DESIGN OF A TARGETED BACTERIAL DRUG DELIVERY VECTOR FOR THE TREATMENT OF CANCER AND METASTASES

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DESIGN OF A TARGETED BACTERIAL DRUG DELIVERY VECTOR FOR
THE TREATMENT OF CANCER AND METASTASES

A Dissertation Presented

by

CHARLES ALEXANDER SWOFFORD

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2014

Department of Chemical Engineering
DESIGN OF A TARGETED BACTERIAL DRUG DELIVERY VECTOR FOR THE TREATMENT OF CANCER AND METASTASES

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Department of Chemical Engineering
DEDICATION

To my family,
whose love and support made this possible
ACKNOWLEDGEMENTS

I would like to thank my advisor Neil Forbes, for giving me the opportunity to work on this project and allowing me the freedom to pursue my own ideas and strategies. His infectious and unbridled enthusiasm for research kept me going even when everything seemed to go wrong. I would not be the engineer and scientist I am today without him. I would also like to thank Mike Henson, for being the first person to give me an opportunity to try my hand at scientific research when no one else would. I would also like to thank Barbara Osborne, for both giving me an opportunity to learn biological techniques in her lab and for providing me the opportunity to sit down with a pioneer of cancer research, Bob Weinberg. I would also like to thank the other members in the Forbes group that have overlapped with me: Raja Venkatasubramanian, Yumei Dai, Bhushan Toley, Miaomin Zhang, Adam St. Jean, Jan Panteli, Carolina Morrell, and Nele Van Dessel. I could not ask for a better group of people to work with. They made coming to work enjoyable every day and their support was invaluable when I was at my wits end.

I would like to thank everyone at the Pioneer Valley Life Sciences Institute and the Baystate Animal Care Facility for all their help with my mice work. Specifically, I would like to thank Alexandre Dufresne, for keeping me calm when tail-vein injections did not go as planned, Brooke Bentley and Jennifer Ser-Dolanksy, for paraffinizing and cutting my tissues at a moment’s notice, and Sally Schneider and Joe Jerry, for giving me a space to do all my bacteria work, despite the concerns of contamination. I would like to
also thank the Jerry lab, for teaching me immunohistochemistry and providing a space for me to run all my IHC experiments.

I would like to give a special thanks to the Institute for Cellular Engineering, for providing me my first research opportunity when I was still an undergraduate at Northwestern University. I would also like to thank them for funding all the interdisciplinary conferences that I attended throughout my graduate career that gave me an opportunity to showcase my research to the scientific community. Specifically, I would like to thank Shana Passonno for providing a place to vent frustration when funding was hard to come by.

I would like to thank all my friends and family that have supported me throughout my career. I have made life-long friends during my time at UMass, Northwestern, and earlier whose support and friendship kept me sane during my graduate career. I would like to thank my entire family for their love and support. Specifically, I would like to thank my Uncle David and Aunt Louise for providing me with a home away from home and giving me a place to write and sleep when finances were tough. Finally, and most importantly, I want to thank my parents. They have always put my needs before their own and their love and encouragement has helped shape the man I am today. This one’s for you.
ABSTRACT

DESIGN OF A TARGETED BACTERIAL DRUG DELIVERY VECTOR FOR THE TREATMENT OF CANCER AND METASTASES

MAY 2014

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Engineered Salmonella possess unique capabilities that make them ideal drug delivery vectors for tumors. Targeted bacterial delivery of anticancer proteins has the ability to overcome therapeutic resistance in tumors that limits the efficacy of chemotherapeutics. In my doctoral research, I identified a protein-drug that can be expressed by bacteria and rapidly kills cancer cells. I also created a density dependent switch that initiates gene expression in tumors and prevents expression in healthy tissue. Combining these two systems has created a potent anti-cancer system that targets tumors with minimal toxicity.

I cloned genes for five potential anti-cancer proteins into Salmonella. Supernatant from cultures was applied to MCF-7 mammary carcinoma cells to identify proteins that 1) were expressed, 2) secreted, and 3) rapidly killed cancer cells. Of the investigated proteins, α-hemolysin from Staphylococcus aureus (SAH) was the most promising because it secreted, caused trauma to cellular membrane, and induced oncosis in 18 minutes. After exposure for six hours, SAH decreased cell viability by 90%. The maximum death rate induced by SAH was a 7.1% reduction in cell viability per minute.
Due to systemic toxicity, bacteria that constitutively express anti-cancer drugs would not be an alternative to standard chemotherapy. To overcome this, I engineered *Salmonella* to initiate gene expression using the *lux* quorum sensing system. Quorum sensing (QS) allows bacteria to change gene expression based on differences in population density. Because *salmonella* preferentially accumulate in tumors, a QS genetic circuit could create an expression switch that only initiates in bacterial colonies in tumors. QS *Salmonella* only expressed GFP in high-density colonies *in vivo*. Gene expression of colonies was also dependent upon the radial distance of neighboring bacteria. At densities above $5 \times 10^{10}$ cfu/g, 84% of the colonies whose neighbors were at an average radial distance less than 103µm expressed GFP, whereas no colonies expressed GFP when their neighbors were at radial distances greater than 108µm. A mathematical model correctly predicted GFP expression in 93% of 84,213 QS colonies based on density and radial distance from adjacent *Salmonella*. QS *Salmonella* will allow for targeted bacterial drug delivery to tumors while minimizing systemic toxicity.
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CHAPTER 1

INTRODUCTION

Current cancer chemotherapeutic regimens have limited efficacy due to therapeutic resistance, which decreases the success rate for treatment of late-stage cancer and metastases (Brown and Giaccia, 1998; Jain, 1998; Tannock et al., 2002). This therapeutic resistance can be explained by a number of factors including 1) intra-tumoral transport limitations, 2) reduced susceptibility of quiescent cancer cells, and 3) protein pumps that expel therapeutics from cells (Jain, 1999; Tannock, 2001; Tannock et al., 2002). The first two factors can be explained by the overall architecture of most tumors. Tumors consist of chaotic vasculature, large intercapillary distances, and variable blood flow (Helmlinger et al., 1997; Jain et al., 1997; Vaupel et al., 1989) that in turn create regions of 1) highly perfused, rapidly proliferating cells, 2) poorly perfused, quiescent cells, and 3) avascular, necrotic or apoptotic cells (Sutherland, 1988; Sutherland and Durand, 1984). Due to perfusion limitations in the two inner regions of a tumor, most blood-born chemotherapeutics cannot diffuse the distance needed to achieve sufficient cytotoxic concentrations (Cowan and Tannock, 2001; Davis and Tannock, 2002; Tannock, 2001; Tannock et al., 2002). Furthermore, most chemotherapeutics target rapidly growing cells and have limited efficacy against quiescent cells (Jain, 1999; Tannock, 2001; Tannock et al., 2002). Since most of the tumor cannot be eradicated even after multiple courses of chemotherapy, the surviving cancer cells can repopulate the tumor leading to recurrence and metastases (Davis and Tannock, 2000; Davis and Tannock, 2002; Fidler et al., 2000).
Bacterial cancer therapies can overcome the many limitations of chemotherapy because they can actively transport into tissue, penetrate tumor tissue, and be genetically engineered to translate and transcribe most proteins. Most cutting edge therapeutics, including viruses, liposomes, and antibodies do not actively transport because they do not expend energy. These therapeutics can be highly specific to cancer cells, but due to high molecular weight, cannot penetrate deep into tumor tissue (Graff and Wittrup, 2003; Pluen et al., 2001). Bacteria can be used in concert with traditional therapies to attack both the quiescent cells and proliferating cells of a tumor thereby eradicating all living tumor tissue as opposed to only the outer region.

The use of bacteria as a therapeutic device for cancer is not a completely novel idea. Over the past 150 years, numerous patients have seen eradication of their tumors following severe bacterial infection (Coley, 1891; Nauts et al., 1946; Nauts et al., 1953). Furthermore, many different genera of bacteria have been shown to specifically accumulate in tumors including Salmonella (Jain, 1998; Pawelek et al., 1997), Clostridium (Lambin et al., 1998; Minton, 2003; Nuyts et al., 2002; Theys et al., 2001), Bifidobacterium (Fujimori et al., 2002; Yazawa et al., 2001), and Escherichia (Yu et al., 2004). In recent years, scientists have developed various strains of bacteria that have potential therapeutic advantages and reduce toxicity to the host. Low et. al has developed an msbB and purI mutant of S. typhimurium (VNP20009) that is non-pathogenic in mice, pigs, and humans (Clairmont et al., 2000; Low et al., 1999; Low et al., 2004; Toso et al., 2002). This strain preferentially accumulates in subcutaneous mouse tumors 2000-fold more than in the liver and spleen (Mei et al., St Jean et al., 2008). In addition, this
strain also retards tumor growth and prolongs survival of tumor-bearing mice (Clairmont et al., 2000; Low et al., 1999; Luo et al., 2001; Minton, 2003). Initial clinical trials further showed that VNP20009 could be tolerated at a high dose and able to effectively colonize human tumors (Nemunaitis et al., 2003; Toso et al., 2002). These trials suggest that VNP20009 would be an ideal candidate strain for use in a bacterial cancer therapy.

Bacterial targeting differs between obligate and facultative anaerobes. *Clostridium* and *Bifidobacterium* are obligate anaerobes and *Salmonella* and *Escherichia* are facultative anaerobes. Obligate anaerobes cannot survive in oxygenated environments and therefore only grow in hypoxic regions of tumors (Lambin et al., 1998; Minton, 2003). While obligate anaerobes are effective at targeting and accumulating in hypoxic regions of tumors, they are ineffective at targeting smaller tumors and micro-metastases. This is due to the fact that smaller tumors have not grown to a size large enough to support a necrotic, hypoxic center. Facultative anaerobes, on the other hand, have been shown to effectively colonize tumors and metastases smaller than 200µm in diameter by more complicated means of targeting. Several possible mechanisms include: chemotaxis toward compounds produced by tumors, preferential growth in tumor tissue, and protection from clearance by the immune system in the poorly perfused environment of tumors (Sznol et al., 2000).

Other than the ability to target tumors and metastatic sites, an effective bacterial therapy must also deliver anticancer drugs to the cancerous site without increasing systemic toxicity effects within the patient. This work aims to solve this problem in two steps by:
i) screening bacterial toxins that can effectively secrete out of VNP200010 and extracellularly kill cancer cells quickly and efficiently.

ii) developing a gene triggering strategy that will induce drug expression only within tumors and metastases while remaining repressed in healthy tissue environments.

Targeted bacterial delivery of anticancer proteins has the ability to overcome therapeutic resistance in tumors that limits the efficacy of chemotherapeutics. The ability of bacteria to specifically target tumors allows for delivery of aggressive proteins that directly kill cancer cells and cannot be administered systemically. However, few proteins have been tested for this purpose. To identify effective molecules, we systematically sorted proteins that have been shown to cause mammalian cell death. The genes for five proteins were selected and cloned into *E. coli* and *Salmonella*. Supernatant from cultures of the transformed bacteria was applied to flasks of MCF-7 mammary carcinoma cells to identify proteins that 1) were expressed, 2) secreted, and 3) rapidly killed cancer cells. Time-lapse images were taken to visualize mammalian cell morphology. Of the investigated proteins, α-hemolysin from *Staphylococcus aureus* (SAH) was the most promising because it was secreted, caused trauma to cellular membranes, and induced oncosis in 18 minutes. After exposure for six hours, SAH decreased cell viability by 90%. In comparison, the positive control, *Pseudomonas aeruginosa* exotoxin A (PEA), required 11 days to achieve a similar effect, when administered at 3,000 times its LC$_{50}$. The maximum death rate induced by SAH was calculated to be a reduction in cell viability of 7.1% per minute, which was 200-fold faster than the PEA control. Two
proteins, Dermonecrotic Toxin and Phospholipase C were active when extracted from the bacterial cytoplasm but were not secreted. This investigation revealed for the first time SAH as a potent anticancer drug for delivery by bacteria because of its ability to be secreted in a fully functional form and aggressively kill cancer cells.

