LLNL-TR-463636

Final Technical Report
09-LW-112

November 29, 2010

Antibiotic Heteroresistance in Methicillin-Resistant Staphylococcus Aureus: Microchemostat Studies at the Single-Cell Level

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Antibiotic Heteroresistance in Methicillin-Resistant Staphylococcus Aureus: Microchemostat Studies at the Single-Cell Level

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Executive Summary

This is the final report on work accomplished under the Lab-wide LDRD project 09-LW-112. This project was awarded to Frederick Balagadde who has left LLNL for a position at Stanford University. This report is prepared by Raymond Lenhoff who assumed the role of PI on the project for the remaining two months in August of 2010. The project accomplished most of its original objectives despite the fact that numerous biosafety related approvals not envisioned in the original proposal had to be obtained. In addition, the original PI left prior to the last two months of the project. A microfluidic device capable of the culture and optical data collection on microcultures of S. aureus was developed. A simpler chip design was developed and produced. New chip-interface and optical-analysis software was written and tested. *S. aureus* was successfully cultured and preliminary data (fluorescence and bright field) was collected. The project has provided valuable expertise in microfluidic culture that can be leveraged for host pathogen interaction studies and has been used in a new $9M DARPA proposal which is now being written for submission by Jan 4, 2011.

Project Description:

Background

Since the development of new antibiotics is out-paced by the emergence of bacterial resistance to existing antibiotics, it is crucial to understand the genetic mechanisms underlying resistance existing antibiotics. At the center of this mystery is a poorly understood phenomenon, heteroresistance: the coexistance of multiple subpopulations with varying degrees of antibiotic resistance. A better understanding of the fundamental basis of heteroresistance could result in sorely needed breakthroughs in treatment options. This project proposed to leverage a novel microfluidic (microchemostat) technology to probe the heteroresistance phenomenon in bacteria, with the aim of restoring the efficacy of existing β–lactam antibiotics.

The clinically important bacteria Methicillin Resistant *S. aureus* (MRSA) was used as the test case of bacteria that exhibits antibiotic heteroresistance. MRSA is difficult to treat because it is resistant to all β-lactam antibiotics, as well as other classes of antimicrobials. Whereas β-lactams such as methicillin and oxacillin are the preferred antibiotics to treat *S. aureus* infections due to their efficacy and low side effects, accurate determination and use of oxacillin/methicillin dosage is hampered by heteroresistance. In fact, invasive MRSA infections now account for about 95,000 deaths per year, a number that exceeds the deaths due to either influenza or HIV (12). In some MRSA strains, two subpopulations of cells may coexist: both populations carry the mecA gene that confers resistance, but mecA is differentially expressed so that only a small number of cells are observed during in vitro testing. Why this occurs is not understood. Prior experiments have sought to explain this phenomenon with conflicting results, with technology being the primary barrier to test the system sufficiently (6,15).

There are several candidate genes thought to be responsible for conferring methicillin resistance to MRSA, key among them, mecA, hmrA and the vraSR operon (7). Moreover, it is notclear whether any one gene is solely responsible or whether they act in synergistic

combinations to confer resistance. Until now, expression studies have been tested at the population level.

Single-cell measurements are important to revealing heterogeneity in gene expression or differences in other phenotypic traits between the cells as these differences are often masked in population level measurements. This project aimed to extend a microfluidic platform (Figure 1) unique to LLNL to observe single cell bacterial antibiotic resistance gene expression. It sought to observe MRSA cells at various antibiotic concentrations to profile expression levels of candidate genes at the single cell level.

Through this approach the project would zero in on the specific gene or gene combinations responsible for conferring resistance. By continually substituting a fraction of bacterial culture with sterile nutrients, the microchemostat presents a near constant environment that is ideal for controlled studies of microbes and microbial communities. The whole system is computer controlled for constant monitoring of the experiment in progress. The miniaturized format greatly reduces reagent costs, and allows single cells to be tracked through time as gene expression occurs. The system is further enhanced by its closed fluidics to minimize contamination and its flexibility to integrate downstream analysis capabilities (such as PCR, cell-sorting) when desired. This system should allow observation of the growth dynamics of MRSA in the presence of antibiotics like never before. To monitor differential gene expression of the aforementioned candidate proteins fluorescent reporter systems (such as green fluorescent protein) were used to determine the cause of resistance.

