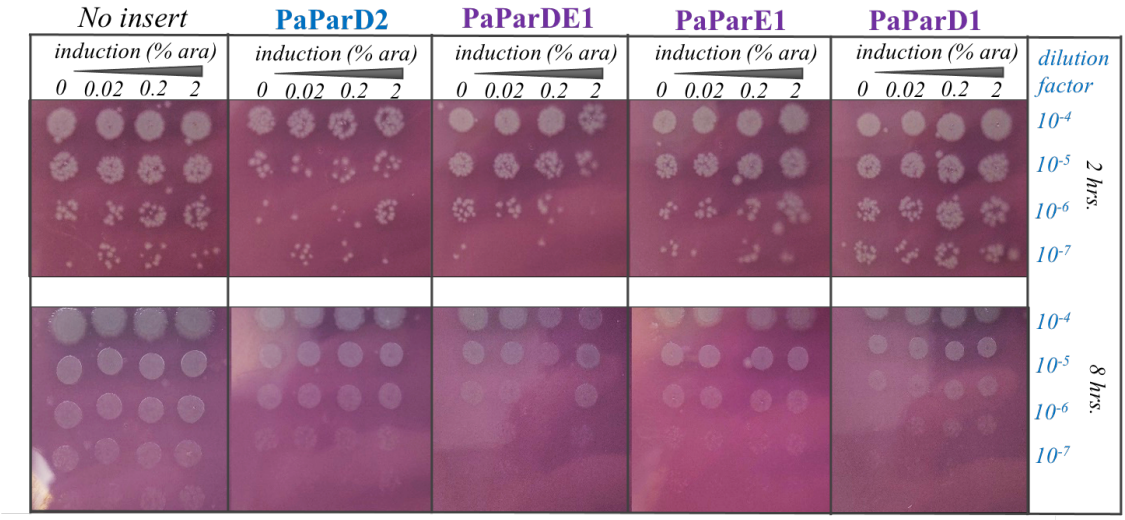

Figure 1. *P. aeruginosa* strain PA14 viability as a function of ParE toxin, ParD antitoxin, or ParDE induction demonstrate the ParDE1 system does not impact viability, while the ParE2 toxin (not shown) is potently toxic and strongly limits bacterial growth. Bacteria are grown in LB media supplemented with selection antibiotic and the indicated percent of inducer sugar arabinose. Serial dilutions are performed at the indicated time points after addition of arabinose, and spotted onto LB agar plates (color of plates arises from background in photograph).

Analogous experiments with the opportunistic pathogen *B. cenocepacia* pinpoint a potent toxicity from the ParDE1 system (**Fig. 2**, red arrow heads), while the more recently identified system (BcParDE2) does not impact viability.

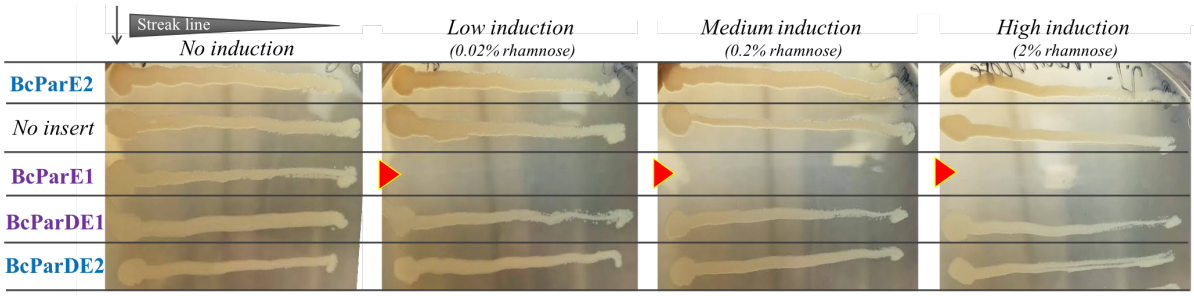

Figure 2. Toxicity of the ParE1 toxin from *B. cenocepacia* is evident by a lack of growth upon induction of expression (red arrow heads), while in the absence of induction or when in complex with the neutralizing ParD antitoxin there is no impact on viability. Cultures were tested on both LB and SOB rich media, with streaks originating on the left side of the image. Plated media contains the inducer (rhamnose, indicated); images were taken after 36 hrs incubation.