Due to low-level bacterial counts in healthy tissue, bacteria that constitutively express an anti-cancer drug, such as SAH, would not be an alternative to standard chemotherapy. Because *Salmonella* preferentially accumulate in tumors, we have engineered *Salmonella* to switch on protein expression in response to high density using the *lux* quorum sensing (QS) system. Fluorescence and density were measured *in vitro* and in a tumor-on-a-chip device. QS *Salmonella* were injected into 4T1 tumor-bearing mice to quantify protein expression *in vivo* using immunofluorescence. At low densities, 3% of QS colonies expressed GFP compared to 41% of constitutive colonies and show statistically similar expression levels at densities above $4.2 \times 10^{10}$ cfu/g. GFP expression from QS colonies was also dependent upon the radial distance of neighboring bacteria. At densities above $5 \times 10^{10}$ cfu/g, 84% of colonies whose neighbors were at an average radial distance less than 103μm expressed GFP. No colonies expressed GFP when their neighbors were at radial distances greater than 108μm. A mathematical model of AI-1 concentration accurately predicted GFP expression in 93% of 84,213 QS colonies. It also showed that GFP expression had a sigmoidal relationship with density and an inverse relationship with the average radial distance of adjacent *Salmonella*. These results suggest that QS *Salmonella* will allow for targeted bacterial drug delivery to tumors and metastases while minimizing the risk of potentially harmful drugs reaching healthy tissue.
CHAPTER 2
IDENTIFICATION OF \textit{STAPHYLOCOCCUS AURUES} \(\alpha\)-HEMOLYSIN AS A PROTEIN DRUG THAT IS SECRETED BY ANTICANCER BACTERIA AND RAPIDLY KILLS CANCER CELLS

2.1 Introduction

Current cancer chemotherapeutic regimens have limited efficacy due to therapeutic resistance (Davies and Hiscox, 2011). Resistance is caused by intra-tumoral transport limitations, reduced susceptibility of quiescent cancer cells, and protein pumps that expel therapeutics from cells (Jain, 1998; Minchinton and Tannock, 2006; St Jean et al., 2008). For example, in triple negative breast cancer (TNBC), a lack of known specific therapeutic targets limits treatment options and reduces rates of pathologic complete response (pCR) to lower than 22% (Isakoff, 2010; Liedtke et al., 2008). Using bacteria that produce therapeutic proteins could overcome multidrug resistance. Anticancer bacteria have already been shown to selectively accumulate in tumors 10,000-fold higher than any other organ in mice while showing no signs of toxicity to the host (Forbes et al., 2003; Ganai et al., 2009). In addition, anticancer bacteria actively penetrate tissue and target distant metastases (Ganai et al., 2011; Kasinskas and Forbes, 2006; Kasinskas and Forbes, 2007; Loeffler et al., 2008; Nuyts et al., 2001a). Native bacterial toxicity has been shown to regress tumors and increase survival in multiple studies, but bacteria alone are unable to completely eliminate tumors (Chen et al., 2009; Jia et al., 2007; Lee et al., 2008; Low et al., 1999; Luo et al., 2001; Nagakura et al., 2009; Pawelek et al., 1997; Thamm et al., 2005; Theys et al., 2006; Zhao et al., 2007). Engineering bacteria to secrete

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anticancer proteins has the potential to improve therapeutic efficacy and localize high concentrations of drugs within tumors and metastatic sites. Identifying an anticancer protein that both kills cancer cells and can be delivered by bacteria will be essential to create a bacterial therapy that can efficiently eliminate tumors and metastases.

An effective protein for bacterial cancer therapy must 1) be easily transcribed and translated by bacteria, 2) be secreted, 3) be extracellularly active, and 4) rapidly kill cancer cells (Figure 2.1). Bacteria must be able to continuously produce and secrete the protein for an extended period of time to overcome systemic clearance and maintain a lethal concentration within the tumor site (Forbes, 2010). Continuous production would also promote diffusion to tumor regions distal from bacterial colonies. The protein must also be extracellularly functional once secreted from bacteria, as bacteria accumulate in the extracellular space of dying tumor tissue (Kasinskas and Forbes, 2006; Kasinskas and Forbes, 2007). Pore-forming proteins are attractive candidates because the majority act externally on cells and do not require endocytosis (Panchal et al., 2002). The protein must also be able to kill cancer cells at a concentration that can be maintained by bacterial secretion. Many toxins derived from bacteria possess the ability to kill cancer cells but few have been implemented with bacterial drug delivery. To date, Cytolysin A is the only bacterial toxin that has been explored for delivery by anticancer bacteria (Jiang et al., 2010; Nguyen et al., 2010; Ryan et al., 2009). Because of their potential, more proteins must be identified.
Bacterial toxins can be grouped into seven families based on their mode of action: 1) pore-forming toxins, 2) ADP-ribosylating toxins (ADPRTs), 3) glycosylating and deamidating toxins, 4) oxidoreductases, 5) cytolethal distending toxins (CDTs), 6) neurotoxins, and 7) superantigenic toxins. Approximately 35% of all toxins are pore-formers (Alouf and Popoff, 2006). These toxins create pores that disrupt and destroy cellular membranes and induce cell swelling and lysis (Bischofberger et al., 2009). ADP-ribosylation toxins (ADPRTs) interrupt G-protein mediated signal transduction pathways by transferring the ADP-ribose moiety of $\beta$-NAD$^+$ to GTP-binding proteins (Wolf and Elsässer-Beile, 2009). These pathways are involved in cell proliferation, tissue differentiation, signal transduction, protein synthesis, protein translocation, vesicular trafficking, and cytoskeleton structure. Glycosylating and deamidating toxins disrupt the actin cytoskeleton of cells by modifying GTPases into an active or inactive form. This results in the deterioration of the epithelial and endothelial barrier, leaving cells vulnerable to pathogen proliferation (Aktories, 2011). Cytolethal distending toxins (CDTs) induce DNA double-stranded breaks and apoptosis (Lemichez and Barbieri, 2013). These toxins have structural and functional homology to mammalian DNase I (Elwell and Dreyfus, 2000). Azurin is an oxidoreductase produced by bacteria that possesses the unique ability to form a complex with tumor suppressor p53, increasing its intracellular concentration and inducing apoptosis (Yamada et al., 2004). Neurotoxins and superantigenic toxins specifically target neural and immune cells, respectively.

Viable protein candidates must have specific attributes to be useful for bacterial cancer therapy. Candidates should be short, have few subunits, and contain minimal rare
codons in their DNA gene sequence. Shorter proteins have higher production rates because of fast folding kinetics and ease of secretion (Baneyx and Mujacic, 2004). Larger proteins and proteins with multiple subunits often require molecular chaperones to aid in secretion (Bogumil and Dagan, 2012). Heat-stable enterotoxin, for example, requires the periplasmic enzyme DsbA to complete its folding (Peek and Taylor, 1992). These chaperones would have to also be engineered into a bacterial vector to enable formation of a functional protein. A protein with a high number of rare codons in its DNA gene sequence will increase burden on the bacterial cell and slow protein translation (Rocha and Danchin, 2004).

To identify a payload for bacterial therapy, we analyzed all protein toxins and tested the most promising in vitro. Proteins were selected using a systematic method based on prevalence in the literature, size, number of rare codons, and protein structure. Selected proteins were inserted into a regulated cloning vector and transformed into an attenuated Salmonella cancer vector VNP20009 (Low et al., 1999). An integrated assay involving both bacterial and mammalian cell culture was used to screen the translation, secretion, and killing ability of each protein. Cell morphology was observed over time using time-lapse microscopy. Dose response curves were determined using a cell viability assay. The proteins identified in this study could be used against resistant tumors and metastases that were previously untreatable. Ultimately, combining these proteins with a bacterial delivery system will allow for localized expression of a broadly effective anticancer drug specifically within tumors.
Figure 2.1 Ideal characteristics of a bacterially delivered protein for cancer therapy. An ideal toxin should be small, secrete out of bacteria, and act externally on cancer cells to cause death, either through membrane disruption or unassisted internalization into cells.
2.2 Materials and Methods

2.2.1 Protein selection

A systematic approach was used to identify proteins that would be used for testing *in vitro*. First, proteins were separated into classes based on their mode of action. A subset of proteins from each family was selected based on their prevalence in the literature, which was quantified by the number of papers that were returned on PubMed (www.pubmed.gov) after a keyword search for each toxin. Families of proteins were eliminated based on cellular targets and number of subunits. Proteins within each family were ranked based on their size and length/rare codon ratio. Smaller proteins and proteins with high length/rare codon ratios were favored. Finally, proteins that were native to gram-negative bacteria were favored because the cancer vector VNP20009 is also gram-negative.

2.2.2 Bacterial strains and plasmids

All plasmids were derived by inserting toxin genes into the two multiple cloning sites downstream of the $P_{BAD}$ promoter in pBAD_MycHis (Figure 2.2). The pBAD_MycHis plasmid consists of the medium copy ColE1 origin, the ampicillin resistance gene, the araC gene and the $P_{BAD}$ promoter used to express the toxins in response to induction with L-arabinose (Guzman et al., 1995). Multiple cloning sites allowed for easy insertion of multiple genes from different species (Table 2.1). Genes were all cloned out of their respective organisms using a genomic DNA purification kit (*Promega*, Fitchburg, WI). Restriction enzymes were all from *New England Biolabs* (Ipswich, MA).
Staphylococcus aureus alpha-hemolysin (SAH) was cloned from Staphylococcus aureus strain MW2, which was a gift from Dr. Voyich-Kane lab at Montana State University. Dermonecrotic Toxin (DNT) was cloned from Bordetella pertussis strain Tohama I, which was a gift from Dr. Stibitz at the Center for Biologics Evaluation and Research in Bethesda, MD, USA. Pseudomonas Exotoxin A (PEA), Azurin, and Phospholipase C (PLC) were cloned from Psuedomonas aeruginosa strain PAO1, which was a gift from Dr. Pastan at the National Cancer Institute in Bethesda, MD, USA. Created plasmids were transformed into both E. coli strain DH5α and Salmonella typhimurium strain VNP20009.

2.2.3 Assay of protein efficacy

Single colonies of pBAD-PEA, pBAD-SAH, pBAD-PLC, pBAD-DNT, pBAD-Azurin, and a control of pBAD-MycHisA were grown in Luria-Bertani (LB) media (Thermo Fisher Scientific, Waltham, MA) supplemented with 100 ng/µL ampicillin overnight at 37˚C and shaken at 225 rpm. Samples were diluted 1:100 into fresh media, grown to mid-log phase (OD₆₀₀ ≈ 0.5), and were induced with 0.02% w/v L-arabinose for 4 hours to induce toxin production. Bacterial cells were pelleted by centrifugation and separated for lysis and supernatant extraction.

Supernatant fractions were purified with a 0.22 µm-filter and then concentrated 10X by centrifugal ultrafiltration (Millipore, Billerica, MA, USA). A 10X dilution of the concentrated supernatant was considered a co-culture equivalent concentration. Pellets were resuspended and incubated for 30 minutes in 500 µL water containing 1X halt
protease inhibitor cocktail (Thermo), 12.5 units/mL of dnasel (Thermo), and 25 µg/mL of lysozyme (Thermo). After incubation, pellets were transferred to 2 mL centrifuge tubes with 600 µm glass beads. These samples were alternately vortexed for one minute and incubated on ice for one minute, for ten cycles. One mL of water was then added, vortexed briefly, and centrifuged for three minutes. The lysis supernatant was removed without disturbing the glass beads.

MCF-7 human mammary carcinoma cells (American Tissue Type Collection, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM); (Sigma-Aldrich, St. Louis, MO) containing 10% Fetal Bovine Serum (FBS); (Atlanta Biologicals, Lawrenceville, GA) at 37°C and 5% CO₂. Cells were seeded at 2,500 cells/well into 96-well plates and allowed to adhere to the culture surface for 24 hours. The media was aspirated and replaced with treatment media consisting of 90% DMEM/FBS and either 10% phosphate-buffered saline (PBS), PEA toxin at 50 ng/mL, cell lysate, or a co-culture equivalent of supernatant. This dilution allowed for protein levels equivalent to direct co-culture without having bacteria in mammalian culture. Each treatment media application was replicated in eight wells.

The comparison of bacterially produced SAH to an SAH standard was performed with MCF-7 cells. Cultures of bacteria containing pBAD-SAH or pBAD-MycHis were induced for four hours after reaching an OD₆₀₀ of 0.5. Mammalian cells were seeded at 7,500 cells/well and treated with 100µl media containing a co-culture equivalent of bacterial supernatants or 500ng/ml pure SAH (Sigma) for one hour.
After 72 hours, transmitted light images (730.43 µm x 556.52 µm) were acquired by light microscopy using an inverted microscope (Olympus, Center, Valley, PA). Cell viability was measured by (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay (Promega, Madison, WI). Treatment medium was replaced with 100 µl DMEM/FBS plus 20 µl MTS reagent and incubated for one hour at 37°C for color development. Absorbance was measured at 490 nm (Bio-Tek Instruments, Winooski, VT). Relative survival values are reported as measured absorbance normalized by PBS controls.

2.2.4 Protein expression analysis

Lysis and supernatant fractions from bacteria transformed with pBAD-PEA and pBAD-SAH were boiled with 4X Laemmli reducing buffer (Boston BioProducts, Ashland, MA) for 5 minutes and run on SDS-PAGE for 45 minutes at 200 V. Proteins were transferred to nitrocellulose membranes that were blocked with 5% milk in tris-buffered saline containing 0.2% tween-20 (TBST) for one hour at room temperature. PEA samples were probed with a 1:1000 dilution of rabbit anti-PEA (Sigma-Aldrich) overnight at 4°C, washed 3 times in TBST, probed with a 1:1000 dilution of polyclonal horseradish peroxidase (HRP)-conjugated anti-rabbit polyclonal antibody (R&D Systems, Minneapolis, MN) for one hour at room temperature, and washed three times in TBST. SAH samples were probed with 1:1000 sheep anti-SAH polyclonal antibody (Abcam, Cambridge, MA) overnight at 4°C, washed three times in TBST, probed with a 1:1000 HRP-conjugated, donkey anti-sheep polyclonal antibody (R&D Systems) for one hour at room temperature, and washed three times in TBST. To visualize and image the
immunoblot, 4-chloro-1-naphthol/3,3’-diaminobenzidine (CN/DAB); (Thermo) was used for chromogenic detection of HRP.

