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14. ABSTRACT Our original proposal was aimed at understanding the mechanism of how Pseudomonas aeruginosa forms drug tolerant persister cells. This is of critical importance because persisters can lead to antimicrobial therapy failure. Specific Aim 1: Transcriptomic analysis of stationary phase persisters. We successfully developed a method to isolate P. aeruginosa persisters and obtained total RNA from these cells. Attempts to obtain a transcriptome with this RNA failed due to the limitations of our samples. However, the methods developed from this work allowed us					
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Report Title

Genetics of persister formation in *Pseudomonas aeruginosa*

ABSTRACT

Our original proposal was aimed at understanding the mechanism of how *Pseudomonas aeruginosa* forms drug tolerant persister cells. This is of critical importance because persisters can lead to antimicrobial therapy failure. Specific Aim 1: Transcriptomic analysis of stationary phase persisters. We successfully developed a method to isolate *P. aeruginosa* persisters and obtained total RNA from these cells. Attempts to obtain a transcriptome with this RNA failed due to the limitations of our samples. However, the methods developed from this work allowed us to obtain the first persister proteome. This achievement is an important technical and scientific advance. The results have given us candidate persister genes that we are now working to validate. Additionally, these results will provide a guide for other groups working on proteomics approaches where material is limited. Specific Aim 2: Genomic Identification of High Persister (hip) Mutants. We completed this aim and obtained genome sequences of hip mutants from clinical isolates.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
12/11/2012	1.00 Lawrence R. Mulcahy, Jane L. Burns, Stephen Lory, Kim Lewis. Emergence of <i>Pseudomonas aeruginosa</i> strains producing high levels of persister cells in patients with cystic fibrosis, <i>Journal of Bacteriology</i> , (12 2010): 6191. doi:
TOTAL:	1

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
TOTAL:	

Number of Papers published in non peer-reviewed journals:

(c) Presentations

“Dormant Persisters: Mechanisms of Formation and Role in Disease” (2009). American Society For Microbiology. Philadelphia, PA.

“Death and Survival in Bacterial Populations” (2009). Leopoldina Symposium Evolution of Programmed Cell Death in Infection and Immunity. Wuerzburg, Germany

“Bacterial Death or Survival: Mechanisms of Antibiotic Killing and Tolerance” (2009) International Conference on Antimicrobial Agents and Chemotherapy. San Francisco, CA.

“Tolerance, Resistance, and Opportunities for Antibiotic Discovery” (2011) Antibiotic Resistance: Past Present, Future. Cold Spring Harbor Laboratory. Banbury, NY.

“Persister Cells, dormant variants highly tolerated to killing by antibiotics” (2011). Life, Death, and Survival of Micro-organisms. Brussels, Belgium.

“Persister Cells and the Mechanisms of Dormancy” (2011). How Dead is Dead II. Tübingen, Germany.

“Persister cells and infectious diseases” (2012) American Society for Microbiology San Francisco, CA

“Antibiotic Tolerance & Microbial Persistence” (2012) Lyme Disease Association. New York, NY.

Number of Presentations: 8.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):	
<u>Received</u>	<u>Paper</u>
TOTAL:	
Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):	

Peer-Reviewed Conference Proceeding publications (other than abstracts):	
<u>Received</u>	<u>Paper</u>
TOTAL:	
Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):	

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Paper

12/11/2012 2.00 Irirs Keren, Lawrence R Mulcahy, Kim Lewis. Persister eradication: lessons from the world of natural products., United Kingdom: Elsevier, (01 2013)

TOTAL: **1**

Patents Submitted

Patents Awarded

Awards

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
Janet Manson	0.75
FTE Equivalent:	0.75
Total Number:	1

Names of Faculty Supported

NAME

PERCENT SUPPORTED

FTE Equivalent:

Total Number:

Names of Under Graduate students supported

NAME

PERCENT SUPPORTED

FTE Equivalent:

Total Number:

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in
science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue
to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for
Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to
work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive
scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME

PERCENT SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

(4) Statement of the problem studied: Drug tolerant persisters are linked to clinical failure to treat chronic infections. *Pseudomonas aeruginosa* is the causative agent of a number of chronic infections. We found that persisters play a role in maintenance of the chronic infection of the cystic fibrosis lung by *P. aeruginosa*. We aimed to better understand the mechanisms of persister formation and maintenance in *P. aeruginosa* in order to better treat chronic infections.