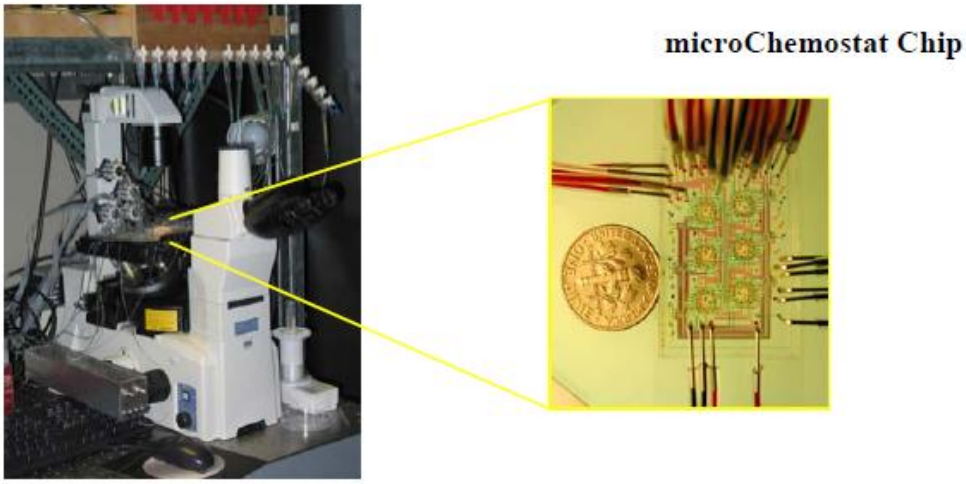


Figure 1. The future microchemostat platform will consist of a microchemostat chip positioned for imaging on an inverted microscope. *In situ*, single cell resolved, real time micrographic images as well as movies of cell cultures can be obtained non-invasively using a CCD camera shown in the sideport.

This platform while configured for the MRSA study, could be adapted to a look at a wide range of clinically relevant antibiotic resistance applications, including cost-effective high throughput screening of microbes against existing antibiotic libraries and novel antimicrobials. This information is of critical financial significance to the drug industry as well as to physicians

in their quest to prescribe effective antibiotics with the least side effects. Preliminary demonstration of this capability makes this work appealing to funding sources such as the NIH, drug companies, and DARPA.

Rationale

Since methicillin is such a highly effective drug in treating methicillin-susceptible *S. aureus*, it was our goal to find schemes that can restore the effectiveness of methicillin for treatment of MRSA. One means to this end is to gain a better understanding of a phenomenon called heteroresistance. Most MRSA strains exhibit a heterogeneous methicillin resistance phenotype, a phenomenon in which subpopulations of bacterial cells within a single strain exhibit varying levels of resistance to methicillin (10,11). In contrast, homogenously-resistant *S. aureus* are comprised of a uniform population of cells that each expresses the same level of resistance. Heteroresistant strains tend to have lower minimum inhibitory concentrations (MICs) for methicillin (MICs in the range of 32 -100 μg/ml) than do homogenously resistant strains (MIC > 1000 μg/ml) and the number of colonies in heterogeneous strains decreases as the bacteria are plated on increasing concentrations of drug. However, stably-derived homoresistant derivatives can be selected and subcultured from heteroresistant strains by incubation in high levels of methicillin or oxacillin, suggesting that this happens during therapy. Moreover, some heterogeneous strains express paradoxically higher levels of methicillin resistance when incubated with very high levels of methicillin (called the eagle effect) (13). Understanding how strains switch from the heteroresistant to homoresistant phenotype could prevent the evolution of highly resistant strains during therapy. By understanding how a subpopulation can be killed by methicillin despite the expression of *mecA,* a more effective therapy might be developed.