2.2.5 Dose response to SAH

Dose response curves were generated for three concentrations of PEA over the course of three days and four concentrations of SAH over the course of six hours. For three-day dose response of PEA, MCF-7 cells were seeded at 2,500 cells/well, grown for 24 hours, and then the media was aspirated and replaced with DMEM/FBS containing 0, 5, 50, 500, 5,000, or 50,000 ng/mL of PEA toxin (Sigma-Aldrich). After one, three, or five days, cell viability was measured after a one-hour treatment with MTS. The growth rate of MCF-7 cells was calculated by exponential regression of the PBS control cultures and used to extrapolate initial cell count at day zero. Cell viability was normalized to this value.

For the six-hour dose response of SAH, cells were seeded at 7,500 cells/well, grown for 24 hours, and then the media was aspirated and replaced with dilutions of 10X concentrated SAH supernatant in DMEM/FBS. The supernatant was applied in relation to bacterial co-culture at a dilution of 1:1, 1:2, 1:10, and a 1:100. After zero, two, four, or six hours, cell viability was measured using a one-hour treatment with MTS. Relative viability was determined by normalizing the absorbance to PBS controls. The 1:1 dilution culture was visualized over time using epifluorescent microscopy. Time-lapse transmitted images were captured every three minutes and tiled using a macro in IPLab (BD Bioscience, Rockville, MD).
The cell death rate induced by SAH was estimated by fitting dose response data to a saturation function:

$$\frac{dC_{Cell}}{dt} = -\mu_d C_{Cell}$$  \hspace{1cm} (1)

where

$$\mu_d = \frac{\mu_{max} C_{SAH}}{K + C_{SAH}}$$  \hspace{1cm} (2)

and the loss of free SAH from the system:

$$\frac{dC_{Cell}}{dt} = -k_{inc} C_{SAH}$$  \hspace{1cm} (3)

Here $C_{Cell}$ is the relative survival of MCF-7 cells exposed to SAH, $C_{SAH}$ is the relative concentration of SAH in bacterial supernatant, $\mu_d$ is the cell death rate, $\mu_{max}$ is the maximum cellular death rate, $K$ is the relative SAH concentration at which cellular death rate is half of the maximum, and $k_{inc}$ is the rate constant for SAH incorporation into cellular membranes. Cellular growth was not included because of the short time scale.

The cell death rate for PEA was estimated by fitting the dose and rate data to a saturation function:

$$\frac{dC_{Cell}}{dt} = (\mu_g - \mu_d) C_{Cell}$$  \hspace{1cm} (4)

where

$$\mu_d = \frac{\mu_{max} C_{PEA}}{K + C_{PEA}}$$  \hspace{1cm} (5)

Here $C_{cell}$ is the relative survival of MCF-7 cells exposed to PEA, $C_{PEA}$ is the concentration of PEA, $\mu_g$ is the cell growth rate, $\mu_d$ is the cell death rate, $\mu_{max}$ is the
maximum cellular death rate, and $K$ is the PEA concentration at which cellular death rate is half of the maximum. Cell growth rate, $\mu_g$, was estimated by fitting the rate data of control MCF-7 cells (not exposed to PEA) to the equation:

$$\frac{dC_{Cell}}{dt} = \mu_g C_{Cell}$$

\[ (6) \]

2.2.6 Cell death and membrane permeability

MCF-7 cells were seeded at 7,500 cells/well, grown for 24 hours. Media was aspirated and replaced with either PBS or a 1:1 dilution of 10X concentrated supernatant from an induced or uninduced culture of pBAD-SAH in DMEM/FBS. The media was supplemented with a 1:800 dilution of ethidium homodimer (EtHd; Invitrogen, Carlsbad, CA). After six hours, transmitted and red fluorescent images were captured within each well. The total number of cells was counted by eye using the transmitted images and cells were considered dead if they contained EtHd in the red fluorescent images.

2.2.7 Efficacy of SAH against resistant breast cancer

Three mammary carcinoma cell lines, BT-549, SkBr3, and MDA-MB-231, a generous gift from Dr. Peyton at the University of Massachusetts Amherst, were cultured in DMEM/FBS. Cells were seeded at 7,500 cells/well into 96-well plates and allowed to adhere to culture surfaces for 24 hours. The media was aspirated and replaced with treatment media consisting of 90% DMEM/FBS and either 10% phosphate-buffered saline (PBS), or a co-culture equivalent of supernatant from pBAD-MycHis or pBAD-
SAH. After six hours, cell viability was measured using a one-hour treatment with MTS. Relative viability was determined by normalizing the absorbance to PBS controls.

2.2.8 Statistical Methods

Data are reported as mean ± standard error. Hypothesis testing was performed using Student’s t-test with significance determined by $P < 0.05$. 
Figure 2.2 Bacterial vector that can produce a toxin after direct induction with L-arabinose. Plasmid contains the ampicillin resistance gene, the pBAD promoter that responds to arabinose, the araC regulator gene, and the gene encoding the desired toxin. Multi-cloning sites (MCS) at the 5’ and 3’ ends were used to insert toxin genes.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Genome</th>
<th>Primers</th>
<th>Restriction Sites</th>
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<td><em>S. aureus</em></td>
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<td></td>
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<td>HindIII</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ATGGATCCGAGCTCGATGCACCTGACAC</td>
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<td></td>
<td></td>
<td>AAGCTT</td>
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</tr>
<tr>
<td>PEA</td>
<td><em>P. aeruginosa</em></td>
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<td>ATGAGCTCTGTGGATAAAGATGAATCGGCGATT</td>
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<td><em>B. pertussis</em></td>
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<td></td>
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<td></td>
<td></td>
<td>CATTACCAGCTGGCTGCCAGTGGCTGCTG</td>
<td>Neol</td>
</tr>
<tr>
<td>Azurin</td>
<td><em>P. aeruginosa</em></td>
<td>PAO1</td>
<td>GCATCTAAGCTTTGACTTCAGGGTCAGGATG</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

Bold Letters Indicate Location of Restriction Site
2.3 Results

2.3.1 Determination of toxin candidates for bacterial anticancer therapy

Four classes of bacterial proteins were considered as candidates for bacterial cancer therapy (Table 2.2). Two classes, neurotoxins or superantigenic toxins, were excluded due to their specificity for noncancerous cell types: neurons or leukocytes. Pore-forming toxins were considered attractive candidates due to their ability to act extracellularly on cells and do not require internalization. Cytolethal distending toxins (CDTs) were eliminated because all proteins in this family consisted of three distinct subunits.

Using a systematic approach, five toxins were identified (Table 2.3). Two toxins, *Staphylococcus aureus* alpha-hemolysin (SAH) and phospholipase C (PLC), were pore-forming proteins. The other three were in three different classes, which ensured that many mechanisms were investigated. Four of the proteins had been previously studied as cancer therapies (Table 2.3). Most of the toxins were short compared to their group, ranging from 149 aa to 1461 aa (Table 2.2). SAH was one of the few pore-forming proteins that was small and did not contain multiple distinct subunits (Table 2.2). Dermonecrotic toxin (DNT) had the highest length/rare codon ratio in the glycosylating and deamidating protein class (Table 2.2). PLC, a pore-forming protein, had a high length/rare codon ratio and did not contain multiple subunits. *Pseudomonas* Exotoxin A (PEA) is one of the most well-studied proteins and has been investigated previously as a cancer therapeutic. Azurin had the unique ability to increase the tumor suppressor p53.
within cells and induce apoptosis. It was also small in size and lacked rare codons (Table 2.3).

2.3.2 Screen for protein efficacy

Two proteins, PEA and SAH, were secreted out of bacteria and killed MCF-7 mammary carcinoma cells in vitro (Figure 2.3). Supernatant from PEA and SAH producing strains significantly decreased cancer cell survival (P<0.05) compared to an empty vector control after a 72 hour exposure (Figure 2.3A). The empty vector control consisted of bacteria containing the pBAD_MycHis plasmid that did not contain any toxin gene. PEA showed a six-fold decrease in cell survival compared to the vector control and SAH decreased cell survival by 80-fold. SAH supernatant was eight times better at decreasing cell survival than the 50 ng/mL PEA positive control (P<0.05). This concentration is above the reported LC$_{50}$ value of 17 ng/mL for PEA (Kuan et al., 1994). PEA, secreted from bacteria, did not reduce survival as well as the positive control (P<0.05). Supernatant from the empty vector control did not reduce cell survival when compared to PBS alone. Supernatant from all other toxin producing strains showed little to no effect at reducing cell survival (Figure 2.3A).

Bacterial cell lysate from PEA-, DNT-, and PLC producing strains significantly decreased cancer cell survival (P<0.05) compared to lysate from the empty vector control (Figure 2.3B). The lysate from the empty vector control also had a significant effect on reducing cell survival compared to PBS (P<0.05). SAH bacterial cell lysate had no effect on cancer cell survival compared to the control. None of the bacterial lysates had a
significant effect on cell survival when compared to the positive control. Bacteria with pBAD-SAH was the only culture that did not exhibit growth over the course of the four hour L-arabinose induction period (Figure 2.3C). SAH and PEA were present in both the cell lysate and supernatant (Figures 2.4A and 2.4B). Bacterially produced PEA was the only toxin that showed a significant effect on cell survival in both the cell lysate and supernatant. SAH showed a greater effect on cell survival than PEA in the supernatant but did not show any effect in the lysate. DNT and PLC only showed a significant effect on cell survival in the lysate but not in the supernatant.

Supernatant from a culture of pBAD-SAH bacteria reduced cell viability similarly to 500ng/ml of pure SAH (Figure 2.4C). Both were significantly less than the vector control (pBAD-MycHisA; P<0.05). Based on this similarity, bacteria with pBAD-SAH, at an OD$_{600}$ of 0.5, produced approximately 6,700 molecules of SAH·bacterium$^{-1}$·hr$^{-1}$.

2.3.3 Cell morphology

Supernatant collected from pBAD-SAH and pBAD-PEA bacteria disrupted cellular integrity and induced cell death in MCF-7 mammary carcinoma cells (Figure 2.5). In cultures treated with PBS and vector supernatant controls, cellular integrity remained intact for more than three days. Nuclei and organelles were present over the entire period (Figures 2.5A, 2.5B). Cells treated with supernatant from pBAD-SAH and pBAD-PEA shrunk and had detached cellular material (Figures 2.5C, 2.5D). There were no visible signs of functional organelles or nuclei. The cells also lost their ability to adhere to both other cells and the surface.
2.3.4 Concentration and time dependence of SAH on cellular death

Supernatant from SAH-producing bacteria significantly reduced MCF-7 cell survival compared to PEA controls (Figure 2.6). The different mechanisms of these toxins affected the rate of cellular death. Pure supernatant from pBAD_SAH reduced cell viability to 68% (P<0.05) in five minutes, and further reduced cell viability to 10% in six hours (P<0.05, Figure 2.6A). Treatment for 72 hours reduced viability by 99% (Figure 2.3A). A two-fold dilution in supernatant concentration also reduced cell viability to 40% after six hours (P<0.05). This reduction of viability was considerably faster than PEA administered at levels higher than its reported LC₅₀ values (Figure 2.6B). PEA administered at concentrations higher than 50 ng/mL took five days to reduce cell viability below 40% (P<0.05). At 50,000 ng/mL, a concentration almost 3,000-fold above the LC₅₀ value, PEA reduced cell viability to 17% in five days (P<0.05).

The dosage and time data for supernatant from SAH-producing bacteria was modeled with a saturation function using equations (1) and (2) and an SAH uptake function using equation (3) (Figure 2.7A). This method determined the maximum cell death rate, \( \mu_{\text{max}} \), to be a 7.1% reduction in viability per minute. The saturation constant, \( K \), was found to be 0.14, indicating that 14% of the concentration in the bacterial supernatant would produce half the maximum cell death rate. Based on the model, the SAH membrane incorporation rate, \( k_{\text{inc}} \), was estimated to be a 7.8% reduction in SAH concentration per minute (Figure 2.7C). The dosage and time data for PEA was also modeled with a saturation function using equations (4), (5), and (6) (Figure 2.7B). For PEA, \( \mu_d \) was found to be a 0.03%
reduction in viability per minute and $K$ was found to be 5.99 ng/mL. Comparison of the maximum cell death rates of SAH supernatant with pure PEA indicates that SAH supernatant kills cells 200 times faster (Figure 2.7D). Based on the model, it would take SAH supernatant from pBAD_SAH one hour to reduce cell viability to 15%, while cells exposed to PEA at 50,000ng/mL would require 225 hours to reduce cell viability by the same amount.

Time-lapse images of MCF-7 mammary carcinoma cells show the rate of cellular death after exposure to SAH supernatant (Figure 2.8). All cells exhibited signs of cellular oncosis after 45 minutes (Figure 2.8A). MCF-7 cells began to die as early as twelve minutes after exposure and complete membrane destruction took six minutes after onset of death (Figure 2.8B). SAH caused the cells to swell 12 minutes after exposure, forcing condensation of the cellular material to one side of the cell. Despite stresses on cells, no formation of apoptotic bodies was observed, indicating that cells were not undergoing apoptotic cell death.