(5) *Pseudomonas aeruginosa* is an opportunistic pathogen which causes serious infections when our immune system is compromised. The leading cause of morbidity in patients with cystic fibrosis is infection with *P. aeruginosa* (Govan and Deretic 1996). We found that persisters likely contribute to the recalcitrance of this infection (Mulcahy, Burns et al. 2010). Cystic fibrosis is not the only clinical situation where *P. aeruginosa* infects the airways. Intubated patients are at risk for developing ventilator-associated pneumonia (VAP), which can develop into a chronic infection (Vincent, Bihari et al. 1995)(Reinhardt, Köhler et al. 2007). *P. aeruginosa* frequently infects burns, where it is also capable of establishing chronic infections that impede healing (Bowler, Duerden et al. 2001; Bjarnsholt, Kirketerp-Møller et al. 2008). We reported that persister cells are a major reason why biofilms of *P. aeruginosa* resist aggressive antibiotic therapy (Spoering and Lewis 2001). Recent studies of the spatial susceptibility of *P. aeruginosa* in biofilms has demonstrated that there are active and dormant cells in biofilms and that these cells exhibit differing tolerance to antimicrobial agents (Haagensen, Klausen et al. 2007; Pamp, Gjermansen et al. 2008). In addition, several antibiotics penetrate biofilms of *P. aeruginosa* effectively, but still do not sterilize the biofilm (Spoering and Lewis 2001; Walters, Roe et al. 2003). These findings strengthen the hypothesis that persisters allow *P. aeruginosa* to escape sterilization by antimicrobial therapy.

While we have gained a good understanding of the mechanisms of persister formation in *E. coli*, the mechanisms behind *P. aeruginosa* persisters have remained elusive. *E. coli* forms persisters in response to gain of function mutations in the kinase HipA (Moyed and Bertrand 1983; Correia, D'Onofrio et al. 2006). We found that this kinase phosphorylates EF-Tu shutting down cellular functions (Schumacher, Piro et al. 2009). Our transcriptomic analyses of *E. coli* persisters demonstrated the importance of toxins in persister formation (Keren, Shah et al. 2004; Shah, Zhang et al. 2006). Overexpression of RNA endonucleases causes cells to enter a persister state (Keren, Shah et al. 2004; Harrison, Wade et al. 2009). We also found that the TisB toxin causes persister formation in response to antibiotic mediated DNA damage (Dorr, Vulic et al. 2010). This toxin shuts down cells by formation of a membrane pore that collapses the proton-motive force (Dorr, Vulic et al. 2010; Gurnev, Ortenberg et al. 2012). It was recently found that at least 6 of the *E. coli* RNA endonuclease toxin-anti-toxin modules must be knocked out before there is an observable effect on persister formation (Maisonneuve, Shakespeare et al. 2011). *P. aeruginosa* has at least three annotated and expressed TA modules, but it is unclear what role they play in persister formation at present.

We proposed to understand the nature of *P. aeruginosa* persisters by utilizing the approaches that have been developed to study *E. coli* persisters. In specific Aim 2 of our original proposal we planned to identify persister genes by isolating high persister mutants (hip). This was the technique utilized nearly three decades ago to identify the first *E. coli* persister gene, *hipA* (Moyed and Bertrand 1983). We recently used this method to identify additional *hip* mutations in *E. coli*. However, this approach did not work for *P. aeruginosa*. Surprisingly, after several rounds of selection for improved survival in the presence of bactericidal antibiotics, no *hip* mutants were recovered. We then turned to clinical isolates to see whether prolonged pulse-dosing with antibiotics selects for *hip* mutants in this pathogen.

We obtained a series of isolates from CF patients who had chronic and clonal infection of the lung with *P. aeruginosa*. Out of 15 patients total, 11 presented with strains that developed a *hip* phenotype over time (Mulcahy, Burns et al. 2010). In many cases the *hip* isolate obtained from the patient exhibited no resistance to antimicrobial therapy. The selection of a *hip* phenotype in vivo indicates the importance of persisters in chronic infections.

We then attempted to identify persister genes responsible for the *hip* phenotype. In a similar study with *E. coli*, we found *hip* mutants among isolates from patients with UTI, and many of these carried gain-of-function mutations in the HipA toxin. These mutations apparently decrease binding of HipA to the HipB antitoxin, releasing active toxin, which creates more dormant cells. However, sequenced genomes of *P. aeruginosa* mutants did not show any notable changes in the toxins. There are over 60 changes in the genome of the clonal series we first analyzed, and no obvious persister gene candidates. Comparison to independent *hip* isolates makes it difficult to identify changes in particular genes, since the pan-genome of *P. aeruginosa* is so large.