Methicillin and its more widely used congener, oxacillin, are chemically related β-lactam antibiotics. All β-lactams act by binding to the peptidoglycan (cell wall) biosynthesis enzymes called penicillin-binding proteins (PBPs), *S. aureus* has 4 housekeeping PBPs. MRSA bacteria have acquired an extra PBP protein that does not recognize the β-lactams, and the antibiotics are rendered useless. This is the mechanism or resistance to methicillin in methicillin-resistant *S. aureus* (MRSA).

This acquired PBP is called PBP2a or PBP2' and is encoded by *mecA* (10). PBP2a confers resistance to all β-lactams due to its poor binding capability for β-lactams and its ability to construct the cell wall despite the presence of β-lactams. PBP2a is not sufficient for methicillin resistance, however. The housekeeping PBP2 is also required as it provides the missing transgycosylase activity lacking in PBP2a (16).

Determining the conditions by which to stimulate MRSA cell death to a greater extent in all cells of the population could make oxacillin more effective. For instance, the subpopulation of bacteria that get killed by Oxacillin despite the presence of PBP2a might produce a compound that interferes with the action of PBP2a or have high levels of autolysis.

Study Objective and Scope

Technical Approach

In the same way that integrated circuits impacted the electronics industry, synthetic biology

(1,8,9,17) promises to spark a revolution in biotechnology and medicine. Recent achievements of synthetic biology include the development of sophisticated non-native behaviors such as programmed population control (3,18) and pattern formation (5), synthetic eco-systems (4), oscillations (3), proteins customized for biosensing, and optimized drug synthesis.

A critical advance in this area is the miniaturization of characterization processes through microfluidics—the science and technology of systems that manipulate small amounts of fluids (10^{-9}–10^{-18} L), using micro-sized channels (3). Particularly exciting is Microfluidics Large Scale Integration (MLSI) that facilitates the integration of complex chemical or biological procedures into a single monolithic process that is faster, more precise and more reproducible than the sum of its stand-alone components. MLSI metering enables ultra-low consumption of biological samples and reagents, allowing high-throughput research at low cost with short analysis time.

The considerable challenges of maintaining, operating and characterizing conventional cultures, including the requirement for large quantities of growth media prompted Balagadde and colleagues to devise and implement a miniaturized 16 nanoliter microchemostat (Figure 1&2). The microchemostat enables automated culturing and single-cell resolved monitoring (cell density, morphology and fluorescence) of small populations (10^2–10^4) of bacteria for hundreds of hours (3,4). Such micro-sized populations undergo proportionately fewer divisions per hour, which effectively insulates the micro-cultures from rapid evolution and promotes genetic stability for prolonged monitoring. Its unique design also allows multiple experiments to be run in parallel on the same chip (Figure 1&2). In addition, the self-contained portability and minimized human involvement due to MLSI automation makes the microchemostat the ideal platform for working with microbes.

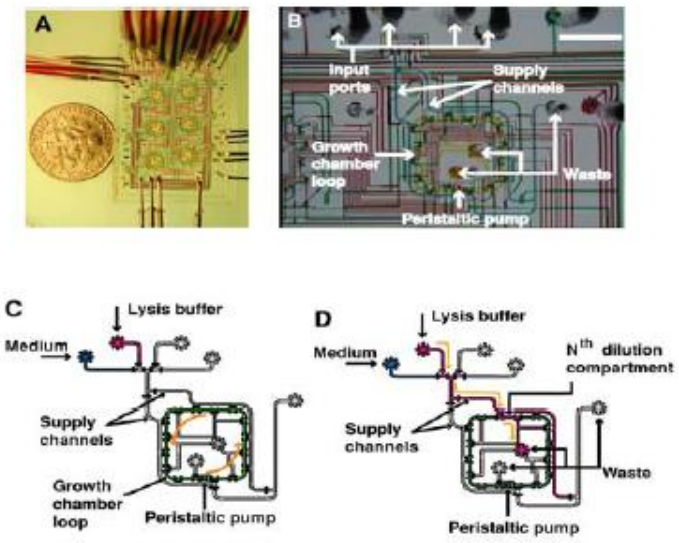


Figure 2. (A) Optical micrograph showing six microchemostats that operate in parallel on a single chip. Various inputs have been loaded with food dyes to visualize channels and sub elements of the microchemostats. The coin is 18 mm in diameter. (B) Optical micrograph showing a single microchemostat and its main components. Scale bar, 2 mm. (C) Schematic