After six hours of exposure to SAH supernatant, cellular coagulation and shrinkage was observed (Figure 2.9). Treated cultures showed increased staining ($P<0.05$) by ethidium homodimer (EtHd), a marker of permeabilized membranes and cell death (Figure 2.9). Supernatant from induced bacterial cultures killed over 98% of the total cell population after six hours (Figure 2.9B). Similarly, treatment with SAH supernatant for six hours reduced the viability of multiple mammary carcinoma cell lines ($P<0.05$), including two
TNBC cell lines, BT549 and MDA-MB-231, as well as a HER2+/ER-/PR- cell line, SkBr3, compared to vector controls (Figure 2.10).
Figure 2.3 Screen for protein drugs for bacterial therapy. A) Supernatant fractions from bacteria expressing *Staphylococcus aureus* alpha-hemolysin (SAH) and *Pseudomonas* Exotoxin A (PEA) significantly reduce cell viability after 72 hours compared to the empty vector control (*, P<0.05). Cell viability was normalized by the PBS control. SAH significantly reduce d cell viability after 72 hours compared to a standard of 50 ng/mL of PEA (positive control, PC; **, P<0.05). Other investigated proteins are azurin, dermonecrotic toxin (DNT), and phospholipase C (PLC). B) Lysis fractions from bacteria expressing PEA, DNT, and PLC significantly reduced MCF-7 cell viability after 72 hours (*, P<0.05). The empty-vector control significantly reduced cell viability compared to the PBS control (**, P<0.05). Values were normalized by the PBS control. C) Change in optical density (ΔOD_{600}) of bacterial cultures after 4-hour induction with L-arabinose. SAH-expressing bacteria were the only culture that did not grow after induction.
**Figure 2.4 Quantification of SAH production.** A,B) Western blots of lysate and supernatant from PEA-expressing (A) and SAH-expressing (B) bacteria. Both PEA and SAH are produced and secreted. C) Supernatant from a co-culture equivalent of SAH-producing bacteria reduced cell viability compared to a vector control after a one hour exposure (*, P<0.05). Resultant survival was similar to treatment with 500 ng/ml of pure SAH.
Figure 2.5 Images of MCF-7 cells after 72-hour exposure to toxins from bacterial supernatant. A) Cells that did not receive any bacterial supernatant appear healthy. B) Cells exposed to bacterial supernatant from the empty vector appear healthy. C) Cells exposed to PEA bacterial supernatant have shrunk and appear dead. D) Cells exposed to SAH bacterial supernatant have shrunk and appear dead. All scale bars are 50µm.
Figure 2.6 Time and concentration dependence of MCF-7 cell viability after exposure to SAH and PEA supernatant. A) Concentration dependence of MCF-7 cell viability after 0.08, 2, 4, and 6-hour exposure to SAH-expressing bacterial supernatant fractions. All dilutions greater than 1:100 showed significant reduction in cell death after two hours of exposure (*, P<0.05). Pure supernatant and a 2X dilution of supernatant showed a significant reduction in cell death after five minutes (*, P<0.05). B) Concentration dependence of MCF-7 cell viability after one, three, and five-day exposure to pure PEA toxin. All concentrations at 50 ng/mL or above showed a significant decrease in cell viability after five days compared to PBS control on day one (*, P<0.05). Cell viability was normalized to an extrapolated initial cell viability value using the growth rate of the 0 ng/mL control.
Figure 2.7 Model of survival response for SAH and PEA  

A) Model of survival response for SAH. The maximum cell death rate, saturation constant and rate of SAH loss were calculated to be \( \mu_{\text{max}} = 0.071 \, \text{min}^{-1} \), \( K = 0.14 \), and \( k_{\text{inc}} = 0.078 \, \text{min}^{-1} \), respectively.  

B) Model of survival response for PEA. The maximum cell death rate and saturation constant were calculated to be \( \mu_{\text{max}} = 0.0003 \, \text{min}^{-1} \) and \( K = 5.99 \, \text{ng/mL} \).  

C) SAH concentration for different starting supernatant dilutions, based on the SAH model in equations (1), (2), and (3).  

D) Comparison of SAH supernatant and PEA models over 6 hours. SAH supernatant is 200 times faster at killing cells than 50,000 ng/mL PEA.
Figure 2.8 Time-lapse images of MCF-7 cells exposed to SAH-expressing bacterial supernatants. A) MCF-7 mammary carcinoma cells undergo cell death in under 45 minutes after exposure to supernatant from SAH-expressing bacteria. Cells swelled and contents condensed to one end of the cell. All scale bars are 150 µm. B) Once initiated, oncosis took approximately six minutes. Each colored arrow (white, black and grey) indicates a different cell undergoing oncosis.
Figure 2.9 Quantification of Cell Death A) Ethidium homodimer (EtHd) staining of cultures exposed to induced pBAD-SAHA supernatant compared to an uninduced control. B) Induced SAH supernatant killed 98% of the total cell population after six hours compared to uninduced and PBS controls (*, P<0.05).
Figure 2.10 Cell viability of mammary carcinoma cells treated with SAH. Supernatant from pBAD-SAH bacteria reduced cell viability in two TNBC cell lines, BT549 and MDA-MB-231, as well as the HER+/ER-/PR- cell line SkBr3. Cells were exposed for six hours and compared to individual vector controls for each cell line (*, P<0.05). Viability was normalized by the PBS control for each cell line.
Table 2.2 Toxin Families

Table 2.2 - Toxin Families

<table>
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<tr>
<th>Class</th>
<th>Total Number</th>
<th>Mode of Action</th>
<th>Paper Count</th>
<th>Number of Distinct Subunits&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Number of Rare Codons&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Length/Rare Codons&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Sizes (aa)&lt;sup&gt;1&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1) Pore-Forming</td>
<td>117</td>
<td>Extracellular</td>
<td>264-2532</td>
<td>1-2</td>
<td>2-23</td>
<td>78.8-15.4</td>
<td>71-1024</td>
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<td>2) ADP-Ribosylating (ADPRT)</td>
<td>21</td>
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<td>659-2228</td>
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<td>9-13</td>
<td>59.4-49.2</td>
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<td>29-75</td>
<td>50.4-17.1</td>
<td>1014-1461</td>
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Bolded classes indicate the families of the selected toxins for *in vitro* studies.

Table 2.3 Selected Toxins

Table 2.3 - Selected Toxins

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<td><em>S. aureus</em> (Gram+)</td>
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<td>1</td>
<td>14</td>
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<td>1</td>
<td>13</td>
<td>49.2</td>
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<tr>
<td>Azurin</td>
<td>Oxidoreductase</td>
<td><em>P. aeruginosa</em> (Gram-)</td>
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<td>(Bernardes et al., 2013)</td>
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<td>0</td>
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<td>149</td>
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Data collected from the NCBI Protein Database http://www.ncbi.nlm.nih.gov/protein
2.4 Discussion

A screen of multiple bacterial toxins identified SAH as a candidate for bacterial anticancer therapy. The goal of the screen was to identify proteins that were 1) easily transcribed and translated by bacteria, 2) secreted, 3) extracellularly active, and 4) could rapidly kill cancer cells (Figure 2.1). A streamlined process was used that tested the ability of proteins to be secreted from bacteria and their efficacy against cancer cells. By directly applying supernatant to cancer cells, any evidence of cell death indicated all three processes: expression, secretion, and efficacy. In addition, any supernatant or lysis fraction toxic to cancer cells indicated that the protein was produced in a functional form and was active against mammalian cells when applied extracellularly. Of all cloned proteins, only SAH and PEA showed a significant reduction in cell viability (Figure 2.3A). Furthermore, SAH was the only cloned protein that rapidly killed cells. Cell viability dropped 85% in less than one hour (Figure 2.6A).

The results from the lysis experiment (Figure 2.3B) indicated that Salmonella were able to express DNT and PLC but were unable to secrete these toxins into the supernatant. While cell viability was reduced in the DNT and PLC lysis fractions, there was no effect on cancer cells when exposed to the supernatant fraction. DNT and PLC from the lysate killed cancer cells when added to culture medium (Figure 2.3B) indicating that the proteins were functional and active when applied extracellularly. Azurin had no effect in either the lysis fraction or the supernatant indicating that it was either not expressed or was not extracellularly functional.
SAH in the supernatant functioned differently from SAH in the cell lysate because of its mechanisms of secretion and pore formation. The first 26 residues on the N-terminus of SAH contains a signal sequence that is cleaved upon secretion (Dinges et al., 2000). Once cleaved, monomers integrate into membranes of host cells and form cylindrical heptameric pores that lyse eukaryotic cells. The now-exposed N-terminus extremity is essential because it interacts with adjacent monomers when forming the pore mouth (Vandana et al., 1997). SAH from the cell lysate could not induce cell death because the secretion signal had not been cleaved from the N-terminus, which would have prevented formation of the heptamer pores.

An important requirement for new cancer therapeutics is an ability to treat multidrug resistant tumors and metastases. SAH has the ability to treat resistant cancers because it indiscriminately kills cells. The mechanism is effective against multiple cell types because SAH forms pores in the membrane and does not require endocytosis to be effective (Berube and Bubeck Wardenburg, 2013). SAH was shown to reduce cell viability in two TNBC cancer cell lines, BT549 and MDA-MB-231, as well as a HER2+,ER-,PR- cell line, SkBr3 (Figure 2.10). When applied to MCF-7 cells (Figure 2.8), cellular membrane disruption caused swelling due to accumulation of water and electrolytes forcing the cellular organelles to one side of the cell. These are all classic signs of oncosis, cellular death due in part to disruption of ionic channels in the cell membrane (Majno and Joris, 1995; Saraste and Pulkki, 2000). This form of cell death is beneficial since it is induced by cellular trauma, as opposed to a pathway-mediated cell death that can be cell-type specific.
For the treatment of aggressive cancers, new therapeutics must kill cells faster than they can replicate. SAH secreted from bacteria can reduce cell viability by 50% in 12 minutes (Figure 2.6A). This is much faster than the doubling times of 0.8-1.2 days for many TNBC cell lines in vitro (Yoon et al., 2002). PEA, at concentrations three orders of magnitude above the reported LC$_{50}$ value, required 82 hours to reduce cell viability by the same amount. This suggests that aggressive cancers could not grow fast enough to overcome continual production of SAH but could continue to grow even with high concentrations of PEA. In culture, the SAH concentration was reduced due to incorporation in the membrane of dying cells (Figure 2.7C). This decrease would not occur within a tumor, where bacteria would continuously produce SAH and overcome loss to cellular uptake. The rate of SAH production and its rate of killing would enable the number of bacteria that typically colonize tumors (Ganai et al., 2011) to effectively kill cells.

Results with this screen suggest that SAH and other potential proteins, previously considered too dangerous for cancer therapy, should be reevaluated in the context of bacterial drug delivery. While SAH has been researched in vitro as a method to overcome cisplatin resistance in certain cancer cell types (Johansson et al., 2008), it has never been considered a therapeutic in its own right due to the potential for high systemic toxicity. Previous studies have shown that cells can reverse membrane damage after exposure to SAH at low concentrations (Thelestam and Möllby, 1983). Attenuated bacteria can provide the localized and persistent drug expression needed to prevent cells from regenerating their membrane by constant exposure to the toxin. The ability of bacteria to
localize to tumors could reduce the systemic effects of SAH while maximizing its concentration at the target site (Ganai et al., 2009). Triggering strategies using a combination of \( \text{L}-\text{arabinose} \) and quorum sensing could help to induce toxin expression once bacterial localization has been achieved (Dai et al., 2012). \textit{In vitro} screening of bacterial toxins for bacterial cancer therapy has the potential to reveal new classes of therapeutics that could be more effective against tumors and metastases than current chemotherapeutics.

2.5 Conclusion

We have discovered SAH as a candidate for bacterial anticancer therapy after screening proteins from seven different toxin families. SAH is produced and secreted out of bacteria in concentrations that rapidly kill cancer cells. It acts externally on cells by damaging cell membranes and inducing oncosis. Combining this potent toxin with the targeted delivery capabilities of bacteria could greatly increase localized toxicity at tumor sites.
CHAPTER 3
QUORUM-SENSING SALMONELLA SELECTIVELY TRIGGER PROTEIN EXPRESSION WITHIN TUMORS

3.1 Introduction

Engineering *Salmonella* to trigger protein expression specifically after tumor colonization has the potential to solve a critical problem with chemotherapy. Current cancer chemotherapeutic regimens have limited efficacy due to therapeutic resistance, which decreases the success rate for treatment of late-stage cancer and metastases (Brown and Giaccia, 1998; Jain, 1998; Tannock et al., 2002), as well as inherent systemic toxicity, which prevents the use of more aggressive dosage schemes (Sakhrani and Padh, 2013). *Salmonella* are capable of overcoming these therapeutic limitations because they preferentially accumulate in tumors, actively penetrate tumor tissue, and can be engineered to produce anti-cancer drugs *in situ* (Forbes et al., 2003; Ganai et al., 2009; Ganai et al., 2011; Kasinskas and Forbes, 2006; Kasinskas and Forbes, 2007; Loeffler et al., 2008; Nuyts et al., 2001b). Due to systemic toxicity, however, *Salmonella* that constitutively express an anti-cancer drug would not be an alternative to standard chemotherapy, due to low-level bacterial accumulation in healthy tissue (Forbes et al., 2003). *Salmonella* engineered to initiate anti-cancer drug expression using a tumor-sensitive switch has the capability to both reduce systemic toxicity and allow for the use of more aggressive therapeutics due to enhanced drug targeting.

