Development of Novel Tools to Study and Combat Pathogenic Microbial Biofilms

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Joshua D. Erickson

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TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

ABSTRACT

An increase in antibiotic resistance along with a decrease in antimicrobial products coming to market has compounded into a global issue that must be addressed. Biofilms are a morphological state of many pathogenic microbes that significantly augments their resistance to antimicrobial agents. Today, there is a need not only for groundbreaking strategies that are effective against drug resistant pathogens in their biofilm form, but also for tools to efficiently grow reproducible biofilms for development of anti-biofilm products. In this work, the design of a novel reactor for biofilm testing and the development of a model live biotherapeutic product that targets and delivers antimicrobial products to specific biofilms are described. The biofilm reactor is a high-throughput laminar flow reactor that is capable of producing uniform biofilm on surfaces for efficient subsequent testing. The live biotherapeutic is an engineered strain of *Lactococcus lactis* that specifically attaches to *Pseudomonas aeruginosa* biofilm structures using surface display technology. Preliminary testing was performed to characterize the anti-biofilm properties of multiple wild-type lactic acid bacteria (LAB) strains in order to choose the most effective strains for further development of the anti-biofilm component.

CHAPTER 1

INTRODUCTION

Antimicrobial resistance has the potential to be one of the most significant public health issues of the twenty-first century. The World Health Organization (WHO) has recently released a global report on antibiotic resistance and stated, "The problem is so serious that it threatens the achievements of modern medicine (WHO, 2014)." The Center for disease control (CDC) estimates that resistant microbes are the cause of over 2 million infections and approximately 23,000 deaths in the United States per year (CDC, 2013). Further compounding the issue is the dearth of new antimicrobial treatments that are coming to market. As of 2013, only 2 new antimicrobial drugs had been approved since 2009, and the number of drugs coming to the clinic continues to decline (Boucher, 2013). Considering the weight of this issue, there is a global responsibility to develop new antimicrobial therapies that will combat the widespread increase in antibiotic resistance.

One characteristic trait of many drug resistant microbes is their ability to form biofilms. Biofilms are complex communities of microbial cells that are attached to a surface. The microbial cells encase themselves in a self-organized extracellular polymeric substance (EPS) that is primarily composed of proteins, polysaccharides, and extracellular DNA (eDNA) (Mann, 2012). When these organisms attach to a surface and encase themselves in a biofilm they are more protected from the immune response (Jensen *et al*., 2010) and external stresses such as antibiotics, chemicals and physical challenges (Mah and O'Toole, 2001). In fact, microorganisms in their biofilm state have been found to be 10 to 1000 times more resistant to antimicrobial treatment than planktonic cells (Nickel, 1985; Prosser, 1987).

Biofilm-associated antibiotic resistance is multifactorial and varies among different organisms. However, there are a few common resistance mechanisms that many biofilm-producing organism possess. The first is the physical protection that the EPS matrix provides for the internal cells. This matrix has been found to slow the diffusion of antibiotics by a factor of 2-3 (Cheema, 1986; Gordon, 1988). This slowed diffusion alone is not enough to account for the drastic increase in resistance, but the combination of enzymatic or chemical inactivation of the antibiotic within the matrix and the slowed diffusion may account for the increased resistance (Anderl, 2000). The integrity of the biofilm matrix is indeed important, as disruption of the matrix has been shown to reverse antibiotic resistance of biofilm microbes (Misagh, 2009). Other biofilm-associated resistance mechanisms may include adaptive response to altered environmental conditions (upregulation of stress-response genes), establishment of persister cells, and microenvironment gradients within the biofilm (low pH, low pO_2, high pCO_2, and low hydration) (Mah and O'Toole, 2001; Pozo and Patel, 2007; Stewart, 2002). It is likely that the enhanced resistance of microbial biofilms is due to a combination of mechanisms that creates a compounded effect.

The economic cost of microbial biofilms is staggering. The Center for Disease Control states that over two thirds of human microbial infections are estimated to be related to the presence of biofilms (Donlan, 2002). While the NIH estimates this number to be even higher, stating that over 80% of microbial infections are caused by organisms growing as biofilms (NIH, 1998). Although it is difficult to quantify, the estimated economic cost of microbial biofilms is consistently in the high billions per year in the US alone. In 2009 the CDC stated that Hospital acquired infections (HAI) cost from $35.7 to

$45 billion in the US (Scott, 2009). If 80% of these infections were biofilm related, that would result in a cost of $28.6 to $36 billion for HAI biofilm-related infections per year in the US. Biofilms related to implantable medical devices alone have been shown to cost the us health care system $3 billion dollars each year (Darouiche, 2004). The financial burden of biofilms in industrial settings is even higher. It is estimated that biofouling and biocorrosion in industrial systems cost approximately $200 billion per year in the US alone (Okabe, 1994). Considering the threat to human health and the financial burden of microbial biofilms, it is critical that new strategies be developed to combat and control them. The goal of this work was to develop novel tools for biofilm testing and design novel antimicrobial therapeutic strategies against microbial biofilms.

One requirement in the development of any anti-biofilm strategy is the reproducible growth of the biofilm *in vitro*. The conditions that are used to grow the biofilm have a significant impact on the architecture of the biofilm and the performance of the antimicrobial therapies. Particularly, the fluid dynamics of the system and the surface that the biofilm is grown on has a major impact on biofilm growth and resistance to antimicrobials. Therefore, it is critical that the laboratory conditions of biofilm growth be similar to the environmental conditions where the antimicrobial will be applied (Buckingham-Meyer, 2007). In order to create the dynamic flow conditions of the true environment, an apparatus or reactor must be utilized. There are a number of biofilm reactors that are currently available to grow biofilms under different fluid flow conditions (Lebeaux, 2013; Buckingham-Meyer, 2007). The American Society for Testing and Materials International (ASTM) has outlined standard methods for use of the drip flow reactor for biofilm growth at the air/water interface under laminar flow conditions

(ASTM E2647-08). This drip flow reactor has been recommended to model multiple disease states such as chronic wound infections, lung infections, and urological infections. However, the current drip flow reactor is only capable of low throughput testing (4 coupons per growth cycle), and the biofilm growth on each coupon is not uniform. Therefore, the design of a novel laminar-flow biofilm reactor that yields uniform, high-throughput biofilm growth would be advantageous for the design of new anti-biofilm treatments. Here the design of a novel laminar flow biofilm reactor that is high-throughput and produces uniform biofilms will be discussed.

Many of the current treatment options for biofilm infections are not effective. There is a need for groundbreaking strategies that target the source of the problem, and go beyond conventional treatments. New strategies such as microbial and cell-based therapeutics have the potential to mitigate this issue. With the advent of synthetic biology we now have the tools to create live bio-therapeutic products that will surpass the functions of small molecules and biologics to solve unmet medical problems. Here we propose a novel strategy that utilizes a probiotic microorganism to target and deliver antimicrobial products to pathogenic biofilms. This task is broken down into two components. The first component involves the design of a lactic acid bacterial (LAB) strain that will specifically bind to *P. aeruginosa* biofilms. The second component involves the engineering of this LAB strain for production of an anti-biofilm therapeutic only in the presence of *P. aeruginosa* biofilms. Although these two components represent the complete vision of this project, the bulk of this work was performed on the development of the binding component. Preliminary testing surrounding the second component will be discussed along with ideas for future development.

Bacterial surface display was utilized to accomplish the first task of enabling the probiotic bacterium to attach to *Pseudomonas* biofilms. Surface display involves the fusion of a protein of interest to a cell membrane protein that is native to bacterial cells. This enables the protein of interest to be displayed on the cell membrane. The protein of interest for this project is an antibody single-chain variable fragment (scFv) that was found to bind specifically to *P. aeruginosa* alginate (Pier, 2004). The cell membrane protein chosen is a sortase-dependent cell membrane protein of *Lactobacillus plantarum*. These genes were fused and added to an *E. coli/LAB* shuttle vector and regulated under a nisin inducible promoter. The characterization of this engineered LAB strain binding to *Pseudomonas* biofilms is discussed in Chapter 3.

The second component will require the addition of genetic machinery that will enable the engineered strain to produce an anti-biofilm therapeutic in the presence of *P. aeruginosa* biofilms. The therapeutic product will disrupt/inhibit *P. aeruginosa* biofilms, and the production of this product will be under the regulation of a *P. aeruginosa* promoter. This regulation will ensure that the anti-biofilm therapeutic is only produced in the presence of *P. aeruginosa* (Saeidi, 2011). This protein could be a biofilm-disrupting enzyme such as a DNase or an alginate lyase, and/or it could be an antimicrobial peptide that kills *P. aeruginosa* cells. Preliminary testing was performed with a combination of *Lactobacillus plantarum* cells and supernatant, alginate lyase, and tobramycin against a clinical strain of *P. aeruginosa*.

Fungal biofilms are also a major concern in public health as the number of anti-fungal agents capable of killing these organisms is small and continually declining. Fluconazole resistant *Candida albicans* is listed by the CDC as one of the major current

infectious disease threats to our society (CDC, 2013). There is a need to develop new anti-fungal agents, or implement new strategies that are effective against fungal pathogens not only in their planktonic form, but also in their biofilm form. Here an isolated *Streptomyces* bacterium from the Soudan mine was found to produce a suite of natural products that were found to be synergistic with copper against *C. albicans* biofilms and planktonic cells. This project will be discussed briefly in Chapter 4.

The following two chapters will include separate background information, materials and methods, results, and discussion sections. Chapter 2 will begin with a description of the biofilm reactor that was designed, and the initial validation testing that was performed. Chapter 3 covers the work that was performed in the development of a microbial therapeutic that targets *P. aeruginosa* biofilms. Finally, Chapter 4 will conclude and cover future directions along with potential applications of each of the developments from this thesis.

CHAPTER 2

DESIGN OF A NOVEL LAMINAR FLOW BIOFILM REACTOR FOR IN-VITRO ANTIMICROBIAL EFFICACY TESTING

Introduction

Biofilm testing is a field of research that is relatively new to microbiology. In the early 20th century, during the time of Robert Koch, a "pure culture" paradigm was established in which microorganisms were studied almost exclusively in free-living, planktonic cell cultures. This became the standard, and was ingrained into microbiology and medicine for many years to come. In the mid 20th century Arthur Henrici and others began to realize that the vast majority of microbes in the natural environment resided as communities attached to surfaces and surrounded themselves by a slimy material. Not until the end of the 20th century did the importance of studying microbes in biofilm-form become firmly established. Now it is known that biofilm-associated bacteria are physiologically different, and are much more resistant to antimicrobial treatment than planktonic bacteria (Cunningham, 2005).

In order to test antimicrobial agents against biofilm-associated bacteria, specific biofilm test methods must be designed. Biofilm test methods can be broken down into subcategories of biofilm growth, treatment, sampling, and analysis. It is important that each of these categories be compatible. For example, the size and material of the coupon that the biofilm is grown on can have an impact on the treatment options. Additionally, the method used to grow the biofilm may limit the options that are available for analysis. Ideally, the method used to grow the biofilm should represent the environmental conditions of interest, while enabling efficient treatment and analysis options. In order

for a method to be useful by a wide audience it should be affordable, not require a high level of specialized training, be performed with readily available laboratory equipment, and be compatible with biosafety concerns (Parshionikar *et al*., 2009).