diagram of a microchemostat in continuous circulation mode. Elements such as the growth loop with individually addressable connected segments, the peristaltic pump, supply channels, and input/output ports are labeled. (D) Isolation of a segment from the rest of the growth chamber during cleaning and dilution mode. A lysis buffer (indicated in red) is introduced into the chip through the lysis buffer port. Integrated microvalves direct the buffer through the segment, flushing out cells, including those adhering to chamber walls. The segment is then rinsed with fresh sterile medium and reunited with the rest of the growth chamber.

Previous research has used the microchemostat device to monitor the dynamics of cell populations containing a synthetic 'population control' circuit (18), which autonomously regulates the cell density via a quorum-sensing (14) based negative feedback system. With the circuit ON, the cell density was broadcasted and detected via the synthesis and sensing of a signaling molecule (acyl-homoserine lactone, or AHL), which in turn modulated the expression of a killer gene (lacZα-ccdB). The killer gene regulated cell density by controlling the cell death rate. The circuit, under control of a synthetic promoter, was inducible with isopropyl-β-Dthiogalactopyranoside (IPTG). In one of the runs, we performed six experiments simultaneously on a single microchemostat chip using *E. coli* MC4100Z1 cells and a dilution rate of 0.16 hr–1 (Figure 2). Cultures in reactors 1-3 with circuit-bearing cells were induced with IPTG (circuit ON), while those in 5 & 6 were not induced (circuit OFF). Reactor 4 contained a circuit-free population with IPTG. Circuit-free and circuit OFF cultures (4, 5 & 6) grew exponentially to a steady-state density of ~3.5 cells/pL. In contrast, circuit ON populations (1, 2 & 3) exhibited oscillatory dynamics before reaching a lower steady-state population density after ~125 hrs. Using the ability to monitor the microchemostat with single cell resolution, we observed that the oscillations in cell density correlated with specific cell morphologies (3).