In specific Aim 1 we proposed that obtaining a transcriptome of *P. aeruginosa* persisters would lead to candidate persister genes. We developed a relatively rapid method for isolation of stationary phase *P. aeruginosa* persisters using fluorescence activated cell sorting (FACS). To isolate persisters we constructed a plasmid with constitutive mCherry expression and inducible GFP expression. The ability to express GFP in response to inducer indicates that a given cell is metabolically active while the presence of mCherry indicates cell viability. Dim cells with mCherry are easily isolated from the bulk population with our BD FACS ARIA II instrument. However, we were surprised to find that *P. aeruginosa* persisters resuscitate during cell sorting. Chemical fixation was then used to preserve the transcriptome. This allowed to isolate persisters, but crosslinking prevented obtaining a transcriptome. We then decided to use these cells for a proteomics analysis.

We established a collaboration with Dr. Joshua Adkins at Pacific Northwest National Laboratory (PNNL) to obtain a persister

proteome. Dr. Adkins is an expert in nano-proteomics, and after extensive optimization his group was able to obtain proteomics data from 1x10⁸ formaldehyde fixed and sorted cells. This sorting requires only a few days to obtain enough sample material for analysis. In an unsorted stationary phase sample where quantity of material is not limiting, ~1500 proteins that map to the PA01 genome are identifiable. This is the full complement of proteins that can be detected during stationary phase. In the first persister proteome obtained, ~1,000 proteins are detected. More importantly, quantitative comparisons between the persister fraction and the susceptible fraction have been made. Not surprisingly, most cellular protein levels decrease in the persister fraction with over 600 proteins showing decreased levels in persisters. This is expected because persisters are not metabolically active. There are 90 proteins that show a significant increase in the persister fraction. The largest change in any single protein is in bacterioferritin, an iron storage protein. This indicates that persisters could shut down due to sequestration of iron. Another interesting finding is that a predicted RNA endonuclease, PA3614, is more abundant in persisters. This class of protein is predicted to play a role in ribosome biogenesis and could potentially shut down persister cells by reducing functional ribosome content.

Persisters are most abundant in non-growing populations, however our previously developed persister isolation methods relied on actively growing cultures (Keren, Shah et al. 2004; Shah, Zhang et al. 2006). We can now isolate persisters from stationary phase, which represents a significant experimental development. The description of the first persister proteome is a major scientific advance and has provided us with new candidate persister genes. In addition, technical advances in proteomics made during our collaboration will provide useful information for future proteomic studies where samples are limited or from archival material.

(6) Bibliography:

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Technology Transfer

(4) Statement of the problem studied:

Drug tolerant persisters are linked to clinical failure to treat chronic infections. *Pseudomonas aeruginosa* is the causative agent of a number of chronic infections. We found that persisters play a role in maintenance of the chronic infection of the cystic fibrosis lung by *P. aeruginosa*. We aimed to better understand the mechanisms of persister formation and maintenance in *P. aeruginosa* in order to better treat chronic infections.

(5) Summary of Most Important Results

Pseudomonas aeruginosa is an opportunistic pathogen which causes serious infections when our immune system is compromised. The leading cause of morbidity in patients with cystic fibrosis is infection with *P. aeruginosa* (Govan and Deretic 1996). We found that persisters likely contribute to the recalcitrance of this infection (Mulcahy, Burns et al. 2010). Cystic fibrosis is not the only clinical situation where *P. aeruginosa* infects the airways. Intubated patients are at risk for developing ventilator-associated pneumonia (VAP), which can develop into a chronic infection (Vincent, Bihari et al. 1995)(Reinhardt, Köhler et al. 2007). *P. aeruginosa* frequently infects burns, where it is also capable of establishing chronic infections that impede healing (Bowler, Duerden et al. 2001; Bjarnsholt, Kirketerp-Møller et al. 2008). We reported that persister cells are a major reason why biofilms of *P. aeruginosa* resist aggressive antibiotic therapy (Spoering and Lewis 2001). Recent studies of the spatial susceptibility of *P. aeruginosa* in biofilms has demonstrated that there are active and dormant cells in biofilms and that these cells exhibit differing tolerance to antimicrobial agents (Haagensen, Klausen et al. 2007; Pamp, Gjermansen et al. 2008). In addition, several antibiotics penetrate biofilms of *P. aeruginosa* effectively, but still do not sterilize the biofilm (Spoering and Lewis 2001; Walters, Roe et al. 2003). These findings strengthen the hypothesis that persisters allow *P. aeruginosa* to escape sterilization by antimicrobial therapy.

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