*Salmonella* can be engineered to trigger protein expression in response to external cues (Hoffman et al., 2008), but these signals are dependent on both diffusion and location of
the tumor. Previous bacterial cancer studies have used external cues such as arabinose or salicylate to induce drug transcription (Dai et al., 2012; Loessner et al., 2007; Nguyen et al., 2010; Royo et al., 2007; Stritzker et al., 2007) in bacteria, but these methods are inherently more difficult because small molecules, analogous to current chemotherapeutics, cannot diffuse deep into tissue to trigger bacterial drug expression (Foley et al., 1978; Loessner et al., 2007; Seri et al., 1996). Radiation has been suggested as another possible trigger (Ganai et al., 2009; Nuyts et al., 2001a; Nuyts et al., 2001b), but radiation-inducible promoters have high uninduced protein expression, possibly leading to harmful effects in healthy tissue. Promoters that respond to hypoxia have also been suggested as a potential triggering strategy (Arrach et al., 2008; Mengesha et al., 2006; Ryan et al., 2009) but many micrometastases do not develop hypoxia that would signal bacteria to initiate drug production.

Integrating nonpathogenic *Salmonella* with a QS genetic circuit would create a robust expression switch that only turns on in bacterial tumor colonies (Figure 3.1A). Quorum sensing (QS) is the ability of bacteria to change their gene expression based on changes in population density (Waters and Bassler, 2005). Because *Salmonella* accumulate in tumors at densities almost 10,000-fold higher than other organs (Forbes et al., 2003; Ganai et al., 2009; Zhang et al., 2013), sensing changes in density would provide a switch to distinguish between healthy and cancerous tissue. Many bacterial populations naturally sense density through QS systems. The *lux* QS system is used by the marine bacterium *Vibrio fischeri* to induce the expression of genes required for bioluminescence once it has colonized the gut of the Hawaiian squid *Euprymna scolopes*. This squid uses
the light provided by the bacteria for counterillumination to mask its shadow and avoid predation (Waters and Bassler, 2005). The lux QS system consists of two genes: LuxI and LuxR (Figure 3.1B). LuxI synthesizes the autoinducer N-3(oxohexanoyl)homoserine lactone, also known as AI-1. LuxR, the transcriptional regulator, activates in the presence of AI-1 and induces transcription by binding to the promoter PluxI (Fuqua et al., 2001; Sitnikov et al., 1995). At low population density, low-level expression of LuxI synthesizes AI-1, which freely diffuses out of cells. As the population density increases, intracellular AI-1 activates LuxR, creating a positive feedback loop which increases the production of any gene incorporated into the operon (Sitnikov et al., 1995). QS systems have been used previously to trigger E. coli invasion into cancer cells (Anderson et al., 2006).

To create a tumor-sensitive gene expression switch, we integrated the lux QS system and a fluorescence reporter into attenuated Salmonella. We hypothesized that QS Salmonella would (1) switch on gene expression in response to high bacterial density and (2) only switch on gene expression in tumor tissue. Fluorescence and density were measured in culture and compared to controls that constitutively expressed GFP. GFP expression was measured in an in vitro tumor-on-a-chip device to assess if QS Salmonella turn on expression within tissue. QS and constitutive Salmonella were injected into tumor-bearing mice to quantify protein expression in vivo. Organ plating was used to quantify bacterial colonization in tumors and livers. Immunofluorescence was used to quantify the spatial distribution and density of bacteria and GFP expression within tumors. A mathematical model was created to predict the density and distribution of bacteria needed
to turn on protein expression within tumors. QS *Salmonella* will improve the use of bacteria for the treatment of cancer by creating a sensitive switch that will express protein therapeutics in tumors without toxic effects in healthy tissue.
Figure 3.1 Schematic of QS bacterial drug delivery. A) QS Bacteria will only turn on drug expression once they have colonized tumor tissue and sense an increase in their localized density. B) The PluxI promoter controls one operon consisting of the genes encoding for the LuxR, GFP, and LuxI proteins. LuxI produces the communication molecule AI-1. The PluxI promoter responds to LuxR protein bound to AI-1. As the density of bacteria increases, AI-1 concentration increases within the cell, creating a positive feedback loop that increases transcription of the operon.
3.2 Results

3.2.1 Quorum-sensing Salmonella behavior in vitro

*Salmonella* transformed with a quorum-sensing system using GFP as a reporter (QS-GFP) turned on GFP expression only at high density in culture and in *in vitro* tumor tissue (Figure 3.2). *Salmonella* were grown from colonies, diluted at either 0.5x10^8 cfu/mL (Diluted Low) or 5x10^8 cfu/mL (Diluted High) down to 10^5 cfu/mL, and then their density and GFP expression was measured over time (Figure 3.2A). At densities less than 0.5x10^8 cfu/mL, QS-GFP remained off compared to LB controls (Figure 3.2B; P<0.05). Once a critical density threshold of 10^8 cfu/mL was reached, QS-GFP turned on GFP expression. Constitutive controls linearly increased GFP expression with density and had low-level expression at densities as low as 2.5x10^7 cfu/mL (Figure 3.2B).

Diluting QS-GFP at high densities increased GFP expression levels but not the critical density that switches on expression (Figures 3.2C-D). Both the Diluted High and Diluted Low cultures turned off gene expression after dilution to 10^5 cfu/mL and turned on GFP expression at 10^8 cfu/mL (Figure 3.2C). The Diluted High cultures, however, had a higher GFP expression compared to the Diluted Low cultures at densities higher than 5x10^8 (P<0.05; Figure 3.2D). Because to this increase in GFP expression, all subsequent experiments were cultured using the Diluted High cultured method.

QS-GFP only turned on GFP expression within high-density colonies in *in vitro* 3D tumor tissue compared to controls (Figures 3.2E-F). Thirty-eight hours post bacterial
injection, tumor tissue infected with QS-GFP exhibited pockets of GFP expression within distinct bacterial colonies while the remaining tissue area contained no GFP expression (Figure 3.2E). The tumor tissue infected with constitutive bacterial controls turned on GFP expression throughout the entire tissue by 53 hours, indicating that bacteria colonize the entire tissue. The area of affected tissue was greater in the constitutive controls than QS-GFP (Figure 3.2F; P<0.05). QS bacteria expressing GFP were present in 45% of the total tissue area after 53 hours compared to 97% area coverage for the constitutive bacteria. The heterogeneity of the GFP expression within the tissues infected with QS bacteria indicates a switch behavior between areas of low and high bacterial densities.

3.2.2 Quorum-sensing salmonella behavior in tumor-bearing mice

Mice injected with QS-GFP expressed GFP within high-density colonies in tumor tissue and showed no sign of GFP expression within livers (Figure 3.3). 84,213 and 133,305 colonies of Salmonella were present in tumor tissue for QS and constitutive controls, respectively, while no Salmonella or GFP was present in corresponding livers using immunofluorescence (Figure 3.3A). Salmonella density was 2.21x10^6 cfu/g and 1.13x10^7 cfu/g in tumors and 2.49x10^4 cfu/g and 2.92x10^4 cfu/g in livers of both the QS and constitutive Salmonella infected mice, respectively. (Figure 3.3B). There was, however, no statistical difference between the QS and constitutive bacterial densities across livers or across tumor tissue. In tumor sections of mice infected with QS-GFP, GFP co-localized with large colonies of bacteria (Figure 3.4A, i-iii) while no GFP co-localized with small colonies (Figure 3.4A, iv-vi). In comparison, GFP expression co-localized with colonies of constitutive controls, regardless of colony size (Figures 3.4A, vii-xii).
QS *Salmonella* did not require an external inducer and GFP expression was observed in tumor tissue as early as 9 days and as late as 24 days post injection.

At low densities, 41% of constitutive colonies express GFP compared to 3% of QS colonies (P<0.05, Figure 3.4B). QS colonies begin to show statistically similar GFP expression levels as low density constitutive colonies at densities above $4.2 \times 10^{10}$ cfu/g (P<0.05). The likelihood of a QS colony expressing GFP statistically increased from 4% to 43%, once the density reached $4.2 \times 10^{10}$ cfu/g (P<0.05, Figure 3.4C). In comparison, the likelihood of constitutive colonies expressing GFP statistically increased from 45% to 72%, once the density reached above $4.2 \times 10^{10}$ cfu/g (P<0.05). No constitutive colonies above a density of $4.8 \times 10^{10}$ cfu/g were found in tissue.

### 3.2.3 Optimization of Density Calculations

To quantify the effect of density and spatial distribution of QS-GFP on GFP expression within tissue, the average radial distance and the number of bacteria were counted within circles of increasing radii surrounding the center of mass of each individual colony (Figure 3.5). The total number of bacteria expressing GFP was calculated as a function of bacterial density and fit to the sigmoidal function:

$$
\alpha = \frac{1}{2} + \frac{1}{2} \tanh \left( \frac{\rho - \rho_{\text{crit}}}{\sigma} \right)
$$

where $\alpha$ is the fraction of bacterial colonies expressing GFP, $\rho$ is the density of salmonella within the circle, $\rho_{\text{crit}}$ is the critical density at which GFP expression occurs, and $\sigma$ represents how sensitive the quorum-sensing switch is to changes in density.
(Figure 3.5A). The percentage of colonies expressing GFP fit Eq. 1 with $\rho_{\text{crit}} = 4.97 \times 10^{10}$ cfu/mL and $\sigma = 2.72 \times 10^{10}$ cfu/mL within a circle size of 125 pixels (161.3 $\mu$m; Figure 3.5B). The majority of colonies below this critical density exhibited little to no GFP expression while the majority of colonies above this critical density co-localized with GFP in tissue. No colonies were found in tissue above a density of $5.65 \times 10^{10}$ cfu/g but the model infers that over 95% of colonies would express GFP at densities upwards of $9.01 \times 10^{10}$ cfu/g.

To calculate the optimal circle size for density optimization, the density of Salmonella surrounding each colony was calculated for circles ranging from a radius of 10 pixels (12.904 $\mu$m) to 200 pixels (258.08 $\mu$m; Figure 3.5C). Varying the circle size around the colony was important for determining the region in which bacteria interacted with their neighbors. Circles that were too small can underestimate the total number of bacteria interacting with the colony of interest while circles that were too large can overestimate the number of bacteria interacting with the colony of interest. A circle with a radius of 125 pixels was used for further analysis because it minimized the standard error of the fitted parameters $\rho_{\text{crit}}$ and $\sigma$ to the data (Figure 3.5D). $\rho_{\text{crit}}$ and $\sigma$ minimized at a circle with a radius of 150 pixels (Figure 3.5E).

### 3.2.4 Effect of spatial distribution on GFP expression

At high densities, the percentage of colonies expressing GFP increases as the average radial distance of neighboring colonies decreases from the colony of interest (Figure 3.6). The number of *Salmonella* were counted for increasing annuli from each colony of
interest and used to calculate the weighted average distance of all neighboring colonies (Figure 3.6A). At densities above $5.28 \times 10^{10}$ cfu/g, 84% of the colonies whose neighbors were at an average radial distance less than 103µm expressed GFP while zero colonies expressed GFP when their neighbors were at radial distances greater than 108µm ($P < 0.05$; Figure 3.6B). Between this range, 58% of the colonies expressed GFP. At densities below $5.28 \times 10^{10}$ cfu/g, radial distance has no effect on GFP expression. Only 43% of colonies expressed GFP when neighbors were at a radial distance of 103µm or less compared to the 84% seen at densities above 0.44 ($P < 0.05$).

### 3.2.5 Mathematical Modeling of AI-1 production and diffusion in tumor tissue

AI-1 concentration in tumor tissue was mathematically modeled by a partial differential equation that related AI-1 production to colony density and diffusion into infinite three-dimensional space.

$$\frac{\partial C}{\partial t} = \frac{D}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial C}{\partial r} \right)$$

$$\left. \frac{dC}{dt} \right|_{r=0} = m \alpha$$

$$C|_{r=\infty} = 0$$

(8)

Here, $C$ is the concentration of AI-1 and $D$ is the diffusion coefficient of AI-1 in tissue. The boundary conditions state that AI-1 is produced by a release rate, $m$, and the fraction of colonies expressing GFP, $\alpha$, which is dependent on bacterial density (Eq. 7), which is dependent on density $\rho$, and the concentration is zero far from the colony. This problem is set up using the density of bacteria and the average radial distance of all the colonies surrounding the colony of interest. Neighboring bacteria are lumped together and considered a point source at $r=0$, while the colony of interest is at the average radial distance ($r=r_{avg}$) away from the point source (Figure 3.7A). The analytical solution of Eq.
2 is dependent on dimensionless distance $\tilde{r}$, time $\tilde{t}$, bacterial density $\rho$, and a dimensionless parameter, $Q$, that relates AI-1 production, to diffusion.

$$\overline{C} = \frac{\alpha Q}{\tilde{r}} \text{erfc} \left( \frac{\tilde{r}}{2\sqrt{\tilde{t}}} \right)$$  \hspace{1cm} (9)

Here, $\overline{C}$, $\tilde{r}$, and $\tilde{t}$ are dimensionless concentration ($C/C_q$), radius ($r/r_c$), and time ($\frac{Dt}{r_c}$).