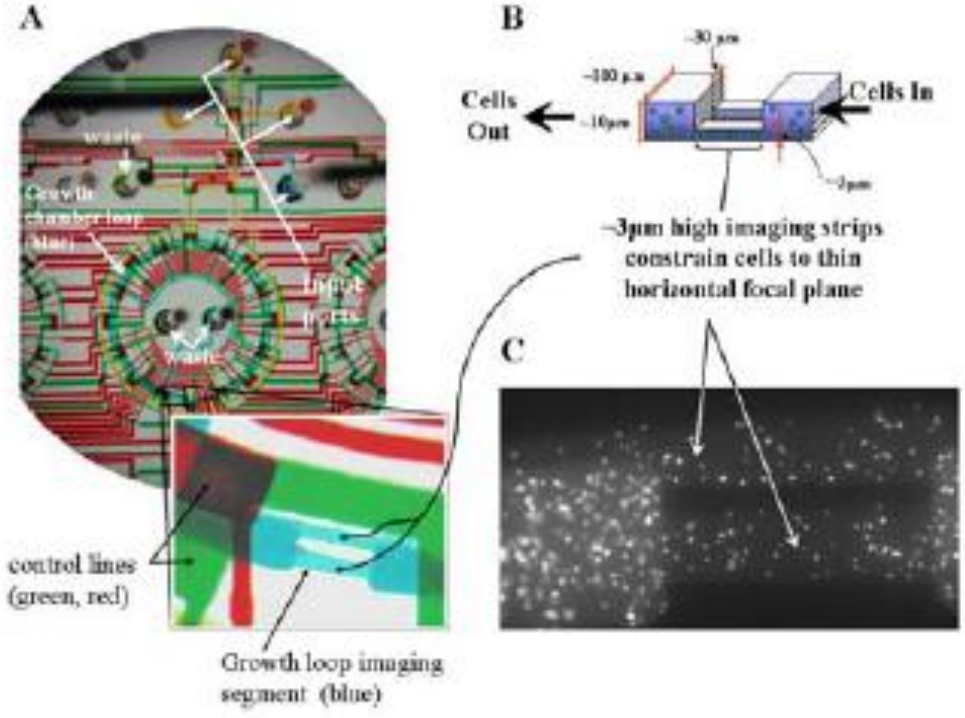


Figure 3. Microchemostat design illustrating modifications that allow for accurate monitoring of bacterial community composition. (A) New microchemostat reactor design with circular growth chamber loop. The imaging section has been enlarged to show the ~3 μm high strips incorporated along the growth loop track (compared to a ~10 μm height otherwise). (B) Three dimensional schematic of the imaging section along the growth loop. (C) Sample fluorescent image, showing well resolved fluorescent bacteria in the ~3 μm tall imaging section (center), compared to the unresolved signal in the ~10 μm tall sections at the edges. The imaged cells are constrained to a thin vertical height to put them all in focus simultaneously.

Proposed Work

The text below is from the original LDRD lab wide proposal for the FY2009 tasks as well as the proposed FY2010 work from the LDRD annual report web page for this project. Text that addresses specific technical difficulties encountered that had to be addressed or additional work that was not envisioned in the original proposal but was accomplished is in *italic text*.

FY 2009

Tasks and Deliverables

1. Set up microchemostat for *S. aureus* studies (LLNL). 4 months
Activities: Experiments will be performed to determine optimum *S. aureus* growth conditions. A fluorescent microscope will be attached to the microchemostat for cell observations of single-cell-resolved gene expression. Advanced automation fluidics will be built to completely eliminate human involvement in the experiment cycle.

The work described in this task took nearly two years as opposed to four months complete. While the experiments to optimize the microfluidic approach for S. aureus growth were completed in a month's time near project completion, the design and construction of the device and the accompanying IWS and IBC paper work allowing experiments with this risk group 2 agent at biosafety level (BSL) 2 took nearly two years. A phased approach suggested by safety personnel was taken for culture experiments which first involved demonstration of the culture and safe operation of the device with risk group 1 E. coli at BSL 1which Dr. Blagadde had previously worked with at Stanford. An inspection of the laboratory prior to the start of BSL 2 S. aureus work at the beginning of the second year revealed several difficiencies in the laboratory infrastructure that had to be corrected prior to approval of work at BSL 2. Correction of the laboratory deficiencies took several months to complete and only then could IWS and IBC approval be sought for S aureus work. Despite these unforeseen delays the project did succeed at the end of the second year in demonstrating optimum S. aureus growth conditions in the microfludics. A picture of the bacteria growing in a microfluidic channel is shown in Figure 4.