Table 2. Summary of results on host viability mediated by induced expression from constructs

Pathogen (strain)	Native Operon(s)	Outcome, Viability
Pa (PA14)	PaDE1	E1 not toxic E2 potently toxic
Pa (PAO1)	PaDE1, PaDE2	*Pending*
Bc (LMG 16656)	BcDE1 BcDE2	E1 toxic, DE1 operon limited toxicity E2, DE2 operon not toxic

Subtask 3: Fluctuation assays
Pending.

Subtask 4: Antibiotic susceptibility assays
Pending.

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.
Subtasks 1 – 3
Pending.

Timeline to Milestones

Milestones Achieved:	***Proposed***	***Actual***
Aim 1		
1. Determine dose-dependence of individual ParE toxicity	6	***In progress*** *Half completed at 12 mo.*
2. Determine impact of ParE expression on mutation accumulation	12	*Pending- Projected completion at 18 mo.*
3. Determine impact of ParE expression on antibiotic susceptibility	12 - 13	*Pending- Projected completion at 22 mo.*
Aim 2		
1. SspB induced degradation experimental test system build complete	17	*Pending*
2. Determine the extent of individual ParD antitoxin degradation in response to SspB induction	20	*Pending*
3. Determine the impact of degrading individual ParD antitoxins on viability and morphology	24	*Pending*

Impact

(reporting period Feb. 14th, 2020 – March 16th, 2021)

- Procured required bacterial strains; cultured and secured stocks
- Procured over-expression plasmids, reagents for construction of genetic tools for three of four organisms.
  - The *V. cholera*-specific constructs are pending request to previous investigator; if resolution not achieved by the end of May we will re-acquire from repository/commercial source and re-build constructs in-house
- Constructs made for Bc and Pa systems; Mt constructs designed
- Initial toxicity tests established for Bc, Pa
  - In each tested system, one ParDE is noted as highly toxic, while the other TA system does not impact viability
  - Moving forward with the project objective, the toxic ParDE systems should be targeted for manipulation, while the non-toxic systems are more likely to promote intrinsic resistance through activation of survival pathways

Training

- Partial financial support for three graduate students total; two have been trained in BSL2 procedures, all have been trained in cloning needed for construct generation
- Two students are focused on one organism each, gaining expertise in microbiology and specific handling procedures

Changes and Problems

- Complete University closure (no access to any university spaces, labs, ordering systems, or support): March 24 – May 22, 2020.
- Limited access (myself and one other individual): May 23 – July 1, 2020.

- Funding was initiated 5 weeks prior to complete closure, with internal fund availability lagging by ~2 weeks. As such, procurement of supplies had just been initiated at the time of closure. Resumption of purchasing was not available until June, with limited capacity for deliveries through July 2020. This caused significant delays of needed over-expression vectors, primers, and reagents for construction of constructs.

- Hiring freeze and limited Visa availability; a budgeted post-doc position remains unfilled. Attempts to hire from mid-Jan through July resulted in three offers; two were refused on the basis of unavailable visa (H1b) processing (the third took an out-of-state offer).
  - To limit the impact on the project, funds were transferred to graduate student support. While a post-doc would require less training, the students are making sufficient progress. I expect to significantly move the project forward for both aims during the summer months using available personnel.

Reportable Outcomes / Products

- Partial support of three graduate students
- Training in handling BSL2 bacteria, carrying out assays, cloning
- Construction of expression clones, bacterial over-expression strains
- Establish toxic versus nontoxic ParDE systems in *P. aeruginosa* and *B. cenocepacia*

Participants and other collaborating organizations

The project is carried out solely by members of the Bourne laboratory. To date this has included graduate students (Chih-Han Tu, Shengfeng Ruan, Michelle Holt), and myself (Christina R. Bourne, PI).

Conclusion

Despite delays in progress due to Covid-19 impacts just as the project was initiated, we have completed just over half of the first and second major subtasks. Additional replicates for these experiments, in conjunction with testing of the remaining targeted systems, will allow completion of Milestone 1 and progression into Milestone 2. Milestone 2 will only be completed for ParE toxins that have modest impacts on viability, as the limited toxicity then presents an opportunity for bacterial cell survival through mutagenesis. We anticipate initiating Aim 2 subtasks concomitantly with completing Aim 1, and this can be achieved by current group members (graduate students).

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 will 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 after low dose toxin-induced DNA damage, 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 potentially contributing to rise of resistance, 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.***

Special Reporting Requirements

Not applicable.

References

Not applicable.

Appendices

None to include.