Laboratory biofilm reactors are commonly used tools in biofilm research. They are necessary to create a specific aquatic environment for the biofilm to grow. The conditions that are used to grow the biofilm have a significant impact on the architecture and resistance of biofilms. For example, a *Pseudomonas fluorescens* biofilm grown under high-shear conditions was much denser and more tightly attached to the surface than a *P. fluorescens* biofilm grown under low-shear conditions (Pereira, 2002). Additionally, a significant difference in colony forming unit (CFU) log reduction of antimicrobial agents was found when *P. aeruginosa* and *S. aureus* biofilms were grown under high-shear, low-shear, or static conditions (Buckingham-Meyer, 2007). Therefore, when testing antimicrobial efficacy against a biofilm, it is critical that the laboratory conditions used to grow the biofilm be similar to the environment in which the antimicrobial will be applied.

There are a number of different biofilm reactors that have been developed for use in a wide range of applications. Two primary categories of biofilm reactors are those used for bioremediation and those used for antimicrobial efficacy testing. The focus of this chapter will be only on biofilm reactors designed for antimicrobial efficacy testing. Two of the primary categories of biofilm growth for antimicrobial testing include either batch cultures or continuous flow systems. A few batch culture systems include the Calgary device (Ceri *et al*., 1999), microtiter assay (O'Toole and Kolter, 1998), cover slip culture (Merritt *et al*., 2003), the colony biofilm (Anderl *et al*., 2000), and biofilm grown on a

coupon suspended in a batch culture (Yu *et al*., 1993). Batch culture systems are beneficial due to the ease of setup and are more amenable to high-throughput testing. A few of the most commonly used continuous flow reactor systems include: the flow cell (Stoodley and Warwood, 2003), annular reactor (Characklis, 1990), rotating disc reactor (Zelver *et al*., 1999), modified Robbins device (Kharazmi *et al*, 1999), drip flow reactor (Stewart, 2002), and biofilm grown in tubing (Sauer *et al*, 2002). The advantage of a continuous flow reactor is that a steady state can be achieved (Goeres, 2006), along with providing a more accurate representation of many natural environments.

Despite the fact that the importance of biofilm testing has been known for many years, standard operating procedures for biofilm testing are just beginning to be developed. The primary reason for the development of many microbiological standard procedures is for antimicrobial efficacy testing claims that are obtained through registration with governmental regulatory agencies such as the EPA or FDA. These product claims are obtained by demonstrating specific antimicrobial efficacy against a panel of microbial pathogens. A number of standard methods have been developed that support disinfectant claims against planktonic cells, but standard methods that support biofilm claims are limited. The American society for Testing and Materials (ASTM) released a series of biofilm test methods in 2002-2011 (ASTM E2647-08, ASTM E2562-12, ASTM E2799-12, ASTM E2196-07). Each of the ASTM methods was designed for biofilm growth under different conditions (For a description of each of the ASTM methods see **Table 2.1**). These ASTM methods are useful for standard testing, but generally do not support product efficacy claims with a governmental agency such as the EPA. The EPA, however, has just released the first SOPs for biofilm testing in August of

2013 that will support biofilm efficacy claims using the CDC reactor for biofilm growth (EPA MB-19-02, EPA MB-20-01). Recognizing that standardized biofilm testing is in the early stages of development, there is room for improvement of current methods and development of new biofilm growth methods that represent additional environments.

Table 2.1. ASTM Standard methods for biofilm growth

Method	Reactor used	Year published	Growth conditions
ASTM E2196-07	Rotating Disk Reactor	2002	Submerged, Continuous flow, Medium-shear
ASTM E2562-12	CDC Reactor	2007	Submerged, Continuous flow, High-shear
ASTM E2647-08	Drip Flow Reactor	2008	Air/liquid interface, Continuous flow, Laminar flow/low-shear
ASTM E2196-07	MBEC	2011	Batch culture, low to medium-shear

The purpose of this collaborative project between the University of Minnesota and Ecolab was to design improved biofilm test methods that could be used for product development of antimicrobial products in the food and beverage industry. In order to create the proper biofilm growth conditions that represent the environment in which these products will be used, biofilm growth under laminar flow conditions at the air/water interface was determined to be the most accurate growth method. The biofilm reactor that fits these criteria is the drip flow reactor (ASTM E2647-08); however, this was determined to be insufficient for product development for a number of reasons. First, the number of individual biofilm test replicates that can be produced from a single cycle of growth in this reactor is too low (4 coupons/growth cycle) (**Figure 2.1**). Second, biofilm growth on each coupon is not uniform, which would result in statistically insignificant

comparisons of antimicrobial products. Additionally, the time required to grow the biofilm is too high for the number of replicates that are produced. According to the published work on this reactor, the total time requirement is 13 h of active work that is distributed over a period of 5 days (Goeres, 2009) yielding 195 min of active work/coupon. Here a novel laminar flow biofilm reactor (designated as the MultiRep reactor) that produces uniform biofilms at the air/water interface and yields high replicates per growth cycle was designed.

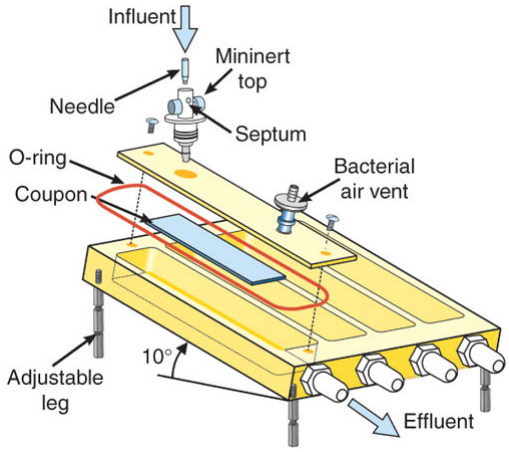

Figure 2.1. Schematic diagram of the drip flow biofilm reactor (Goeres 2009).

Materials and Methods

Strains and growth conditions

For routine growth, *Pseudomonas aeruginosa* ATCC 15442 was taken from a frozen glycerol stock (-80°C) and plated on TSA. Single colonies were used to inoculate trypticase soy broth (TSB) (30 g/L), and cultures were grown at 37°C for 18-24 h in a

shaker (200 rpm). For growth in the MultiRep reactor, TSB media was made at a concentration of 6 g/L and the temperature was 23°C.

MultiRep biofilm reactor method

Reactor preparation:

Autoclaved stainless steel coupons (5 mm diameter) were rinsed twice with de-ionized water and placed into the wells of the MultiRep reactor with a forceps. The tubing was then assembled and attached to the reactor vessel and autoclaved. Silicone tubing was used for the effluent port attachments (VWR 1/8" X 1/4" Cat. #89068-432) and the influent port attachments (Masterflex L/S 14 tubing Cat. # 96400-14). This is the correct size tubing for the inlet and outlet adaptors of the reactor, and also enabled the low flow rate that was desired for the system. A glass flow break was added to the system upstream from the peristaltic pump. Glass flasks (4 L) were used for nutrient supply and waste. TSB medium (6 g/L) was autoclaved in 2 L volumes and added to the sterilized glass flask used for nutrient supply. The waste flask was attached to a vacuum line in order to efficiently pull the waste media from the reactor (see **Figure 2.2** for image of reactor system). The system was set up inside of a biological safety hood with controlled airflow to minimize contamination.

Reactor inoculation:

A 5 mL culture of *P. aeruginosa* (ATCC 15442) was inoculated with an isolated colony from trypticase soy agar (TSA). The 5 mL culture was incubated overnight at 37°C and 200 rpm for 18-24 h, and then diluted 1:10 into fresh TSB media. The tubing on both the inlet and outlet ports of the reactor was clamped off and 4 mL of the diluted

culture was added to each test channel in the reactor. The inoculated system was incubated at 23°C for 4 hours to allow the cells to adhere to the surface.

Continuous flow phase:

The clamps were then removed from the tubing and the reactor was set to an angle by adding a 5 mm spacer underneath the inlet side of the reactor. The pump used in this study was a MasterFlex Pump 3 (Model #7553-71) with an Easy Load II pump head (Model # 77202-60). The pump speed was set at level 1, which resulted in a flow rate of ~ 0.7 mL/min. The continuous flow system was then run for 24 hrs. If the biofilm needed to be grown for a longer period of time (48 – 72 h), the waste was removed and sterile media was added to the feed flask every 24 hrs.

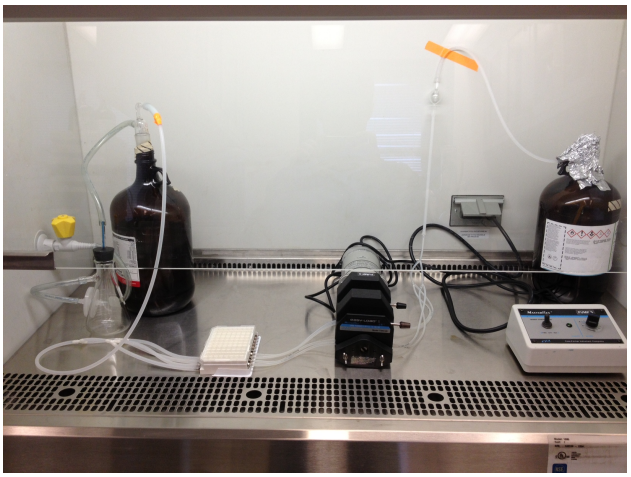

Figure 2.2. Image of the MultiRep reactor system setup.

Crystal violet assay:

This method was adapted from a previous method (O'Toole, 2011). Briefly, discs were transferred to a round bottom 96-well plate and washed 1x with 160 µL of sterile PBS (pH 7.2) using a multichannel pipette. 150 µL of crystal violet (0.1%) was then

added to each well. Discs were soaked in crystal violet for 10-15 min, and washed 3x with 160 µL of PBS. The discs were then transferred to clean wells and washed 1 final time with 160 µL of PBS. 160 µL of glacial acetic acid (30%) was then added to each of the wells and incubated at room temperature for 10-15 min. Following this incubation period, the acetic acid solution was pipetted up and down 2 times and transferred to clean wells of a 96 well flat-bottom plate. Absorbance was read in a Bio-Tek plate reader at 550 nm.

XTT assay:

Following treatment of the discs, discs were transferred to a round bottom 96-well plate and washed 1x with 160 µL of sterile PBS (pH 7.2). 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) sodium salt (XTT) was added to warm PBS (55°C) at a concentration of 0.8 mg/mL. This solution was vortexed and centrifuged for 1 minute to pellet the insoluble material. Menadione was added to DMSO at a concentration of 0.2 mg/mL. 25 µL of the XTT solution, 1 µL of the menadione solution, and 74 µL PBS were added to each well of the plate. The plate was incubated in the dark for a minimum of 6 h at 37°C. Following the incubation period, the XTT solution was pipetted up and down twice and transferred to a new microtiter plate. The absorbance was then read at 450 nm using a Bio-Tek plate reader. (any concern about cell debris interfering with the absorbance readings?)