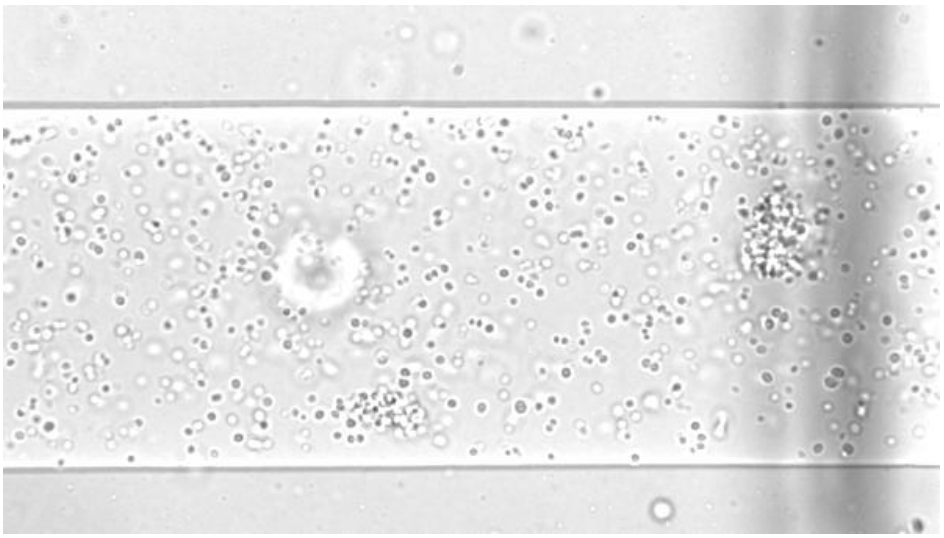


Figure 4*. Phase contrast (63X) image of S. aureus strain 923 bacteria in a microfluidic channel after 16 hours of growth. The channel depth is approximately 20 microns while the bacteria are 1 micron spheres. As can be seen due to the channel depth some cells are in focus (dark spherical dots) while others are out of focus (bright dots). An imaging window was designed to allow for efficient image collection.*

Deliverable: Fully functional system for MRSA observations.

2. Construction of mecA-GFP chromosomal fusions and pLac-mecA fusions (U.Chicago). 4 months.
Activities: use existing mecA-GFP plasmid fusions and place in MRSA chromosome for 1:1 tracking of gene expression. Place fusions in 3 predominant strains of MRSA (USA100, USA300 and USA400).

S. aureus strains received from U. Chicago consisted of a GFP expressing strain that was sensitive to antibiotic (MSSA) as well as two GFP expressing strains that were antibiotic resistant (MRSA 923 and MRSA COL). Because a staged approach to growth of S. aureus at BSL 2 that first involved work with E. coli at BSL 1, E. coli strains that expressed GFP were received in the first year from the University of Chicago and imaged in the microchemostat. In 2009. delays caused the receipt of the S aureus strains to only occur in 2010. In addition there were several issues regarding shipping of frozen cultures on dry ice that required the strains to be shipped on three separate occasions before fully frozen viable cultures of S. aureus were received at LLNL. Once received we demonstrated comparable growth in bulk culture and in the mcirofluidics of the strains received from U. Chicago.

Deliverable: Strains ready for microchemostat experiments.

3. Chemostat System Experiment: Direct single-cell observation of heteroresistance. (LLNL, U Chicago). 3 months
Activities: To determine if differential gene expression in mecA is driving heteroresistance. We will grow MSRA cultures in the microchemostat to steady-state at a fixed dilution rate and then introduce oxacillin through the influent (feed medium) at different concentration in different reactors. Exposure of oxacilllin to a continuous heterogeneous population will preferentially target and drive the sensitive subpopulation to extinction. The remnant resistant subpopulation will be profiled for mecA gene expression. During the course of antibiotic exposure we will track trends in single-cell-resolved mecA gene expression through fluorescence microscopy with unprecedented temporal resolution. A salient shift in

fluorescence distributions from heterogeneous to homogeneous will implicate mecA as a major contributor to the observed heteroresistance. Repeat experiment for the 3 predominant MRSA strains.

While we were able to demonstrate growth of mecA strains and collect limited GFP fluorescence data we were only able to carry out preliminary experiments in the presence of antibiotic before the project ended.

Deliverable: A time course of fluorescence distributions of mecA gene expression. A conclusive result regarding the role of mecA in heteroresistance.

4. Chemostat System Experiment: Determination of mecA as sole contributor to heteroresistance phenomenon. (LLNL, U Chicago). 3 months.
Ordinarily, the expression or mecA is driven by an oxacillin sensitive promoter, such that the mecA produced is proportional to the oxacillin concentration. In this phase, we will decouple the mecA production from the oxacillin concentration, and observe the dependence of resistance on mecA concentration. We will use a synthetic mecA gene driven by an IPTG inducible promoter. For a given concentration of oxacillin, we will vary the mecA produced in different reactors by varying the IPTG induction level and perform the fluorescence characterization described in 3, above. A salient positive correlation between the IPTG concentration (mecA production) and the level of resistance conferred will implicate mecA as a major contributor to the observed heteroresistance. Repeat experiment for the 3 predominant MRSA strains.