$C_q$ is the minimum AI-1 concentration necessary to turn on GFP expression and $r_c$ is the radius of the circle of interest. The dimensionless number, $Q$, describes the relative contributions of AI-1 production to AI-1 diffusion ($\frac{m}{4\pi D C_q r_c}$). The steady state solution of Eq. 3 is

$$\overline{C} = \frac{\alpha(\rho)Q}{\tilde{r}}$$  \hspace{1cm} (10)

The model was assumed to be at steady state since all mice were sacrificed greater than 9 days post-injection, depending on the growth rate of the individual tumors. $Q$ was determined to be 1.1 by non-linear optimization and predicted the GFP expression in 93% of individual QS colonies found in tissue. $Q$, which is greater than one, indicates that the system is diffusion limited when $\alpha$ is equal to one. When $\alpha$ is below 0.68 (8.17x$10^{10}$ cfu/g), the system becomes production limited as the product of $\alpha*Q$ falls below 1.

Mathematical modeling predicted that the AI-1 concentration decreases with distance from the colony point source (Figure 3.7B). The concentration profiles increase sigmoidally as the density of the source colony increases (Figure 3.7C). The density at the colony would have to reach 8.17x$10^{10}$ cfu/g to turn on a colony if it was at the very edge of a 161.3$\mu$m circle.
As the density decreases, the maximum radial distance at which neighboring colonies expresses GFP, $r_q$, increases in a sigmoidal shape (Figure 3.8A). At the critical density predicted by Eq. 1 ($\rho_{\text{crit}} = 4.97 \times 10^{10}$ cfu/g), $r_q$ is 87.1 $\mu$m. Colonies that have a high density and are packed tightly together are most likely to express GFP (Figure 3.8B, i). The model predicts the differences in GFP expression between two colonies with the same density and a 2 $\mu$m difference in radial distance (Figure 3.8B ii,iii). The model also predicts the difference between two colonies with the same radial distance and a $9.9 \times 10^9$ cfu/g difference in density (Figure 3.8B ii,iv). There was no colony below a density of $4.92 \times 10^{10}$ cfu/g that had a radial distance small enough to be predicted as having GFP expression.
Figure 3.2 *In vitro* Behavior of QS *Salmonella*. A) QS cultures were grown to either $0.5 \times 10^8$ cfu/mL or $5 \times 10^8$ cfu/mL and then diluted to $10^5$ cfu/mL. B) QS *Salmonella* only expressed GFP at densities above 0.2 compared to constitutive controls (*, $P<0.05$). C) Low dilution and high dilution cultures only showed significant expression compared at densities above 0.2 (*, $P<0.05$). D) High dilution cultures showed a significant increase in expression compared to low dilution cultures at similar bacterial density (*, $P<0.05$). Fluorescence was normalized to constitutive controls at $6 \times 10^8$ cfu/mL. E) After 31 hours post-injection, QS salmonella express GFP only within distinct bacterial colonies. Lac salmonella express GFP throughout the entire tissue regardless of bacterial concentration. F) Area fraction of tissue with GFP expression over time. Tissue infected with QS *Salmonella* showed a significant difference in area with “on” bacteria after 40 hours (*, $P<0.05$). White bounding boxes indicate the area analyzed for a representative tissue at 53 post-injection. Scale bars are 100µm.
Figure 3.3 QS Salmonella distribution in tissue A) Salmonella and GFP were observed in tumors injected with either QS Salmonella or constitutive controls. No Salmonella were observed in any liver section using immunofluorescence. Blue indicates a DAPI stain. Scale bars are 1mm. B) Tumors showed a statistical increase in the number of Salmonella compared to livers (*, P<0.05). Presence of Salmonella in livers was confirmed by plating (inset).
Figure 3.4 GFP expression of QS Salmonella within tumor-bearing mice. A) Colonies of QS and constitutive Salmonella (red) in 4T1 tumors and associated GFP (Green) for areas of low and high density. Yellow indicates areas of co-localization between Salmonella and GFP. Scale bars are 100µm. B) GFP expression dependence on Salmonella density. The percentage of QS Salmonella colonies expressing GFP at all densities less than $4.2 \times 10^9$ cfu/g was statistically less than the percentage of constitutive Salmonella colonies expressing GFP at densities below $1.2 \times 10^9$ cfu/g (*, P<0.05). C) The fraction of QS and constitutive colonies expressing GFP increased from 0.06 to 0.43 (*, P<0.05). The fraction of constitutive colonies expressing GFP was statistically greater than the percentage of QS colonies for densities above and below $4.2 \times 10^9$ cfu/g (**, P<0.05).
Figure 3.5  Density Optimization of GFP Expression  

A) Profile of GFP expression in relation to bacterial density where $\rho_{\text{crit}}$ is the critical density at which GFP expression occurs, and $\sigma$ represents how sensitive the quorum-sensing switch is to changes in density. 

B) Sigmoidal function modeling the percentage of GFP expression in relation to Salmonella density in a 125 pixel radius circle. Eq. 1 was used to fit the model to the experimental data and calculated $\rho_{\text{crit}} = 0.41 \; (4.97 \times 10^{10} \; \text{cfu/g})$ and $\sigma = 0.23 \; (2.72 \times 10^{10} \; \text{cfu/g})$. 

C) The amount of Salmonella was counted for increasing circle sizes surrounding each colony. 

D) The standard errors of the two parameters, $\rho_{\text{crit}}$ and $\sigma$, in Eq. 1 were minimized at a circle size of radius 125 pixels. 

E) The values of $\rho_{\text{crit}}$ and $\sigma$ for increasing circle radius.
Figure 3.6 Spatial Dependence of *Salmonella* on GFP Expression  A) The number of *Salmonella* were counted for increasing radial annuli from each colony and used to calculate the weighted average distance of all neighboring colonies. B) Dependence of the average radial distance of neighboring colonies on the GFP expression of the QS colony of interest. The percentage of QS colonies expressing GFP with a density of 5.28x10^{10} cfu/g increased as the average radial distance of neighboring colonies decreased (*, P<0.05). At densities less than 0.44, there was no radial distance dependence on GFP expression. The percentage of QS colonies decreased from 72% to 45% at an average radial distance less than 103µm once the density reached less than 5.28x10^{10} cfu/g (**, P<0.05).
Figure 3.7 Mathematical Model of AI-1 Concentration  A) Definition of radial distance in model. The neighboring bacteria are lumped together and considered a point source at r=0, while the colony of interest is placed at the average radial distance (r=r_{avg}) away from the point source. B) Predicted AI-1 concentration dependence on radial distance of neighboring colonies for different bacterial densities. As the radial distance increases, the concentration of AI-1 experienced by the colony of interest decreases. C) Predicted AI-1 concentration dependence on density of neighboring colonies for different averages of radial distance. As the density increases, the AI-1 concentration increases sigmoidally.
Figure 3.8 Predictive capability of AI-1 mathematical model  
A) Critical average radial distance necessary for turn on increases sigmoidally as the density of neighboring colonies increases. The model predicts that colonies that fall below this line turn on the QS system.  
B) Predictive capability of mathematical model. Colonies with a high density and packed together express GFP (i). The model predicts the differences in GFP expression between two colonies with the same density and a 2 µm difference in radial distance (ii and iii). The model also predicts the difference between two colonies with the same radial distance and a 9.9x10⁹ cfu/g difference in density (iii, iv).
3.3 Discussion

Administering *Salmonella* with the ability to change gene expression in a density-dependent manner has the ability to improve the targeting capability of bacterial cancer therapy and reduce systemic toxicity. We have shown that *Salmonella* integrated with a quorum-sensing trigger turns on protein expression in high-density colonies within tumors while remaining off low-density colonies. In addition, the likelihood of GFP expression of high-density colonies increased as the radial distance of neighboring *Salmonella* decreased. A mathematical model of AI-1 concentration predicted the likelihood of GFP expression in 93% of QS colonies found in tumor tissue based on density and radial distance from adjacent *Salmonella*. In contrast, when *Salmonella* was administered with a constitutive trigger, protein expression was observed in low-density colonies and individual *Salmonella* with no surrounding neighbors. These results demonstrate that a bacterial cancer therapy with a QS triggering system will prevent therapeutic protein release in healthy tissue and maximize therapeutic effect in tumors.

The density of QS *Salmonella* in livers and the critical density needed to trigger the QS system render the possibility of gene expression almost impossible in healthy tissue. While no *Salmonella* were seen in livers by immunohistochemistry, approximately $2 \times 10^4$ cfu/g of *Salmonella* was observed in plating experiments (Figure 3.3B), 2 million-fold lower than the density at which the QS system turned on expression in tumor tissue (Figure 3.5B). Theoretically, if all the *Salmonella* found in the liver were located within a $161.3 \mu m$ radial circle, the density would be $4.8 \times 10^{10}$ cfu/g, which would still be below
the critical density \( \rho_{\text{crit}} = 4.97 \times 10^{10} \text{ cfu/g} \) to turn on the QS system. Mathematical modeling predicts that at this density, *Salmonella* would need to be at a radial distance less than 82\( \mu \text{m} \) apart for expression to occur (Figure 3.8A). No QS colony at \( 4.8 \times 10^{10} \text{ cfu/g} \) in tumor tissue had an average radial density less than 82\( \mu \text{m} \). Protein expression was seen in 45\% of constitutive *Salmonella* at the lowest possible detectable density in tumor tissue (Figure 3.4B). Constitutive expression of toxic proteins in livers or other healthy organs, even at low numbers, has the potential for detrimental effects on the patient. In comparison, only 0.06\% of QS *Salmonella* exhibited protein expression at low densities in tumor tissue (Figure 3.4B). This data suggests that none of the QS *Salmonella* in the liver expressed GFP.

The sensitivity of this density-dependent switch suggests that QS *Salmonella* have the ability to treat undetected metastatic legions and remain off in surrounding healthy tissue. The QS system turns on at a density of 41\% coverage \( (4.97 \times 10^{10} \text{ cfu/g}) \) in tumor tissue. In previous work, *Salmonella* accumulate in liver metastases at a density of 44\% (Ganai et al., 2011), suggesting that all colonies would be active. In the surrounding hepatic parenchyma, *Salmonella* colonies accumulate with an average density of 0.5\% (Ganai et al., 2011) and would all be inactive. The average metastases in this previous study had a cross-sectional area of 5.3mm\(^2\), which is 66 times greater than 0.08mm\(^2\) (Figure 3.5E), the region large enough for sufficient neighbors to turn on QS expression.

Diffusion and bacterial spatial distribution within tumors plays an important role in triggering QS system. QS *Salmonella* turn on protein expression at densities of \( 10^8 \)
cfu/mL in flasks (Figure 3.2B), but mixing ensures that AI-1 is well distributed and not detected by diffusion. In tumor tissue, however, the QS switch turned on at densities of 4.97x10^{10}, almost 500-fold higher than flasks (Figure 3.5B). The increase in density was caused by the distance necessary for AI-1 to diffuse throughout the tissue once the system was turned on. At densities above 5.28x10^{10} cfu/g, protein expression was dependent on bacteria being packed closer than 103 µm to each other (Figure 3.7B). Mathematical modeling predicted that as the density of bacteria decreased, the average radial distance of the surrounding neighbors must decrease as well for the QS switch to turn on (Figure 3.8A). Decreasing the distance between bacteria decreases the distance AI-1 must travel between individual bacteria and increases the overall AI-1 concentration in a bacterial cluster. Below a critical threshold of 5.28x10^{10} cfu/g, however, there are not enough individuals producing AI-1 to turn on expression, no matter how tightly packed they are (Figure 3.6B).

QS Salmonella have other important advantages over other proposed mechanisms of bacterial drug delivery. No autoinducer was used to induce expression of GFP after colonization. Previous strategies with autoinducers are problematic, as an autoinducer must overcome both the clearance from the body and diffusion limitations far from vasculature. Without the need for an autoinducer, the system is not reliant on the presence of a small molecule to maintain therapeutic expression levels. Persistent gene expression was observed in tumor tissue as late as 24 days post injection. From a clinical standpoint, this means fewer injections for the patient and a quicker therapeutic effect.
Salmonella integrated with a robust QS triggering system opens up the possibility for the use of aggressive therapeutic proteins, previously deemed too toxic for both systemic and bacterial drug delivery. Potential therapeutics such as Staphylococcus aureus α-hemolysin kill multiple cancer cells quickly (St Jean et al., 2014; Swofford et al., 2014), but might be too toxic if delivered to healthy tissue. Salmonella integrated with a QS switch could prevent expression in healthy tissue, despite the low-level bacterial accumulation in healthy tissue (Figure 3.3B). In addition, QS Salmonella could maintain therapeutic expression levels due to continuous production. Overall, the QS system creates a robust switch that will allow for targeted bacterial drug delivery to tumors and metastases without the need for an external inducer while minimizing the risk of potentially harmful drugs reaching healthy tissue.