CFU enumeration:

Following treatment of the discs, the discs were transferred to a round bottom 96-well plate and washed 1x with sterile PBS (pH 7.2). 150 µL of sterile PBS was added to each well that contained a disc. The plate was then sealed inside of a plastic bag, and

placed in a water bath sonicator (sonicated on high for 30 +/-5 min). A serial 10 fold dilution of each disc was then carried out in additional 96-well microtiter plates. After sonication, the content of each well was pipetted up and down 2 times. Then, 100 µL from each well containing a disc was transferred to the top row of a sterile flat-bottom 96-well microtiter plate. 180 µL of sterile PBS was added to each well in rows B-H of the plate. The transferred 100 µL samples were then serial diluted ($10^0 – 10^{-7}$) by transferring 20 µL from each well into the next using a multichannel pipette. Each well was mixed by pipetting 2 times and swirling the pipette tips in the well a total of ten revolutions. Fresh pipette tips were used for each subsequent transfer. The contents of each dilution were then spot plated on TSA using a multichannel pipette by first mixing each well and spotting 10 µL of the sample onto the TSA. Plates were incubated at 35°C +/- 2°C for 16-18 h. This method was adapted from the MBEC ASTM method (ASTM E2799-12).

Calculation of CFU/disc:

$$\text{Log10(CFU/disc)} = \text{Log10}[(A/B)(C)(D)]$$

Where:

A = CFU counted in the spot

B = Volume plated

C = Well volume

D = Dilution

Reynolds number calculation:

Calculation of the Reynolds number for the MultiRep reactor was based on an equation developed for fluid flow through an inclined plane channel (Bird *et al,* 2002).

The calculations were based on the bulk fluid being water at 20°C. The fluid flow was determined to be 0.7 mL min^{-1}. The fluid thickness was determined to be 1.2 mm based on the flow rate and the geometry of the channel.

Statistical analysis

The data generated from the crystal violet assay, XTT assay, and CFU enumeration was statistically analyzed using a one-way ANOVA test. The results were generated with 3 degrees of freedom between groups, and 28 degrees of freedom within groups for the comparison of the channels. For the comparison of the rows, the results were generated with 7 degrees of freedom between groups, and 28 degrees of freedom within groups.

Results and Discussion

Design of the MultiRep reactor

A novel laminar flow biofilm reactor that produces a uniform biofilm at the air/water interface was designed (**Figure 2.3**). The initial design of the reactor was made using Google SketchUp. The first series of prototypes were 3D printed with a variety of plastics. Multiple adjustments were made to the initial design to optimize uniform biofilm growth over the coupon surface. 3D printing was not ideal for the end product due to the porosity that results from the layering of the plastic material that 'builds' the object. The final model (made in SolidWorks) was CNC machined out of an autoclavable medical grade plastic. The material is not compromised after repeated autoclaving procedures. Further, sterility testing revealed that the device is sterile after autoclaving for 15 min at 121°C using a dry cycle (data not shown). This reactor has

capacity to grow 80 biofilms on separate coupons during a single run (10 channels, with 8 coupons per channel). The wells of the reactor are designed to accommodate coupons that are 5 mm in diameter. This size is ideal for transfer of the discs into a 96-well plate for subsequent testing. The Reynolds number was determined to be 29 when considering a flow rate of 0.70 mL/min, which is considered laminar flow.

A B

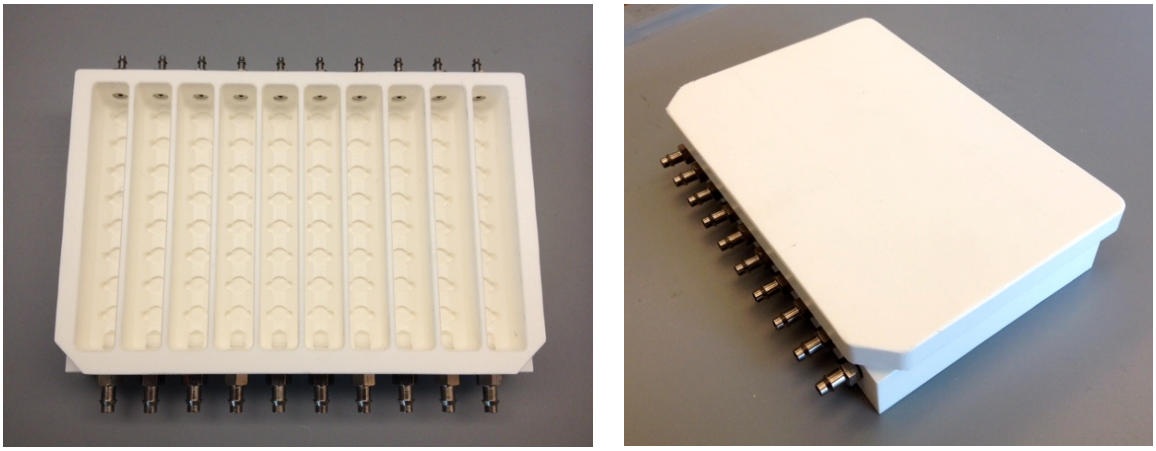

Figure 2.3. Image of the MultiRep reactor. Tope image without cover (A). The inlet ports are at the top of the reactor and the outlet ports are shown at the bottom of the reactor in this image. There are 10 channels with 8 coupon wells in each channel. Total coupon capacity is 80. Side image with cover (B).

Uniformity of biofilm growth in the MultiRep Reactor

In the design of this biofilm reactor, one important characteristic was that a uniform biofilm could be produced over the surface of the coupons. This is important because it maximizes the amount of biofilm on the coupon and minimizes variation between coupons. **Figure 2.4** shows images of *P. aeruginosa* biofilm grown on stainless steel discs. Biofilms were grown on the discs in a microtiter plate, or in the MultiRep reactor and stained with crystal violet. A uniform biofilm over the surface of the coupon could be achieved using the Multirep reactor (**Figure 2.4C**). The biofilm produced on

the steel discs in the 96-well plate grew only around the periphery of the coupon (Figure 2.4B). **Figure 2.5** shows a quantitative comparison of the amount of biofilm produced when grown in the MultiRep reactor compared to a microtiter plate. It is important to note that these two methods of growth are fundamentally different. Growth in the microtiter plate is a batch, submerged culture, while growth in the MultiRep reactor is at the air/water interface with continuous flow conditions. However, this comparison does indicate that the MultiRep reactor is an effective tool for uniform, robust growth of *P. aeruginosa* biofilms at the air/water interface.

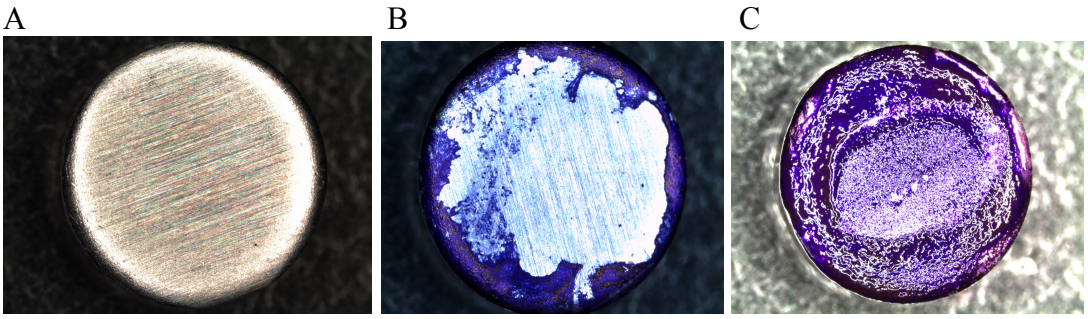

Figure 2.4. Images of biofilm growth on steel discs. Steel disc with no biofilm growth, negative control (A). *P. aeruginosa* biofilm grown on steel disc in a 96-well microtiter plate for 48 h and stained with crystal violet (B). *P. aeruginosa* biofilm grown on a steel disc in the MultiRep reactor for 48 h and stained with crystal violet (C). Discs are 5mm in diameter.

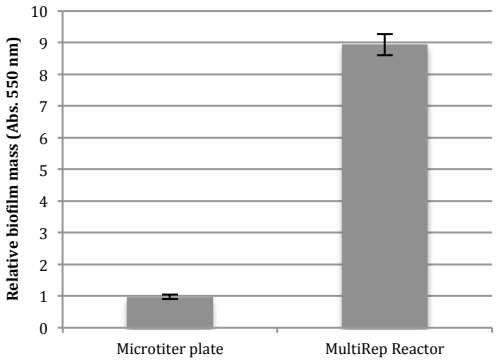

Figure 2.5. Quantification of biofilm mass produced from growth in MultiRep. Quantitative comparison of *P. aeruginosa* grown on steel discs in a microtiter plate or the MultiRep reactor for 24 h using the crystal violet assay.

Validation testing for MultiRep reactor

To determine if the coupon position has an effect on biofilm growth, a study was performed that compared two measurements of biofilm growth. The first was a measurement of biofilm mass that was produced on each disc using the crystal violet assay. In this test, the crystal violet stain absorbs into the biofilm matrix and cells, and is dissolved/extracted with an acid. The resulting absorbance represents the amount of biofilm that was initially present (O'Toole, 2011). The second measurement of biofilm growth was cell viability. For this measurement, both the XTT assay and CFU enumeration were performed. The XTT assay is a colorimetric test that detects metabolically active cells (Smith, 2008). See the supplemental information section for data that shows the correlation between CFU and XTT absorbance for *P. aeruginosa*. Due to the peristaltic pump that was used for this study, only four channels were tested. This generated a sample population of 32 coupons (4 channels x 8 coupons/channel). The MultiRep reactor was capable of generating biofilms on each of the 32 coupons that were tested. The biofilm growth across the four channels and down each row were analyzed and compared.

The average values obtained for each channel are shown in **Figure 2.6**. The results for the crystal violet assay are shown in **Figure 2.6A**, and the results for the XTT assay and CFU enumeration are shown in **Figure 2.6B**. The data generated from these assays were statistically analyzed with an ANOVA test. The crystal violet, XTT, and CFU enumeration values statistically correlated and indicate that there was no difference between biofilm mass or cell viability across the channels. The crystal violet assay data

resulted in a p-value of 0.52, and the XTT assay resulted in a p-value of 0.65. Therefore, the coupon position across each channel has no statistically significant impact on biofilm growth.