Experiments carried out by our collaborators at U. Chicago demonstrated that the experiments described in this task were not needed as mecA was found to not be the sole determinant of oxacillin resistance. We now have demonstrated the technical capability to verify this finding using our microfluidic system..

Deliverable: A graph of final steady-state cell density as a function of IPTG concentration for a given oxacillin concentration. A conclusive result regarding the role of mecA in heteroresistance.

FY 2010

Tasks and Deliverables

1. Grow MSRA cultures in the microchemostat to a steady state and at a fixed dilution rate, then introduce oxacillin at different concentrations in different reactors and track trends in single-cell-resolved mecA gene expression through fluorescence microscopy with unprecedented temporal resolution;

New microfluidic chips that were simpler to operate but functional for our studies had to be designed and synthesized due to the departure of Dr. Balagadde in July of 2010. This required several weeks of time to execute this task. New chip synthesis skill had to be acquired since all of this expertise resided in Dr. Balagadde. However the new team of engineers was able to successfully manufacture functional microfluidic chips at the end of this period and new chips were successfully utilized for the culture of S. aureus at the end of this project.

In addition new software had to be written to operate the new chips as well as the reliably control the camera and microscope to collect data from an experiment. The camera for collecting images had to be optimized for this new software as it was not able to stably collect data over 16 hours without this new software optimization. This task was successfully completed in approximately one week.

During the time when new chips and software were produced, growth curve data and culture methods were optimized in the lab at LLNL to allow seamless transition of S. aureus culture from U. Chicago to LLNL. This expertise was leveraged to allow a rapid acquisition of the ability to transition from culture of E. coli to S. aureus in the new microfluidic chips.

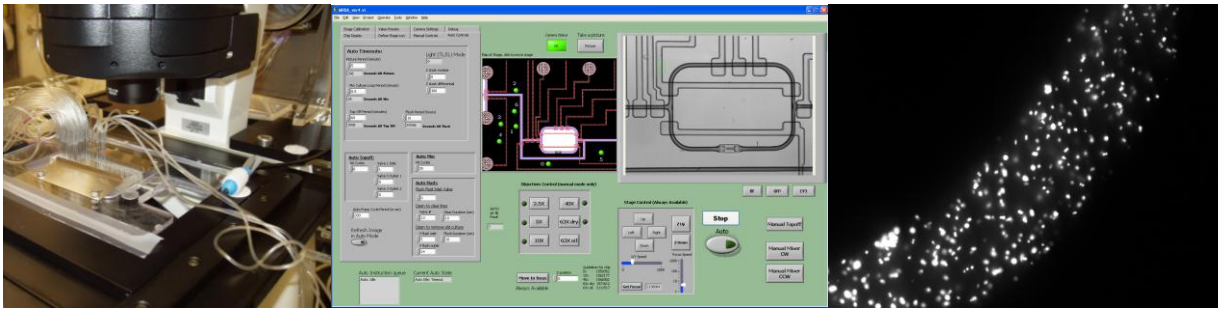


Figure 5. Left: *new microfluidic chip on microscope stage.* ***Middle:*** *new microfluidic chip software interface.* ***Right:*** *fluorescent image of GFP S. aureus grown in microfluidic bioreactor collected at 16 hours enabled by camera interface optimization.*

In addition to these scientific/technical tasks a major change to IWS (14662.03) for microfluidic culture work at BSL 2 had to be written and approved (July of 2010) as well as an amendment to the IBC (2008-027) for E. coli in to allow work with S. aureus (MRSA).

2. Determine a time course of fluorescence distributions of mecA gene expression

To execute this task fluorescence analysis software had to be written to analyze both the phase contrast and fluorescent images collected during and experiment. The analysis software was written in approximately two weeks and used successfully to analyze fluorescence and bright field images and allow the generation of fluorescence histograms and growth curves from the bright filed images.