3.4 Materials and Methods

3.4.1 Bacterial strains and plasmids
The QS architecture used in this study has the luxI, luxR, and gfp genes under one side of the PluxI bidirectional promoter to create an on/off switch (Figure 3.1B). Two plasmids were created that contained the green fluorescent protein GFPmut3 under the control of either the constitutive lac promoter or a quorum sensing circuit. These plasmids also contained the gene encoding for aspartate-semialdehyde dehydrogenase (asd) that allows for plasmid retention in the nonpathogenic msbB, purF, xyI, asd Salmonella strain, VNP200010. Plasmid cloning was performed in Escherichia coli DH5α (Invitrogen, Carlsbad, CA, USA) and transformed into VNP200010. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). QS-GFP was created by
PCR amplifying the luxI gene out of pACluxInv, a gift from Dr. Voigt (University of California, San Francisco, CA), using the forward primer 5’-TTCGGCGGAATAAACGCAAG-3’ (EagI site underlined) and the reverse primer 5’-GCACATGTATCTTGAATCATTCATCCAT-3’ (PciI site underlined) and subcloned into PluxI-Gfpmut3-luxR, a gift from Dr. Sun (University of Massachusetts Amherst, MA) using the EagI and PciI sites. The asd gene was PCR amplified from pYA3332 (Vion) using the forward primer 5’-GCTCATGACGTACGTTTCTGGTCCATTG-3’ (BspHI site underlined) and the reverse primer 5’-ATACTAGTATCTGGTCTTACTCTTGATTACG-3’ (SpeI site underlined) and subcloned into pQSGFPmut3 using BspHI and SpeI sites to create QS-GFP.

placGFPmut3-ASD was created by PCR amplifying the asd gene from pYA322 using the forward primer 5’-GCGAATTCTGACGTTTCTGGTCCATTG-3’ (EcoRI site underlined) and the reverse primer 5’-ATGACGTCATCTGCTTTACTCTTGATTACG-3’ (AatII site underlined). Asd was subcloned into placGFPmut3, a gift from Dr. Sun, to create a constitutive control.

3.4.2 Expression of QS promoter using fluorimetry

QS-GFP and a constitutive control were grown overnight in 3mL cultures and diluted to 10^5 cfu/mL in 20mL of LB media. Every hour, the optical density (OD) of a 200μL aliquot was measured at 600nm. Fluorescence was measured using an excitation wavelength of 465nm and an emission wavelength of 505nm.
3.4.3 Measurement of QS expression levels based on changes in culture conditions

QS-GFP and constitutive controls were grown from a colony in 3mL cultures to an OD of 0.1. An aliquot was diluted to $10^5$ cfu/mL in 20mL of LB media (Diluted Low) while the original 3mL culture was allowed to continue growing. After the original 3mL culture reached an OD of 1.0, another aliquot was diluted to $10^5$ cfu/mL in 20mL of LB media (Diluted High). Every hour, the optical density and fluorescence of both cultures were measured.

3.4.4 Measurement of protein expression within in vitro tumor tissue

A microfluidic tumor-on-a-chip device containing cancer cells was used to measure bacterial protein expression in tumor tissue. Human LS174T colon carcinoma cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) at 37 °C and 5% CO2. Multicellular tumor spheroids were formed by seeding 2.5 x 104 cells/ml on poly(2-hydroxyethyl methacrylate; Sigma-Aldrich) coated T25 flasks for 12-14 days. The methacrylate coating prevents cell adhesion to the flask surface. Devices were fabricated using soft lithography techniques described previously (Toley et al., 2011; Toley and Forbes, 2012; Walsh et al., 2009). Prior to inserting cells, devices were sterilized by flushing with 70% ethanol, followed by phosphate buffered saline (PBS) to remove air and residual ethanol. Spheroids were inserted into the 1000µm x 300µm x 150µm cuboidal chambers by trapping them in the filters at the distal ends (Walsh et al., 2009). The medium flow rate was set to 3.0 µl/min, which is similar to the average linear velocity in tumor vessels (1.7 mm/sec) (Forbes et al., 2003). Devices were incubated for 24 hours at 37°C in an enclosed, humidified
environment on the microscope stage. pH was maintained by continuous perfusion of HEPES (25 mM) buffered DMEM.

QS-GFP and constitutive controls were grown overnight, centrifuged and re-suspended in DMEM with 10% FBS at a density of $10^5$ CFU/mL. Bacteria-containing medium was administered to devices for 1 hour, stopped, and switched to bacteria-free medium. Images of each chamber were acquired for 72 hours (Olympus, Center Valley, PA). To capture an entire chamber (1000 µm x 300 µm), 2 images (867.15µm x 660.68µm each) obtained at 10x were tiled together using a macro in IPLab (BD Bioscience, Rockville, MD). Transmitted light and green fluorescence images were captured at 1-hour intervals, using 470nm excitation and 525nm long pass emission filters (Chroma, Rockingham, VT). ImageJ (NIH Research Services Branch) was used to quantify green fluorescence. Temporal fluorescence profiles were measured for each tissue as a function of time and subtracting background fluorescence. Area fraction of tissue with GFP expression above a constant threshold was measured across all images and divided by the total area of tissue. The zero time point was set to be immediately after bacterial inoculation.

### 3.4.5 Tumor formation in murine models

4T1 mammary carcinoma cells (American Tissue Type Collection, Manassas, VA) were grown in RPMI-1640 with 10% FBS. Tumors were formed by subcutaneously injecting 50,000 4T1 cells, suspended in PBS, into the flank of BALB/c mice at 8 weeks of age. Caliper measurements were taken regularly to monitor tumor growth. Tumor volume was calculated by $(\text{width})^2(\text{length})/2$. Implanted tumors were allowed to grow until volumes reached 2000mm$^3$. All animal procedures were approved by Baystate Medical
Center Institutional Animal Care and Use Committee (IACUC). Experiments were conducted in accordance with the National Institute Health (NIH) guidelines for care and use of laboratory animals.

3.4.6 Administration of Salmonella

A dose of 2x10^6 colony forming units (CFU) mid-log phase VNP200010, transformed with either pQSGFPmut3-ASD or pLacGFPmut3-ASD, was suspended in 100µl PBS and injected intravenously into mice with size-matched tumors of 500mm^3 via the tail vein. Mice were sacrificed when tumors reached 2000mm^3 for tissue collection, which took place 2-3 weeks after bacterial injection. Tumor and liver samples were cut in half. One half was fixed in 10% formalin and embedded in paraffin, while the other half was minced in saline and plated. LB agar plates were counted after growth at 37ºC for 24 hr.

3.4.7 Immunofluorescence labeling

Salmonella and GFP were identified by immunofluorescence. Five micron thick equatorial sections were cut from excised tumors and livers, deparaffinized and rehydrated. Antigen retrieval was performed on each tissue by soaking sections in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 20 minutes. Sections were then blocked with protein block (Agilent Technologies, Santa Clara, CA) for 20 minutes. Sections were probed with a 1:200 dilution of rabbit anti-Salmonella (Abcam, Cambridge, MA) and a 1:50 dilution of goat anti-GFP (Abcam) overnight, followed by incubation with a 1:200 dilution of Alexa Fluor 546-conjugated donkey anti-rabbit antibody and a 1:50 dilution of Alexa Fluor 488-conjugated donkey anti-goat antibody at room temperature for 1 hour. Processed tissue sections were counter-stained
with VECTASHIELD® Mounting Media with DAPI (Vecta Labs, Burlingame, CA), mounted with coverslips, stored at 4°C and protected from light.

3.4.8 Image acquisition

Images were acquired using epifluorescent microscopy techniques described earlier using a 10X objective. *Salmonella*, labeled with Alexa 546, were identified using 546nm excitation and 590nm long pass emission filters (Chroma). GFP, labeled with Alexa 488, were identified using 470nm excitation and 525 long pass emission filters (Chroma). DAPI counterstaining was identified with a UV excitation filter set (Chroma). Images of entire tissues were captured by tiling 250-650 adjacent 867.15µm x 660.68µm frames using a macro in IPLab.

3.4.9 Bacterial and GFP distribution analysis

*Salmonella* and GFP distribution was quantified using imageJ. Red-fluorescence and green-fluorescence were thresholded and converted into binary images to identify regions (pixels) that contained *Salmonella* and GFP. Artifacts were manually eliminated based on morphology and fluorescence intensity from each individual tile comprising the entire tissue. Individual binary tiles were then reconstructed to create red-fluorescence and green-fluorescence images for the entire tissue. The number of *Salmonella* colonies and their center of mass was determined by analyzing each binary image using particle analysis in ImageJ. Different densities of *Salmonella* surrounding each colony were determined by counting the number of red-fluorescence pixels in increasing circle sizes, ranging from a 10 to 200-pixel radius, surrounding each center of mass. An individual red pixel was assumed to be equal to 1 *Salmonella* bacterium. Each colony was
considered co-localized with GFP if a green-fluorescence pixel was within a 25-pixel radius circle surrounding the center of mass. This process assigned a binary result regarding the presence of GFP for each colony: a value of 1 if GFP was present or a 0 if not present.

A sigmoidal function was used to fit the percentage of colonies with GFP at each density for various circle sizes (Eq. 7). The function consisted of two parameters, \( \rho_{\text{crit}} \), the critical density at which GFP expression occurs, and \( \sigma \), the robustness of the QS switch. The data was fit using nonlinear regression in MATLAB to calculate the two parameters and their standard error for various circle sizes. An optimal circle size was determined by minimizing the standard error of the two parameters. The average radial distance of all neighboring colonies to the colony of interest was determined by determining the number of Salmonella in a 25-pixel radial annulus with varying distances from the colony of interest. The radial distance of the annulus to the center of the colony was assigned to each Salmonella pixel located within it. The weighted average of all radial distances of neighboring bacteria was then assigned to each colony of interest.

### 3.4.10 Mathematical modeling of AI-1 diffusion

A mathematical model was used to interpret the concentration of AI-1 in tumor tissue (Eq. 8). The model consisted of a single partial differential equation that balanced AI-1 production based on density with diffusion. The optimized parameters determined in Eq. 1 were used to model GFP production in relation to density. This model had an analytical solution (Eq. 9) that was dependent on distance, time, density, and a dimensionless parameter, Q, which related AI-1 production at maximum density to...
diffusion. This analytical solution was assumed to be at steady-state for further analysis (Eq. 10). The value of $Q$ was determined from the immunofluorescence data by non-linear optimization. Its value was adjusted until the model could predict the GFP expression in the maximum amount of colonies, based on the density and average radial distance of all neighboring colonies to the colony of interest.

### 3.4.11 Statistical analysis

For all *in vitro* work, data are reported as means with error bars representing standard errors of the mean. Hypotheses were tested using Student’s t-test with a significance level indicated by $P<0.05$. For all *in vivo* colony GFP expression work, data are reported as means with error bars representing a 95% Clopper-Pearson binomial confidence intervals. Hypotheses were tested using a Fisher’s exact test with a significance level indicated by $P<0.05$. Parameters from Eq. 7 are reported with error bars representing the asymptotic standard error estimated using the Jacobian matrix and weight function from the nonlinear regression.
CHAPTER 4
INTEGRATION OF THE QUORUM-SENSING SWITCH WITH

*STAPHYLOCOCCUS ALPHA-HEMOLYSIN*

4.1 Introduction

Bacterial cancer therapy has the potential to overcome many of the challenges associated with current chemotherapy, including transport limitations, systemic delivery, and reduced susceptibility of quiescent tumor cells, due to the inherent ability of bacteria to localize in both tumors and metastases (Forbes, 2010). Despite this, many challenges lie ahead before an optimized bacterial therapy will be realized in the clinic. Native bacterial toxicity has been shown to regress tumors and increase survival, but bacteria alone are unable to completely eliminate tumors (Chen et al., 2009; Jia et al., 2007; Lee et al., 2008; Low et al., 1999; Luo et al., 2001; Nagakura et al., 2009; Pawelek et al., 1997; Thamm et al., 2005; Theys et al., 2006; Zhao et al., 2007). In addition, low-level bacterial accumulation in healthy tissue prevents constitutive expression of an anti-cancer drug, reducing the efficacy of a bacterial therapy over standard chemotherapeutics. The research described in this thesis has pushed bacterial cancer therapy closer to the clinic by overcoming these challenges.

In the first part of this work, a novel bacterial cancer therapeutic, *Staphylococcus* alpha-hemolysin (SAH), was discovered to secrete from anticancer bacteria and rapidly kill cancer cells. SAH was shown to be effective against multiple cancer cell types, including two triple-negative breast cancer cell lines. While incredibly effective, SAH could prove
lethal to patients if small amounts of bacteria within healthy tissue were to secrete the protein. The second part of this work aimed to solve this problem by creating a tumor-specific triggering system, which has the capability to both reduce systemic toxicity and allow for the use of more aggressive therapeutics, such as SAH, because of enhanced drug targeting. Therapeutic *Salmonella*, integrated with the *lux* quorum-sensing system from *Vibrio fischeri*, were able to selectively trigger protein expression within high-density colonies in tumor tissue while exhibiting little to no expression at lower densities.