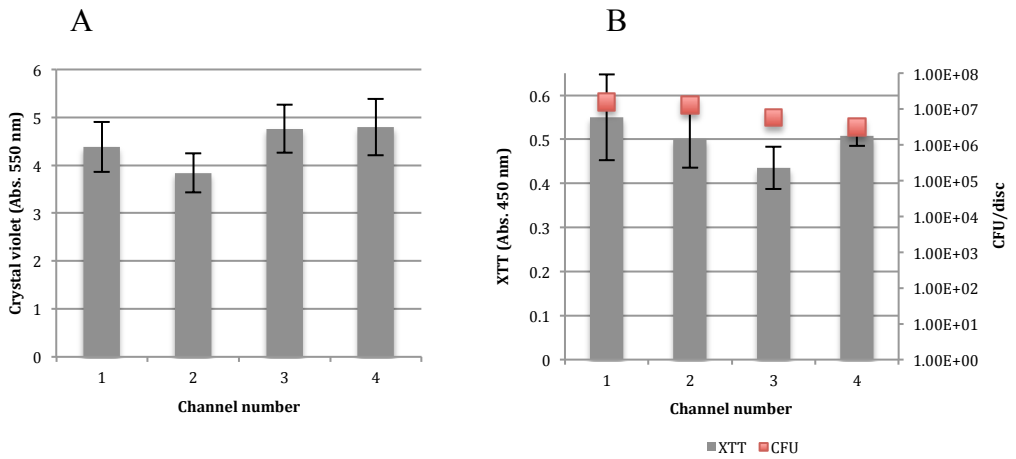

Figure 2.6. Quantification of biofilm mass and cell viability across channels. Results from crystal violet (A) and XTT/CFU enumeration (B) assays following biofilm growth in the MultiRep reactor. Crystal violet assay results indicating the biofilm mass on each steel disc (A). XTT assay results (bars) indicating the abundance of viable cells recovered from each disc (A – left axis). The red squares are the average values obtained for CFU enumeration from each disc (B – right axis).

Next, a comparison of biofilm growth for each row of the reactor was performed. The data generated for this comparison is shown in **Figure 2.7**. The crystal violet assay comparison of each row shows more variation than the comparison of each channel shown in **Figure 2.6**. This is most likely due to each group in each row only having 4 replicates, while each group per channel has 8 replicates. There appears to be a general trend downward from row number 1 to row number 8 in both biofilm mass and cell viability; however, this trend is not statistically significant for biofilm mass (p-value >0.05 for CV assay). This trend is likely due to the fact that the first row receives the fresh nutrient supply, while the last row receives a more depleted nutrient supply. Additional testing should be performed to fully characterize this effect down each

channel. Despite the slight decrease in biofilm growth as the coupon position moves away from the nutrient supply, it appears that each channel has this same variation. Therefore, a comparison of disinfectant efficacy can still be effectively made as long as each channel is used as a study group rather than each row.

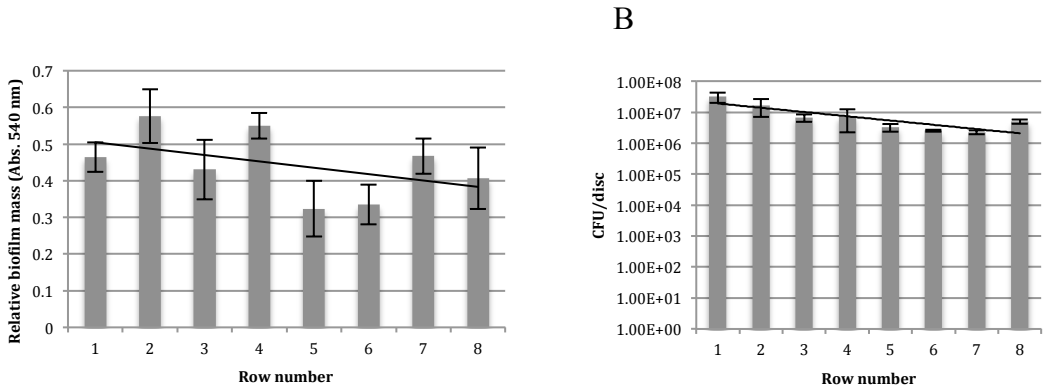

Figure 2.7. Quantification of biofilm mass and cell viability for each row of the reactor. Crystal violet comparison of each row in the MultiRep reactor (A). CFU enumeration comparison of each row in the MultiRep reactor (B).

In order to properly validate this reactor for standard method use, additional testing would need to be provided. According to the US Environmental Protection Agency regarding validation of microbiological methods of analysis, multiple method attributes would need characterization (Parshionikar *et al*., 2009). These attributes include specificity and sensitivity, precision, accuracy, limit of detection, and linearity. Specificity is the ability to differentiate between the target organism and other organisms, and sensitivity is the proportion of the target organism that can be detected. Precision is a variation of independent results that are obtained under specific conditions. This is broken down into both within-lab and between lab repeatability and reproducibility. Accuracy is the closeness of a test results to that of an accepted reference value. Limit of detection is the minimum amount of test substance that can be detected. Finally, linearity

is the ability of the method to produce results within a set range and is directly proportional to the concentration of a test substance (Parshionikar *et al*., 2009). The validation of these attributes for the MultiRep reactor was not yet performed due to time constraints.

As mentioned in the introductory section of this chapter, the EPA has only released a method regarding the CDC biofilm reactor (EPA MB-19-02). To date, there is no EPA method released for the drip flow reactor (ASTM E2647-08). The low replication of this reactor (4/growth cycle) may be a limiting factor for use of this reactor in an EPA method for biofilm growth under laminar flow conditions at the air/water interface. The MultiRep reactor; however, with much higher replication (80/growth cycle) and uniform biofilm growth over the surface of the coupon holds significant promise for standard method development.

Subsequent anti-biofilm testing in a 96-well plate

As mentioned previously, the MultRep reactor is designed to accommodate coupons that are the proper size for transfer to a 96-well microtiter plate. To demonstrate this utility, biofilms grown on steel discs in the MultiRep reactor were transferred to a 96-well plate, treated with anti-microbial products provided by Ecolab, and compared using the XTT assay. From the total coupons used in this study, 4 (1 from each channel) were CFU enumerated, 4 (1 from each channel) were measured using the crystal violet assay, and 16 were used to compare the impact that multiple treatments had on cell viability. The CFU enumeration and crystal violet assays were performed to compare the starting CFU and biofilm mass from coupons across different channels. **Figure 2.8** shows that

following biofilm growth in the MultiRep reactor, coupons can be transferred to a 96-well plate, treated with antimicrobials (A, B, and D), and analyzed to differentiate the antimicrobial effect on the biofilm. This demonstrates the feasibility of this reactor for antimicrobial product development against microbial biofilms.

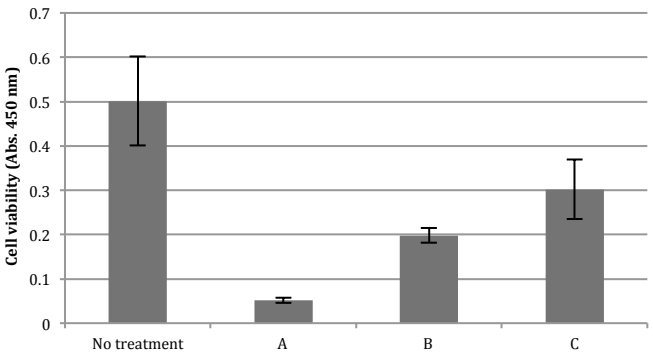

Figure 2.8. Subsequent anti-biofilm testing in a 96-well plate. Comparison of multiple treatments (provided by Ecolab) against *P. aeruginosa* biofilms using the XTT assay.

Time and cost comparison of the MultiRep and the Drip Flow reactors

To determine the affordability of the MultiRep reactor, a cost comparison was made with the drip flow reactor. The cost of the drip flow reactor is $998 from Biosurface Technologies Corporation. The rough cost estimate of the MultiRep reactor is $200. Therefore, the cost per replicate ($998/4) for the drip flow reactor is ~ $250, while the cost per replicate ($200/80) for the MultiRep reactor is $2.50. The total time requirement for the drip flow reactor procedure is 13 h of active work that is distributed over a period of 5 days (Goeres, 2009), yielding 195 min of active work/coupon. Assuming that the MultiRep reactor would require around the same amount of time to perform the procedure; this would result in 9.75 min of active work/coupon. Therefore,

the economic and time costs are substantially lower for the MultiRep reactor in comparison to the drip flow reactor.

Conclusion

With a growing understanding of the importance of biofilms in antimicrobial efficacy testing there is a need for reactors that support biofilm growth under a wide range of conditions. Current reactor options for continuous laminar flow biofilm growth at the air/water interface are limited to low replication and less than ideal biofilm uniformity over the surface of the coupon. This results in a high cost per replicate along with a high time requirement per replicate. In collaboration with Ecolab, we engineered a novel biofilm reactor that produces biofilm growth at the air/water interface under laminar flow conditions. This reactor yields a high number of replicates per growth cycle with uniform biofilm growth over the surface of the coupon. The high replication per growth cycle drastically lowers the cost per replicate and the time required per replicate. The coupon position within this reactor was found to have no statistically significant impact on biofilm growth across each channel and the ease of subsequent testing in a 96-well plate was demonstrated. This reactor holds the potential to be used in a standard method for biofilm growth under laminar flow conditions at the air/water interface. The versatile utility of this reactor will be demonstrated in the following chapter through characterization of a live biotherapeutic agent that targets microbial biofilms.

CHAPTER 3
ENGINEERING A PROBIOTIC MICROBE TO TARGET AND DESTROY *PSEUDOMONAS AERUGINOSA* BIOFILMS

Introduction

Acute microbial infections in humans are primarily caused by free-living (planktonic) cells that rapidly replicate, while many chronic infections are due to the establishment of biofilms within the host (Furukawa *et al*., 2006). These chronic infections are especially concerning because they are often resistant to conventional antimicrobial strategies such as antibiotics. Chronic infections associated with cystic fibrosis patients, endocarditis, burn wounds, chronic prostatitis, otitis media, periodontitis, and in-dwelling medical devices are examples of disease states caused by biofilms that are of high concern in health care (Donlan, 2002).

According to the CDC, gram-negative bacteria such as *Pseudomonas, Acinetobacter*, and Enterobacteriaceae are some of the most prominent concerns due to pan-resistance to nearly all the drugs that could be considered for treatment (CDC, 2013). These pathogens are notorious biofilm-forming microbes, and are known to have high-levels of antimicrobial resistance especially in their biofilm state (Bales *et al*., 2013). *P. aeruginosa* is a biofilm-forming opportunistic organism that plagues patients with immune-deficiencies such as cancer and cystic fibrosis (Saeidi *et al.*, 2011). This organism is known to colonize and cause disease in burn wounds, respiratory, gastrointestinal, and urinary tracts (McCue and Kahan, 2006; Jensen *et al*., 2010). Notably, *P. aeruginosa* infections are the most common infection for patients that have been hospitalized for more than 1 week (McCue and Kahan, 2006).