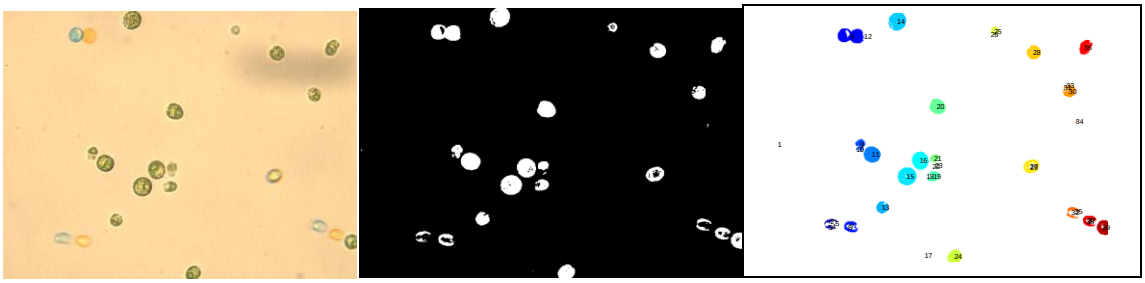
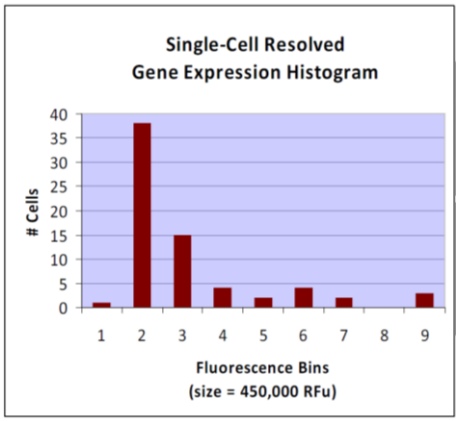


Figure 6. *Steps taken by in image analysis software* ***Left:*** *Raw image of cells* ***Middle:*** *Extracting individual cells from image* ***Right:*** *Calculating area and or image brightness (fluorescence) of each cell.* ***Below:*** *Histogram generated from compiled brightness of individual cells in an image.*



3. Examine the role of mecA in heteroresistance.

While we were able to demonstrate the culture of S. aureus in the microfluidic device and collect preliminary fluorescence data from GFP expressing antibiotic resistant S. aureus,the project ended before we were able to carry out sufficient experiments in the presence of oxacillin to determine the role of mecA in oxacillin resistance. The experiments that remained to be completed were to culture heteroresistant strain (923) and homoresistant strain (COL) of S. aureus in the presence of 0, 4, 8, 16, 32, 64 and 128 ug/ml of oxacillin and measure the fluorescence histogram collected from many cells at each antibiotic concentration over a 16 hour culture period. At project end the laboratory modifications and IWS and IBC approvals, microfluidics, culture system, and data collection software all was functional, optimized, and demonstrated..

Dissemination/Exit Strategy

The original dissemination/exit strategy for the project follows:

Our increased technical capability will benefit LLNL programs and position us to do additional work in the pharmaceutical drug development arena. This work could be applied to our programmatic biosecurity portfolio, for example to gain a better understanding of which antibiotics should be used for select agents when taking under consideration a variety of unique circumstances. This work will contribute to the career development of the coPI, a postdoctoral researcher at LLNL and a graduate student at U. Chicago. We expect to publish at least two papers of results of these experiments in high profile medical and technical journals.

The expertise gained in microfluidic culture of bacteria in the presence of antibiotic should have value to the commercial pharmaceutical screening activities. A new IBC has been written and approved to study the culture of biothreat bacteria using a microfluidic system which should be applicable to several host pathogen efforts ongoing at LLNL. The work contributed to the career development of the coPI who obtained a new postion at Stanford. While no publications were possible due to the project end with approximately one month of work remaining, this work has enabled the writing of a $9M full proposal to DARPA to be submitted on Jan 4 2011.

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