Individually, each part of this thesis provide a necessary framework for improving upon bacterial cancer therapies. The logical next step would be to combine the aggressive therapeutic SAH with the quorum-sensing system. We hypothesized that *QS-SAH* Salmonella *would only secrete SAH at high densities*. To test this hypothesis, we integrated the *lux* QS system with SAH and transformed this system into VNP200010. SAH production in response to density was measured by western blot. An integrated assay involving both bacterial and mammalian cell culture was used to screen the translation, secretion, and killing ability of SAH in response to the bacterial density in culture. Cell viability was determined using a cell viability assay. *Salmonella* integrated with the QS-SAH system were injected in tumor-bearing mice to quantify tumor volume over time.
4.2 Materials and Methods

4.2.1 Bacterial strains and plasmids

QS-SAHA S. salmonella was created by PCR amplifying the SAH gene out of the genome of Staphylococcus aureus strain MW2, using the forward primer 5’-TAGCTAGCTCGTTAAAAATAGAAG-3’ (NheI site underlined) and the reverse primer 5’-ATCCTGCAAGTTAATGTCATTCTTCT-3’ (Shfl site underlined). SAH was then subcloned into pBAD_SAH using the NheI and Shfl sites to create pQS-SAHA.

The pQS-SAHA plasmid consists of the high copy PUC origin, the ampicillin resistance gene, the lux QS system, and the asd gene, used for selection pressure in the asd-VNP200010 S. salmonella strain. pQS-SAHA was then transformed into VNP200010. pQS-GFP was previously designed as outlined in Chapter 2.

4.2.2 Supernatant and lysis collection of QS-SAHA

QS-SAHA S. salmonella was grown overnight in 3mL cultures and diluted to 10⁵ cfu/mL in 200mL of LB media. After the culture reached an OD of 0.01, a 5mL aliquot was removed every half an hour. The optical density was measured at 600nm on a µQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Bacterial cells were then pelleted by centrifugation and separated for supernatant extraction. Supernatant fractions were purified with a 0.22 µm-filter and concentrated 10X by centrifugal ultrafiltration (Millipore, Billerica, MA). Eventual 10X dilution of dilution of the concentrated supernatant was considered a co-culture equivalent concentration. Pellets were resuspended and incubated for 30 min in 500 mL water containing 1 halt protease inhibitor cocktail (Thermo Fisher Scientific), 12.5 units/mL of
DNAaseI (Thermo Fisher Scientific), and 25mg/mL of lysozyme (Thermo Fisher Scientific). After incubation, pellets were transferred to 2 mL centrifuge tubes with 600 mm glass beads. These samples were alternately vortexed for 1 min and incubated on ice for 1 min, for 10 cycles. One milliliter of water was then added, vortexed briefly, and centrifuged for 3 min. The lysis supernatant was removed without disturbing the glass beads. pBAD-SAH supernatant controls were collected by the methods outlined in Chapter 1.

4.2.3 Western blot analysis

Supernatant fractions from each OD were boiled with 4X Laemmli reducing buffer (Boston BioProducts, Ashland, MA) for 5 min and run on SDS-PAGE for 45 min at 200V. Proteins were transferred to nitrocellulose membranes that were blocked with 5% milk in tris-buffered saline containing 0.2% tween-20 (TBST) for 1 hour at room temperature. SAH samples were probed with 1:1,000 sheep anti-SAH polyclonal antibody (Abcam, Cambridge, MA) overnight at 4°C, washed three times in TBST, proved with a 1:1,000 HRP-conjugated donkey anti-sheep polyclonal antibody (R&D Systems) for 1 hour at room temperature, and washed three times in TBST. To visualize and image the immunoblot, 4-chloro-1-naphthol/3,3'-diaminobenzidine (CN/DAB); (Thermo Fisher Scientific) was used for chromogenic detection of HRP.

4.2.4 Assay of protein efficacy

MCF-7 human mammary carcinoma cells (American Tissue Type Collection, Manassas, VA) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM); (Sigma-Aldrich, St. Louis, MO) containing 10% Fetal Bovine Serum (FBS); (Atlanta Biologicals,
Lawrenceville, GA) at 37°C and 5% CO₂. Cells were seeded at 7,500 cells/well into 96-well plates and allowed to adhere to the culture surface for 24 hours. The media was aspirated and replaced with treatment media consisting of 90% DMEM/FBS and either 10% phosphate-buffered saline (PBS) or bacterial supernatants from increasing densities. This dilution allowed for protein levels equivalent to direct co-culture without having bacteria in mammalian culture. Each treatment media application was replicated in eight wells.

After 1 hour, cell viability was measured by assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolum (MTS); (Promega, Madison, WI). Treatment medium was replaced with 100µL DMEM/FBS plus 20µL MTS reagent and incubated for 1 hour at 37°C for color development. Absorbance was measured at 490nm (Bio-Tek Instruments, Winooski, VT). Relative survival values are reported as measured absorbance normalized by PBS controls.

4.2.5 In vivo efficacy of QS-SAH

4T1 mammary carcinoma cells (American Tissue Type Collection, Manassas, VA) were grown in RPMI-1640 with 10% FBS. Tumors were formed by subcutaneously injecting 50,000 4T1 cells, suspended in PBS, into the flank of BALB/c mice at 8 weeks of age. Caliper measurements were taken regularly to monitor tumor growth. Tumor volume was calculated by (width)²(length)/2. Implanted tumors were allowed to grow until volumes reached 2000mm³. All animal procedures were approved by Baystate Medical Center Institutional Animal Care and Use Committee (IACUC). Experiments were conducted in accordance with the National Institute Health (NIH) guidelines for care and
use of laboratory animals. A dose of $2 \times 10^6$ colony forming units (CFU) mid-log phase VNP200010, transformed with either pQS-GFP or pQS-SA, was suspended in 100 µl PBS and injected intravenously into mice with size-matched tumors of 500 mm$^3$ via the tail vein. Mice were sacrificed when tumors reached 2000 mm$^3$ for tissue collection, which took place 2-3 weeks after bacterial injection.

### 4.3 Results

#### 4.3.1 Density dependence on the concentration of SAH

SAH was only present in the supernatant of QS-SA Salmonella at very high densities (Figure 4.1). SAH was detectable once Salmonella grew to densities higher than $1.16 \times 10^9$ cfu/mL (Figure 4.1A). Below this critical threshold, no presence of SAH was detected. QS-SA Salmonella secreted 13% the concentration of SAH at $1.16 \times 10^9$ cfu/mL compared to pBAD-SA supernatant induced at a density of $2.5 \times 10^8$ cfu/mL (Figure 4.1B). QS-SA lysis had 40% the concentration of SAH as pBAD-SA lysis controls.

#### 4.3.2 Efficacy of QS-SA Salmonella on cancer cell death

SAH secreted from Salmonella at high densities did not show a significantly decrease cell death compared to PBS controls (Figure 4.2). pBAD-SA supernatant controls reduced cell viability to 22% after one hour, while supernatant from QS-SA Salmonella at densities at $1.16 \times 10^9$ cfu/mL did not reduce cell viability at all. There was also no effect on cell viability as the density of Salmonella increased.
4.3.3 Effect of QS-SAH Salmonella on in vivo murine models

Mice administered with QS-SAH *Salmonella* did not show a significant decrease in tumor volume compared to QS-GFP *Salmonella* controls (Figure 4.3). 4T1 tumors exposed to QS-SAH and QS-GFP *Salmonella* both had doubling times of 7.3 days. After the administration of *Salmonella*, mice did not exhibit any signs of toxic side effects.
Figure 4.1 Density dependence of QS-SAHA) Western blots of lysate and supernatant from QS-SAHAH and pBAD-SAHAH. SAH is secreted from QS-SAHAH at densities of OD=2.3 (1.15x10^9 cfu/mL). B) Relative protein expression was quantified using densitometry. The QS system only secreted 13% the amount of SAH secreted from the pBAD system after induction at 2.5x10^8 cfu/mL. Values were normalized to the supernatant of pBAD-SAHAH.
Figure 4.2 Density dependence of QS-SAH on cell viability. QS-SAH supernatant from increasing densities had no effect on cell viability compared to PBS controls. Supernatant from induced pBAD-SAH controls reduced cell viability (*, P<0.05).
Figure 4.3 Effect of SAH secreted by QS-SAHS on in vivo tumor volume. QS-SAHS did not affect volume of subcutaneous 4T1 tumors compared to QS-GFP controls. 4T1 tumors infected with either QS-SAHS or QS-GFP both had doubling times of 7.3 days.
4.4 Discussion

Salmonella expressing SAH integrated with a QS expression switch only initiated SAH expression at very high densities in vitro. The reduced concentration of SAH was not enough to reduce cell viability of cancer cells in vitro. In addition, QS-SAHI did not reduce tumor volume in mice. SAH produced by QS Salmonella was only 13% of the amount produced by the pBAD-SAHI controls, which induced expression in the presence of arabinose. These results show that a VNP200010 bacterial cancer therapy integrating SAH with a QS triggering system did not produce enough SAH to reach a therapeutic effect both in monolayer culture and in murine tumor models.

SAH was most likely unable to reduce tumor volume in mice because of the low SAH expression produced. In chapter 2, SAH from pBAD Salmonella was shown to reduce cell viability by over 88% in just six hours (Figure 2.6A), but this therapeutic effect quickly dropped if secreted SAH was diluted 10-fold. At a 1:10 dilution, cell viability only reduced by 41%, 2-fold less than the non-diluted sample (Figure 2.6A). SAH from QS Salmonella, at high densities of $1.16 \times 10^9$, only produced 13% of the pBAD-induced sample (Figure 4.1A), similar to the 1:10 diluted pBAD-SAHI sample. This would reduce the therapeutic effect of SAH.

Because the QS system was unable to produce enough SAH, SAH would benefit from a different expression system to induce enough for a therapeutic effect. Inducing SAH using the pBAD system would be the first obvious choice since we have shown its efficacy in previous experiments (Chapter 2). Arabinose has also already been shown to
control gene expression in tumors (Loessner et al., 2007; Nguyen et al., 2010) and infarcted myocardium (Le et al., 2011). Due to the need for high concentrations of SAH for therapeutic effect, a system integrating both the pBAD and QS system created in our lab could be used to amplify the production of SAH and trigger the expression of SAH in regions of tissue unreachable by arabinose alone (Dai et al., 2012). Other inducible systems using salicylate (Royo et al., 2007) or IPTG could also provide a stronger expression system that would boost the concentration of SAH within tissue.

The QS system, despite its inability to produce therapeutic concentrations of SAH, still has the potential to treat cancerous tissue if combined with another therapeutic protein. PEA, for example, was another protein discovered from the toxin screen that was secreted from *Salmonella* and reduced the cell viability. Secreted PEA reduced cell viability 6-fold compared to an empty vector control (Figure 2.3A) after a 72-hour exposure. Furthermore, pure PEA as low as 50ng/mL was able to reduce cell viability even after a five-day exposure (Figure 2.6B). SAH secreted from *Salmonella* produced concentrations of approximately 500ng/mL (Figure 2.4B), suggesting that PEA could be secreted at similar amounts using the pBAD system. If the QS system were to secrete 10% of that amount, it could potentially still produce a therapeutic effect with PEA. Results with the toxin screen suggest that many more potential proteins, previously considered too dangerous for cancer therapy, should be reevaluated in the context of the QS delivery system.
CHAPTER 5

IMPACT

This work has solved two critical problems associated with bacterial cancer therapy: 1) the discovery of a therapeutic drug that can be expressed by bacteria for the treatment of cancer and 2) a gene triggering strategy that will prevent expression within healthy tissue. Bacterial cancer therapy holds great promise as a clinical alternative to chemotherapy due to the inherent ability of bacteria to localize within cancerous tissue. However, colonization within tumors alone is not enough to eliminate cancerous tissue. For the first time, we have discovered SAH as a potential therapeutic protein for bacterial cancer therapy, due to its ability to secrete out of nonpathogenic Salmonella in concentrations that rapidly kill cancer cells. The toxin also indiscriminately kills multiple types of cancer cells because it forms pores in cellular membranes and does not require endocytosis to be effective. Using our toxin screen, we have also discovered PEA as a potential therapeutic, since it also secretes and kills cancer cells, although at a much slower rate than SAH. This screen suggests that the hundreds of bacterial toxins considered too toxic for consideration should be reevaluated in the context of bacterial therapy.

This work has also demonstrated, for the first time, a QS-gene-triggering strategy that only switches on expression in high-density colonies within tumors without the need of an external autoinducer. Previous strategies with autoinducers are problematic, as an autoinducer must overcome both the clearance from the body and diffusion limitations far from vasculature. Another distinct advantage is that the QS system exhibits persistent
gene expression once the density is above a critical threshold. Without the need for an autoinducer, the system is not reliant on the presence of a small molecule to maintain therapeutic expression levels. From a clinical standpoint, this means fewer injections for the patient and a quicker therapeutic effect. Finally, due to the density of bacteria being significantly lower than the critical density needed for QS gene expression, the small amount of bacteria in healthy tissue remain off. This will be critical if the QS system is connected to an aggressive therapeutic that only requires a small concentration in healthy tissue to harm the patient. In conclusion, coupling the QS system developed in this thesis with an aggressive protein drug similar to SAH has the potential to be more effective than standard chemotherapeutics, due to its ability to target expression only within tumors.