The primary treatment option for *P. aeruginosa* infections is antibiotics. One challenge associated with antibiotic treatment for *P. aeruginosa* infections is that many strains are multi-drug resistant even in their planktonic form, and become even more drug-resistant in the biofilm state. This leads to a requirement for high doses of the antibiotics over long periods of time that can result in the selection of more resistant strains. Further, treatment of *P. aeruginosa* infections with high doses of antibiotics, especially broad-spectrum antibiotics, can have a negative effect on the healthy human microbial flora leading to further issues such as chronic *Clostridium difficile* infections (Aloush, 2006).

Bacteriophage therapy, the therapeutic use of bacteriophage for treatment of pathogenic bacterial infections (Darouiche, 2004), is another treatment that has been considered and tested extensively. Although it has a longer history than antibiotics, and can be effective against specific antibiotic resistant strains of bacteria even in their biofilm form, there are multiple important limitations to this therapy. First, phage therapy many times cannot be reapplied to a patient once antibodies have been raised against the virus (Hausler, 2006). Second, to be effective, phage therapy requires very specific identification of the infectious organism in order to select the proper phage for treatment, which can be costly, unreliable, and require cocktails of bacteriophage (Keen, 2012). It is evident that current treatment options for biofilm-associated infections are limited, and there is a need for new strategies.

Synthetic biology combines principles of biology and engineering to design and construct biological devices that serve useful purposes. The power of synthetic biology combined with a new understanding of the human microbiome has created a new area of

biopharmaceutical therapies called live biotherapeutic products (Olle, 2013). This new, exciting field harnesses the ability that individual microbes and microbial communities have in the modulation of human health to solve medical problems that are otherwise untreatable. Our focus here will be on individual engineered microbes for therapeutic applications.

These individual live biotherapeutic products or microbial therapeutics have multiple attributes that make them the ideal 'robot factories' for drug delivery (Forbes, 2010). First, they have the capability of performing superior tasks compared to small molecules or biologics alone. Not only can these microbial vehicles use small molecules and biologics as tools or weapons, but they can also deliver, produce and regulate the deployment of these accessories. One major advantage of microbial therapies is that they are programmable. Through manipulation of their genetic machinery, special functions can be added or taken away (Wang *et al*., 2013). Additionally, many bacteria are motile and can sense stimuli within their environment and make adjustments accordingly. This ability could be harnessed to enable intelligent delivery and production of drugs at hard to reach sites through signal recognition or production (Forbes, 2010). Another advantage of microbial therapy is that microorganisms that normally inhabit human bodies can be utilized; thus minimizing the potential for an immune response to the treatment (Steidler and Rottiers, 2006).

There are a few challenges to using bacteria as drug delivery vehicles that must be considered and addressed before these organisms can be used in the clinic. One challenge is insuring that the organism is producing the drug at the proper dose. It must be a high enough dose to perform its purpose against the pathogen, but it must not be too

high of a dose where it will be toxic to the host. Another challenge is that bacteria are prone to mutate, which could hinder the efficacy of the therapeutic organism. An additional concern is that the engineered microbes may escape into the environment (Steidler and Rottiers, 2006). This issue can be dealt with by designing the organism to have a 'kill switch' were it will be inactivated once its task has been implemented (Callura *et al*., 2010). Despite these challenges, the potential benefits of using microbes as therapeutics could outweigh the challenges.

The use of engineered bacterial cells for therapeutic purposes has gained wide interest, and a number of groups have demonstrated and developed some of the key attributes of bacteria as therapeutic vehicles. Duan and March demonstrated that bacteria could modulate the virulence of pathogens through the production of signaling molecules by engineered probiotic organisms (Duan and March, 2010). Hwang, *et al*. have recently shown that bacterial chemotaxis can be programmed to direct probiotic organisms toward human pathogens and produce proteins that eradicate biofilms (Hwang *et al*., 2013). The Forbes lab is developing strains of bacteria that target cancerous tumors and are programmed to deploy proteins that kill the cancer cells once they are inside the tumor (Jean, 2014)**.** There are also a few examples of companies that are developing microbial therapeutics. A company called Oragenics (http://www.oragenics.com/) has design a cavity prevention therapy that employs a novel strain of *Streptococcus mutans* that no longer produces lactic acid (lactis acid production lead to dental caries) and has a competitive advantage over wild-type *S. mutans* strains (Hillman, 2002). Another example, designed by a company called Osel (http://www.oselinc.com/), involves the prevention of HIV infections in females via an engineered *Lactobacillus*. In this system,

the *Lactobacillus* is engineered to produce a HIV microbicide that will inhibit the virus as it enters the vagina (Lagenaur, 2011).

The rapidly growing number of studies in the field suggests that microbial therapies hold great promise for application in human health. One additional component that could enhance some of the example systems discussed is the design of a probiotic organism that attaches to specific surfaces of interest for localized production of the therapeutic products. To demonstrate this idea, and to provide a novel strategy against pathogenic biofilms, we chose to direct the attachment of a probiotic organism to the exopolysaccharide matrix of biofilms. In this study, *P. aeruginosa* was chosen as the target pathogen because it is a model organism for biofilm testing and it is a gram-negative pathogen of great concern in health care. *L. lactis* was chosen as the therapeutic vehicle because it is considered a GRAS (define) organism (FDA, 2014), it is motile, and the genetic manipulation has been well established (Mierau and Kleerebezem**,** 2005).

Surface display has been used extensively in both phage and bacteria over the years for a wide range of applications in biotechnology. Phage display has been a fundamental tool in combinatorial protein engineering because it allows the phenotypic mutational optimization of a protein to be easily linked to the genotype of the protein (Rader and Barbas, 1997). The use of bacteria for surface display enables the display of larger proteins on the surface and broadens the potential applications such as vaccine-delivery vehicles, antibody display for diagnostics, combinatorial protein engineering, whole-cell biocatalysis, and metal binding protein display (Wernerus, 2004; Bloois *et al*., 2011). The advantage of using bacteria as vaccine delivery vehicles is that the bacteria can greatly increase the half-life of the antigen in the host, and create a local immune

response near the mucosal surface of interest. Antibody surface display can be combined with a vaccine delivery vector to enhance delivery to specific immunoreactive locations. Whole-cell biocatalysis has been used in industry as a cost effective method of immobilizing enzymes that perform specific industrially relevant reactions (Wernerus, 2004).

For our model system, a native cell-membrane protein found in *L. plantarum* was used to display and anchor the alginate-binding scFv protein on the cell membrane of the engineered lactic acid bacteria (LAB) strain. In bacteria as a whole, surface proteins play an important role in mediating interactions with the environment. In LAB, the sortase enzyme is responsible for covalently attaching a subgroup of proteins called sortase dependent proteins (SDPs) to the cell membrane. This is a two-part process that involves both protein targeting to the membrane and covalent anchoring of the protein to the cell membrane. The targeting of the protein usually involves the secretory (Sec) pathway or the twin-arginine translocation (TAT) pathway. The Sec pathway appears to be much more common in LAB. In the Sec pathway, unfolded proteins that contain an N-terminal leader peptide, a hydrophobic core, and a specific C-terminal sequence that is recognized by the Sec machinery are targeted to the cell exterior (Call and Klaenhammer, 2013). One group of SDPs that are covalently attached to the cell membrane of LAB is the LPXTG-anchored proteins. These proteins contain a specific C-terminal motif (LPXTG), a positively charged tail, and a C-terminal hydrophobic region that is recognized by the sortase enzyme (**Figure 3.1 A**) (Call and Klaenhammer, 2013). However, it has been found that in most *Lactobacilli* the motif is actually LPQTXE (Kleerebezem *et al*., 2003), and this motif is also functional in *Lactococcus* spp. (Cortes-Perez *et al*., 2005).

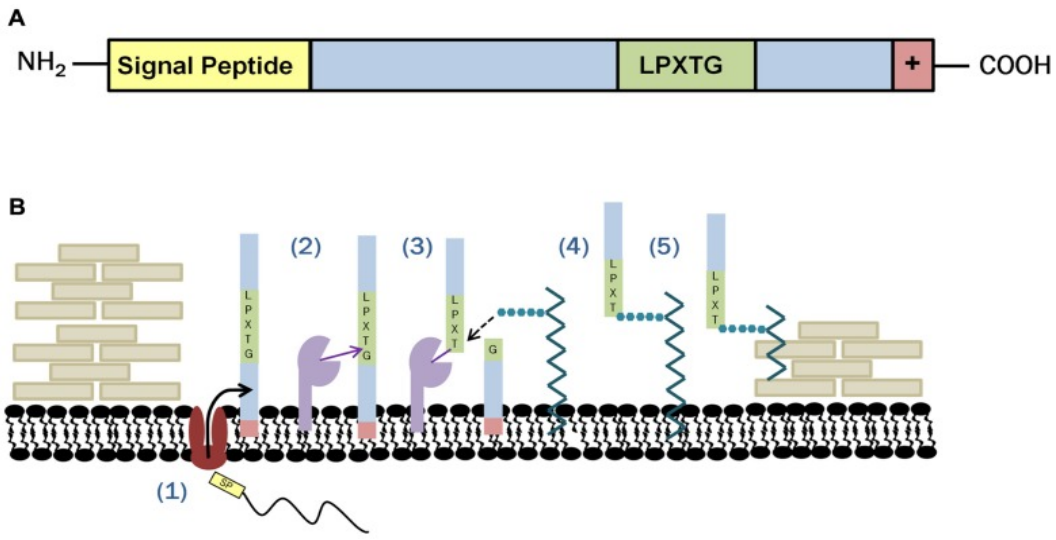

Figure 3.1. Illustration of the sortase-dependent anchoring in Gram-positive bacteria. (A) Shows the two components that are necessary for sortase anchoring, the signal peptide and the LPXTG motif. (B) The signal peptide is important for localization to the membrane. The Sec system recognizes the signal peptide and exports the protein to the exterior (1). The sortase then recognizes the substrate (LPXTG)(2), cleaves between glycine and theonine (3), threonine then forms a bond with the pentapeptide crossbridge (4). Finally, the sortase substrate becomes part of the normal cell-wall construction (Reprinted with permission from Frontiers in Microbiology Journal (Call and Klaenhammer, 2013)).

The cell-membrane protein used in this study is an LPQTXE-anchored SDP identified as lp_2578. Previously, this protein was used to display an oncofetal antigen (OFA) on the surface of *L. plantarum* for a mucosal cancer vaccine (Fredriksen *et al*., 2010). To display the OFA on the cell membrane, the OFA gene was fused to the anchor sequence on the N-terminal side of both the hydrophobic region and the LPQTXE motif, but on the C-terminal side of the signal peptide. Fredriksen et al. compared the surface expression of this protein using a short, medium, or full-length anchor sequence. They found that the highest level of surface display was found with the truncated medium-length anchor (Fredriksen *et al*., 2010). Therefore, the medium truncated version was chosen for this study.

