A. 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
ABSTRACT
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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)

Received  Paper

12/11/2012  1.00 Lawrence R. Mulcahy, Jane L. Burns, Stephen Lory, Kim Lewis. Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis, Journal of Bacteriology, (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)

Received  Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations


“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


Number of Presentations: 8.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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(d) Manuscripts
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Books


TOTAL:  1

Patents Submitted

Patents Awarded

Awards

Graduate Students

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Names of Faculty Supported
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### 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

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### Names of personnel receiving PHDs

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### Sub Contractors (DD882)
Inventions (DD882)

Scientific Progress
We established a collaboration with Dr. Joshua Adkins at Pacific Northwest National Laboratory (PNNL) to obtain a persister transcriptome. We then decided to use these cells for a proteomics analysis. Chemical fixation was then used to preserve the transcriptome. This allowed to isolate persisters, but crosslinking our BD FACS ARIA II instrument. However, we were surprised to find that P. aeruginosa persisters resuscitate during cell sorting. Dim cells with mCherry are easily isolated from the bulk population with inducible GFP expression. The ability to express GFP in response to inducer indicates that a given cell is metabolically active activated cell sorting (FACS). To isolate persisters we constructed a plasmid with constitutive mCherry expression and genes. We developed a relatively rapid method for isolation of stationary phase P. aeruginosa persisters using fluorescence activated cell sorting (FACS). In specific Aim 1 we proposed that obtaining a transcriptome of P. aeruginosa persisters would lead to candidate persister genes. However, sequenced genomes of P. aeruginosa mutants did not show any notable changes in the toxins. There are over 60 mutations apparently decrease binding of HipA to the HipB antitoxin, releasing active toxin, which creates more dormant cells. Mutations in HipA are associated with clinical failure in chronic infections. microarray analysis of stationary phase Hip mutants of P. aeruginosa showed the expression of several genes involved in stress responses, including heat shock proteins, DNA repair enzymes, and nutrient uptake systems. 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 persistor 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, not 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. 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 transcriptome.
proteome. Dr. Adkins is an expert in nano-proteomics, and after extensive optimization his group was able to obtain proteomics data from 1×10^8 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 60% of 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:
(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-Moller 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, not 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.
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