In designing the binding component of this project, it was important to target a unique biofilm component not commonly found in many biofilm structures to enable specific binding of the engineered LAB to the biofilm of interest even in the presence of other natural biofilms. Mucoid strains of *P. aeruginosa* contain a unique EPS component called alginate. Alginate is a high-molecular weight polysaccharide composed of non-repeating D-mannuronic acid and L-guluronic acid residues (Linker and Jones, 1964). It is only found in some brown algae species, and *Azatobacter* and *Pseudomonas* bacteria. The algal alginate is, however, different from the bacterial form in that the bacterial version is O-acetylated (Donati and Paoletti, 2009).

Our system utilizes a short chain fragment variable (scFv) fragment derived from human monoclonal antibodies (mAbs) that bind to *Pseudomonas* alginate (Pier *et al*., 2004). These mAbs mediated immunological killing of *Pseudomonas* mucoid strains as well as non-mucoid, low alginate producing strains. One specific mAb (F429) was found to have the broadest overall alginate binding activity over the widest range of *Pseudomonas* isolates from cystic fibrosis patients. For this project, we utilized the scFv region of the F429 mAb (Pier *et al*., 2004), and joined the V_H and V_L chains of the scFv with a linker peptide $(GGGGS)_3$ that enables proper linkage and flexibility between the light and heavy chains of the scFv (Shen *et al*., 2008). In order to display this alginate binding protein on the cell membrane, the synthesized F429 scFv DNA sequence was fused to the N terminal side of the SDP anchor peptide DNA sequence and C terminally to the signal peptide DNA sequence. This construct was then cloned into an *E. coli*/LAB shuttle vector and is regulated by a nisin inducible promoter.

The impetus for designing a microbe that can attach to biofilms is to ultimately inhibit or eradicate that biofilm through deployment of extracellular products. Five strategies have been suggested to treat biofilm-associated infection: substances able to destroy the biofilm matrix, substances that destroy persister cells, quorum-quenching enzymes, substances that cause biofilm self-destruction, and strategies to boost antimicrobial action (Del Pozo and Patel, 2007). With our system, any one of these strategies could be employed. It is also possible that the probiotic strain could natively have the capability of inhibiting or destroying the biofilm. An *L. plantarum* supernatant was shown to inhibit *P. aeruginosa* through a quorum quenching mechanism (Ramos *et al*., 2012). Additionally, LAB strains are known to modulate the immune system in a number of ways (Matsuzaki and Chin, 2000; Wells, 2011) and prevent infections (Reid and Burton, 2002). It is possible that attachment of the probiotic organisms to the biofilm structures could enhance the immune systems ability to eradicate the infection. To enhance the native ability of the probiotic to eradicate biofilms, heterologous expression of anti-biofilm components could also be implemented. For example, alginate lyase and DNase, both alone and separately have been found to enhance the antibiotic killing of *P. aeruginosa* biofilm cells (Alipour *et al*., 2009). Additionally, high molecular weight protein antibiotics such as pyocin S2 that not only kill the pathogenic cells, but also destroy the biofilm matrix could be utilized (Smith *et al*., 2012).

In this project, bacterial surface display was utilized to express the F429 scFv on the cell membrane of *L. lactis* by fusing it to a native LAB sortase-dependent cell membrane protein to enable the attachment of *L. lactis* to the biofilm structure of *P. aeruginosa*. The EPS component alginate was chosen as the target for binding because it

is a unique polysaccharide component not commonly found in microbial biofilms. *P. aeruginosa* was chosen as a target for binding because it is a model organism for biofilm testing and is a known antibiotic resistant human pathogen. This is a model system designed to test the feasibility of directing the attachment of probiotic cells to biofilm structures. Further, this is one tool in the overall goal of enhancing biofilm eradication by developing a microbial therapeutic that deploys anti-biofilm products at the location of the biofilm. The bulk of this chapter focuses on the binding aspect of this system, but preliminary testing was performed to characterize the effect that LAB strains alone and in combination with biofilm degrading enzymes have on *P. aeruginosa* biofilms.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in **Table 3.1**. *Lactococcus lactis* was cultured statically at 30 +/-2°C in M17 broth (Oxoid Ltd. Basingstoke, UK) supplemented with 0.5% (w/v) glucose. *Lactobacillus plantarum* was cultured statically at 37°C in MRS broth (Oxoid Ltd. Basingstoke, UK). *Pseudomonas aeruginosa* strains were propagated at 37°C with 200 rpm shaking in LB broth for routine growth. *Escherichia coli* DH5a was cultured at 37°C with 200 rpm shaking in LB broth. Agar plates were made by adding 1.5% (w/v) agar to the broth media. For maintenance of plasmids, erythromycin (Em) was added to the growth media of *E. coli* and LAB strain at a concentration of 150 μg/mL and 6 μg/mL respectively.

Table 3.1 Strains and plasmids

Strain	Description	Source and/or reference
E. coli DH5α	Host for cloning: F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17(rk-, mk+) phoAsupE44 -thi-1 gyrA96 relA1	Saltikov and Newman, 2003
L. plantarum ATCC 14917	Wild Type	ATCC bacteriology collection
L. plantarum WCFS1	Wild Type	Kindly provided by Dr. Juan Borrero at the University of Minnesota
P. aeruginosa NH57388A	Stable mucoid CF mouse sputum isolate, hyperproducing alginate, functional AHL-based QS, mutation in mucA.	Kindly provide by Dr. Bryan Williams at the University of Minnesota
P. aeruginosa PAO1 ΔalgD	Alginate knockout strain	Kindly provided by Dr. Katharina Ribbeck at the Massachusetts Institute of Technology
P. aeruginosa PAO26	Mucoid clinical isolate from cystic fibrosis patient	Kindly provide by Dr. Bryan Williams at the University of Minnesota
L. lactis subsp. *cremoris* NZ9000	Plasmid-free strain, derivative of *L. lactis* MG1363; pepN::nisRK, non bacteriocin producer; $EntA^r$	Kindly provided by Dr. Juan Borrero at the University of Minnesota

Plasmid	Description	Source and/or reference
pMSP3545	Em^r; inducible expression vector carrying the nisA promoter and the nisR and nisK genes	Kindly provided by Dr. Juan Borrero at the University of Minnesota
pMG36e	Em^r; p32 constitutive promoter	Kindly provided by Dr. Juan Borrero at the University of Minnesota
pUC57-algscFv	Amp^r; pUC57 containing the	GenScript (Piscataway, NJ)

	alginate scFv (Alginate binding antibody variable region with linker peptide) synthesized by GenScript	
pDR111-GFPsp	Amp^r; pDR111 containing the GFPsp gene optimized for expression in gram positive bacteria	Kindly provided by Dr. Claudia Schmidt-Dannert at the University of Minnesota
pJE1	Em^r; pMSP3545 containing the signal peptide sequence (cloned from *L. plantarum* 14917)	This study
pJE2	Em^r; pJE2 containing alginate scFv (synthesized by GenScript) and anchor peptide (cloned from *L. plantarum* 14917)	This study
pJEGFPsp	Em^r; pMSP3545 containing the GFPsp gene (cloned from pDR111-GFPsp)	This study
pJE7	Em^r; pJEGFPsp containing the binding cassette from pJE2 with a RBS site in front of the cassette	This study

Table 3.2 Primer sequences

Primer	Nucleotide sequence (5'-3')
PrimF-Sp(SphI)6-9	TTAGCATGCTATA**AGGAGG**CACTCAACATGGGGG AGGAGCGTATGCGAAGA
PrimR-Sp(SpeI)6-9	TAAGTTACTAGTTCAAGCACGACGGCGATAACC
PrimF-OptalgscFv	CACATGCTAAAGAAATCTTCTAGACAGTTACAGTT ACAAGAAAGTG
PrimR-OptalgscFv	GGCCATGGAACTGTCAATTTCGTGCC
PrimF-Anchor	TTGACAGTTCCATGGCCGGTCACTGAACCAGGA

PrimR-Anchor(SpeI)pMSP	TAAGTTACTAGTTCAAGCACGACGGCGATAACC
PrimF-GFPsp(NcoI)	CATGCATGCCATGGTTTCTAAAGGTGAAGAATTG
PrimR-GFPsp(SphI)	CCGGCATGCTTATTTATACAATTCATCCATACCAT G

[a] Restriction enzyme cleavage sites are underlined in the primers; RBS site shown in bold

Basic genetic techniques and enzymes

Qiagen kits were used for all purification of genomic DNA, PCR products, and plasmids (QIA-GEN, Hilden, Germany). Restriction enzymes, T4 DNA ligase, Taq polymerase, and Antarctic phosphatase were purchased from New England Biolabs (NEB, Beverly, MA). Taq polymerase (NEB) was used for colony PCR, and TaKaRa Ex Taq DNA polymerase (Clonetech) was used for PCR amplification. *E. coli* cells were made compotent according to standard protocols with some adaptations (Sambrook, 2001), and LAB strains were made electrocompetent and transformed using a Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA) as described previously (Aukrust *et al*., 1995). Primers were purchased and DNA sequencing was performed at the University of Minnesota Biomedical Genomics Center (Minneapolis, MN).

Construction of plasmids

Construction of the binding cassette

For a list of primers and plasmids used in this study see **Table 3.1**. The design for the binding cassette was based on previous studies (Fredriksen et al., 2010), but the specific construct for this work was developed during this project. The binding cassette, which included a signal peptide (Sp), an Alginate binding scFv (algscFv), and an anchor

peptide (Anchor) were PCR amplified and joined together via Gibson assembly. The signal peptide and the anchor peptide were obtained from *L. plantarum* ATCC 14917 genomic DNA using PrimF-Sp(SphI)6-9 and PrimR-Sp(SpeI)6-9 for the signal peptide, and PrimF-Anchor and PrimR-Anchor(SpeI)pMSP for amplification of the anchor peptide sequence. The anchor peptide is a truncated version of lp_2578, accession no. YP_004890243. The algscFv binding protein sequence design included the heavy and light chain of the F429 mAb (F429 IGHV-D-J accession no. AY626664.1, and F429 IGLV-J accession no. AY626662) that was developed by the Channing Laboratory (Pier *et al*, 2004). The heavy and light chains from this mAb were fused together with a linker peptide sequence ($GGGGS_3$) in order to enable proper folding and display of the scFv. The algscFv was codon-optimized by GenScript for expression in *Lactobacillus* and synthesized by GenScript (Piscataway, NJ). GenScript originally sent this algscFv gene product in pUC57. PrimF-OptalgscFv and PrimR-OptalgscFv were used to amplify the algscFv gene from pUC57. The binding construct was cloned into pMSP3545 and regulated by the pNisA nisin inducible promoter.

Construction of pJEGFPsp and pJE7

The GFPsp gene was provided by Dr. Claudia Schmidt-Dannert (St. Paul, MN). The GFPsp gene was amplified from the pDR111-GFPsp plasmid using PrimF-GFPsp(NcoI) and PrimR-GFPsp(SphI). This PCR product was cloned into pMSP3545 using the NcoI and SphI restriction enzyme sites, which yielded the pJEGFPsp plasmid. pJE7 was obtained by amplifying the binding cassette with PrimF-sp(SphI)6-9 and PrimR-Anchor(SpeI) and cloning this PCR product into the pJEGFPsp using the sphI and speI site, which yielded the pJE7 plasmid. The PrimF-sp(SphI)6-9 primer also contained

a RBS binding site that was identical to the RBS site that is found in the pNisA promoter region.

Characterization of nisin induction

Overnight cultures of *L. lactis* (pJEGFPsp or pJE7) cells were diluted 1:100 in 5 mL of M17+ glucose (0.5%) supplemented with 6 μg/mL erythromycin. After 2 h of growth at 32°C the cultures were induced with various concentrations of nisin (0 – 40 ng/mL). Optical density measurements (600 nm) were recorded every hour for 7 h, and GFP expression was measured using a fluorescent plate reader.

Protein characterization

Cultures were started by adding 100 μL of an overnight culture to sterile M17 + glucose (0.5%) (10 mL) supplemented with 6 μg/mL erythromycin. After 2 h of growth at 32°C the cultures were induced with 10 ng/mL nisin. Cells were harvested by centrifugation at 4,000 rpm for 10 min after an additional 4 h of growth at 32°C. The supernatant was removed and the pellet was re-suspended in 10 mL of PBS (pH 7.2). Cells were then sonicated with a Branson 250 sonicator set at an output power of 6 for 8 min. Sonication pulses were set to sonicate 30% of the total time (for every 3 seconds of sonication there was a 7 second intermission time period). Following sonication, the lysed cells were centrifuged at 10000 g for 10 min to remove the cell debris. The pellet was re-suspended in 100 μL of PBS, and the supernatant was centrifuged at 100000 g for 30 min. The resulting pellet, which contained the cellular membrane components, was re-suspended in 30 μL of PBS. Protein concentrations were determined according to the Coomassie Plus ^{TM} (Bradford) Assay (Thermo Scientific, Rockford, IL).

Samples were treated with Bolt™ (Life technologies, Carlsbad, CA) non-reducing sample buffer and incubated at 70°C for 10 min. To visualize the expression and the location of the binding cassette product and GFP, the samples were separated on a 10% Bis-Tris polyacrylamide gel. Gels were then stained with coomasie for 2 h followed by a minimum of 3 h of de-staining with 50% methanol and 10% glacial acetic acid.

Quantification of alginate

Alginate was precipitated from 1 mL aliquots taken from 5 mL *P. aeruginosa* cultures that were grown at 37°C for 48 h. The 1 mL aliquots were centrifuged at 23,000 X g for 30 min at 4°C. The resulting pellet was discarded, and the supernatant was added to 3 mL of 99% ice-cold ethanol. The precipitate was centrifuged at 5,000 X g for 5 min at 4°C, and the pellet was dissolved in 0.9% saline (Hoffmann *et al*., 2005). Quantification of alginate was then carried out according to the carbazole-borate method developed by Knutson and Jeanes with some modifications (Knutson and Jeanes, 1968). Briefly, 190 μL of boric acid (100 mM) was added to the wells of a 96-well plate. An aliquot (30 μL) of the purified alginate sample was then added to the wells with boric acid and mixed by pipetting up and down twice. Carbazole solution (30 μL , 0.1% in ethanol) was added to the wells and mixed as before. The plate was then sealed and placed in a 55°C incubator for 40 min. The resulting absorbance values were read at 530 nm. A standard curve based on known concentrations of pure alginate (3.125 – 50 μg/mL, final concentration in wells) was used to determine the concentration of the unknown samples.

Characterization of binding to *P. aeruginosa* biofilms

P. aeruginosa growth in MultiRep reactor

P. aeruginosa NH57388A and *P. aeruginosa* PAO26, was grown in LB (20 g/L) + glycerol (1%) overnight at 37°C and 200 rpm shaking. LB media is commonly used for *Pseudomonas* alginate production, and glycerol has been found to promote alginate production (Wingender *et al*., 2001; Hoffman *et al*., 2005). This culture was then diluted 1:10 in sterile LB (20 g/L) + glycerol (1%). The channels of the MultiRep reactor (See Chapter 2 for description of MultiRep reactor setup) were inoculated with 4 mL of the diluted culture and held statically for 4 h at room temperature. Growth in the reactor was then carried out over a period of 6 days (48 h of flow at 0.7 ml min^{-1}, then held static for 48 h, followed by an additional 24 h of flow) with LB (6 g/L) + glycerol (1%). The long growth period was used due to the slow growth of *P. aerguinosa* NH57388A. Sample discs were pulled from the reactor throughout this cycle to quantify the biofilm growth using the crystal violet assay (see chapter 2). Following the 6 days of growth in the MultiRep reactor, discs were removed and transferred to a 96-well plate.

P. aeruginosa growth in 96-well plate

P. aeruginosa NH57388A, was grown in LB (20 g/L) + glycerol (1%) overnight at 37°C and 200 rpm shaking. This culture was then diluted 1:10 in sterile LB (20 g/L) + glycerol (1%). 150 μL of this diluted culture was then added to the wells of a 96-well plate and incubated for 72 h at 35±2°C and 100 rpm shaking. After 72 h, the media was removed from the wells and sterile LB (20 g/L) + glycerol (1%) was added to the wells. This was done to supply the established biofilm with fresh media nutrients. The plate was incubated for an additional 24 h. *P. aeruginosa* (PAO1 ΔalgD) biofilms were grown as described for PA NH57388A except that the biofilm was grown for 24 h. This difference in biofilm growth time was due to the difference in biofilm growth rates of these *P.*

aeruginosa strains. After biofilm growth, the spent media was removed from the wells and the wells were washed 1 X with sterile PBS (pH 7.2) to remove unattached planktonic cells.

Preparation of L. lactis cells for binding studies

L. lactis cells were prepared and induced as described in the 'Characterization of nisin induction' section of this chapter. The induced cultures were then diluted 1:3 in sterile M17+glucose broth supplemented with 6 μg/mL erythromycin and 10 ng/mL nisin. This diluted cell culture was then added to the wells of a 96-well plate that either had the biofilm grown on the walls of the wells, or the biofilm grown on the steel discs using the MultiRep reactor. The induced *L. lactis* cells were exposed to the biofilm for a period of 3.5 h at room temperature. Discs and wells were washed a series of times (1 – 6 X depending on the assay performed) with sterile PBS (pH 7.2) to remove unattached cells. The plate was then sonicated in a water bath on high for 30 min to detach the biofilm from the surface. The contents of the wells were then mixed using a multichannel pipette by pipetting up and down 2 X and swirling the pipette tips for 10 revolutions.

Fluorescence microscopy

Steel discs treated as described in the previous section (washed 5 X with PBS) were viewed under a conventional epifluorescence microscope (Olympus BX51, Center Valley, PA) with an Xcite light source. An FITC filter source was used, and the study was performed in duplicate. Multiple images were taken of each surface, and representative images were chosen for display in the results section.

Quantitative binding assay

After *L. lactis* exposure to PA biofilms and washing (as described above), the plate was sonicated in a water bath on high for 30 min to detach the biofilm from the surface. The contents of the wells were then mixed using a multichannel pipette by pipetting up and down 2 X and swirling the pipette tips for 10 revolutions. For wells that contained the steel discs, aliquots of the re-suspended cell solution were transferred to clean wells for analysis. Relative fluorescent units were measured using a fluorescent plate reader (Top read, Excitation 485 nm/Emission 528 nm, sensitivity 70). To determine the percentage of fluorescence retained after washing, the fluorescence measured for each test well was divided by the average initial fluorescence measurement for each GFP producing strain of *L. lactis*.

Preliminary testing of LAB strains against *P. aeruginosa* biofilms

Lactobacillus sp. supernatant and cell culture study

P. aeruginosa (PAO26) biofilms were grown at 37°C in TSB for 24 h in a 96-well plate at 90 rpm. The planktonic PAO26 culture was removed from the wells of the 96-well plate. Overnight cultures of *L. plantarum* strains (WCFS1, NC8, 14917, and 10241) were grown at 37°C in MRS. To obtain the supernatants, cultures were centrifuged at 4000 rpm for 10 min. A portion of each supernatant was pH neutralized (pH 7) with NaOH. The un-neutralized supernatant from each sample was acidic (pH ~ 3.7). Following neutralization, the supernatants were filter sterilized. The supernatants were added to the wells containing the biofilms in triplicate with final concentrations ranging from 5% - 25% (total well volume was 160 µL). The base media in the wells was TSB media. The supernatants were exposed to the biofilms for 18-24 hrs at 37°C. For the cell culture study, a 2 µL aliquot from each culture was used to inoculate the wells containing

the PAO26 biofilms and fresh TSB (performed in quadruplicates). The plate was then incubated for an additional 24 h at 37°C. Following the supernatant and cell culture treatments, the crystal violet assay was performed for each test.

L. plantarum WCFS1 and tobramycin study against P. aeruginosa biofilms

P. aeruginosa (PAO26) biofilms were grown at 37°C in TSB for 24 h in a 96-well plate at 90 rpm. The planktonic PAO26 culture was removed from the wells of the 96-well plate. Overnight cultures of *L. plantarum* WCFS1 was grown at 37°C in MRS. An aliquot (5 μL) of the WCFS1 cell culture was then added to the wells containing the biofilms and fresh TSB. The plate was incubated overnight, and the following day wells were treated with tobramycin (0 – 360 μg/mL) for an additional 24 h. Following the treatments, CFU enumeration was performed as described in the materials and methods section of chapter 2 with one exception. TSB plates were supplemented with 5 μg/mL chloramphenicol to inhibit the growth of *L. plantarum* (WCFS1). Prior to performing this test, *P. aeruginosa* verified to be resistant to this concentration of chloramphenicol.

Results and Discussion

Characterization of the engineered *L. lactis* strain

Genetic construction of the binding cassette

The genetic construct for the binding cassette (scFv F429 DNA sequence fused between the signal peptide and the anchor peptide of lp_2578 sequences) was successfully made and verified by sequencing (**Figure 3.2**). Initially, the signal peptide was cloned into pMG236e following the strong constitutive p32 promoter. Then the algscFv fragment and the anchor peptide PCR products were fused after the signal

peptide using the Gibson assembly method. However, this failed to produce *E. coli* DH5a clones that incorporated the entire binding cassette. A possible reason for this issue is that the constitutively expressed proteins were toxic to *E. coli*. The binding cassette; however, was obtained by using the Gibson assembly product and PCR amplifying the entire cassette with PrimF-Sp and PrimR-Anchor primers. This construct was then cloned into the pMSP3545 vector after the nisin inducible pNisA promoter, and sequenced to verify proper gene orientation and sequence integrity. Any additional cloning was carried out in the nisin inducible pMSP3545 vector to avoid any toxicity issues in *E. coli*.

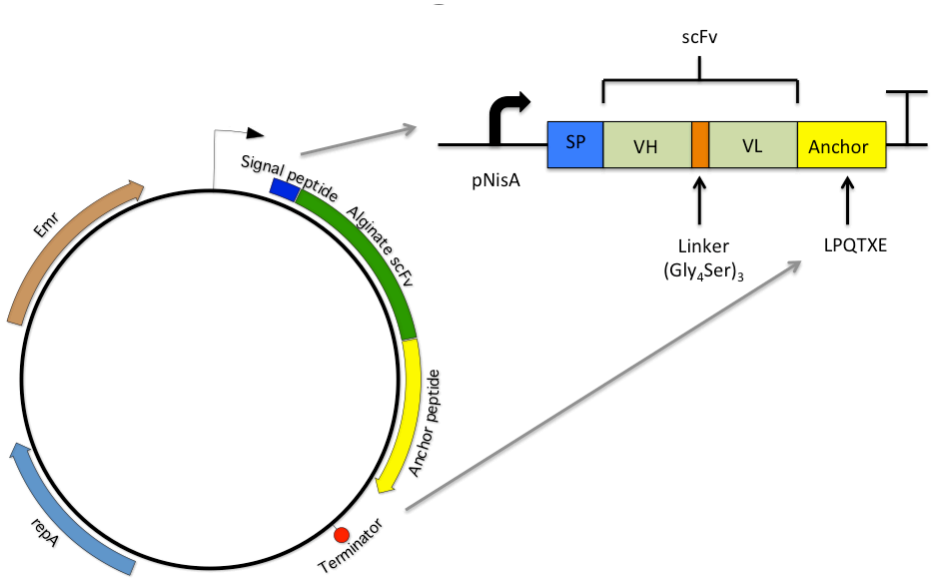

Figure 3.2. Binding cassette construct. pNisA, nisin inducible promoter within the pMSP3545 vector. SP, signal peptide sequence. VH, heavy chain variable region sequence. VL, light chain variable region sequence. Anchor, sortase dependent cell membrane protein containing a hydrophobic region N terminally to the LPQTXE motif.

Characterization of nisin induction

Due to the difficulty of direct expression characterization of the binding protein construct, GFPsp was added to the vector as a reporter protein. The expression of the GFPsp gene is under the regulation of the same promoter as the binding cassette. To gain

an understanding of the optimal nisin concentration necessary for expression of these proteins in *L. lactis*, a study was performed with a range of nisin concentrations (0 – 40 ng/mL)(**Figure 3. 3**). The GFP expression from each of these cultures was measured using a fluorescent plate reader, and compared to the cell density after 6 hours of incubation after the time of induction. The optimal nisin induction concentration falls between 5 and 20 ng/mL, and higher concentrations (40 ng/mL) inhibited growth of the cultures. 10 ng/mL was chosen as the induction concentration going forward, which is in agreement with previous work (Desmond *et al*., 2004).

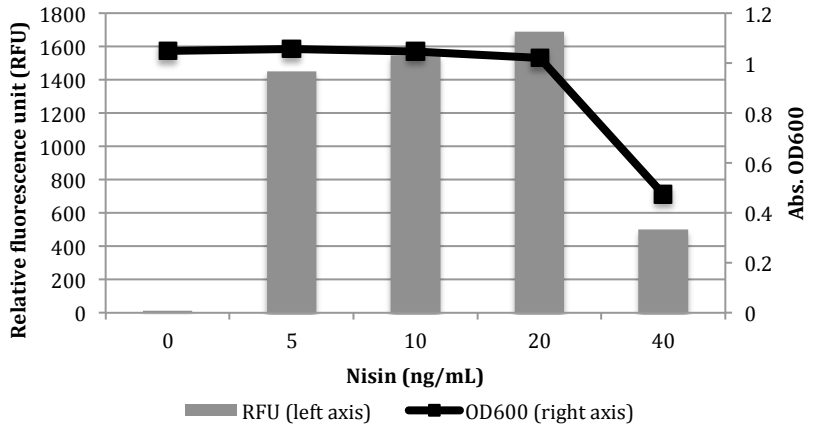

Figure 3.3. Effect of nisin concentration on GFP expression and cell density for *L. lactis* (pJE7).

Characterization of protein expression

The scFv F429 was successfully fused to the SDP lp_2578 and displayed on the cell membrane of *L. lactis*. Cytoplasmic and cell membrane fractions of both un-induced (regular font) and induced (bold font) *L. lactis* cultures containing both GFPsp and the binding cassette were separated and prepared. The proteins from these cell fractions were then compared via LDS polyacrylamide gel electrophoresis (**Figure 3.4**). The results indicate that GFP (expected size 28 kDa) was found only in the cytoplasmic fraction

(**Figure 3.4A**), which is expected due to the fact that there is no signal peptide attached to the GFP for secretion outside the cell. The binding cassette protein (expected size 47.23 kDa) was found primarily in the cell membrane fraction (**Figure 3.4B**). The binding cassette proteins were not found in the culture supernatant (data not shown), indicating that the scFv F429 fused to the lp_2578 SDP was anchored to the peptidoglycan of the cell wall of the *L. lactis* cells. The more intense band seen around 62 kDa in the un-induced cell membrane faction (**Figure 3.4A**) is most likely due to a difference in total protein concentration between the un-induced and induced cultures.

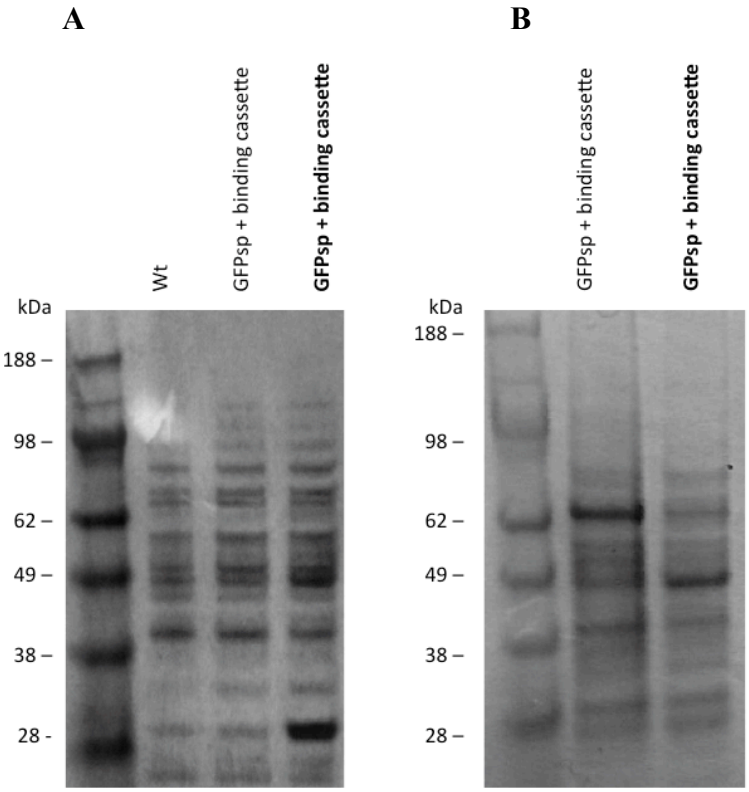

Figure 3.4. LDS-PAGE separation of membrane fractions. (A) cytoplasmic fractions of cellular components (pellet from 10000 x g centrifuge step). (B) Cell membrane fractions (pellet from 100000 x g centrifuge step). Un-induced cultures are in regular font. Induced cultures are shown in bold font. Expected size of GFP - 28 kDa; Expected size of binding cassette protein - 47.23 kDa.

Attachment to Pseudomonas aeruginosa biofilms

Next, to determine if the engineered cells could bind to *P. aeruginosa* alginate-containing biofilms, the *L. lactis* cells expressing GFPsp alone or GPFsp and the binding cassette were exposed to *P. aeruginosa* NH57388A cultivated as biofilms on steel discs, washed five times, and viewed with a epifluorescence microscope using an FITC filter (**Figure 3.5**). Samples were prepared in duplicate, and the images were taken of areas that represented the majority of the sample surface. The images show that a higher number of cells expressing the binding cassette (**Figure 3.5B**) were recovered than cells that did not have the ability to make the binding cassette protein (**Figure 3.5A**). This indicates that the binding cassette protein was not only expressed on the cell membrane of *L. lactis*, but also enhanced binding to *P. aeruginosa* alginate producing biofilms. As mentioned previously, not all strains of *P. aeruginosa* produce alginate; therefore, it was important to verify that *P. aeruginosa* NH57388A does indeed produce alginate prior to performing binding studies. By performing the carbazole assay (Knutson and Jeanes, 1968), the amount of alginate produced by *P. aeruginosa* NH57388A biofilms grown in a 96 well plate was determined to be approximately 12 μg/well. See **Figure S2** for standard carbazole curve.

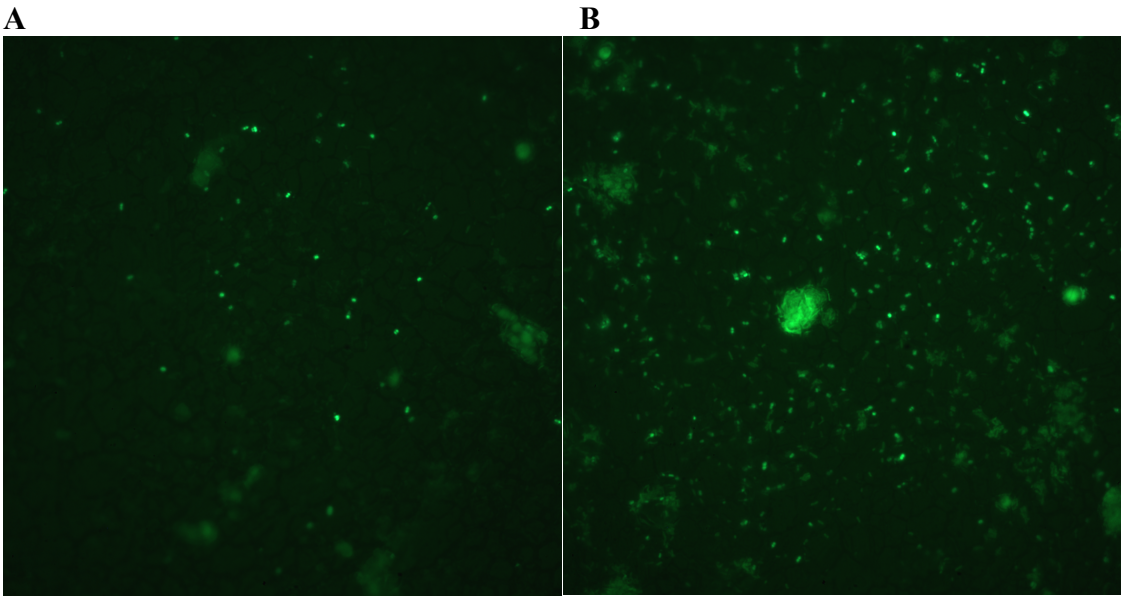

Figure 3.5. Characterization of attachment to *P. aeruginosa* NH57388A biofilms on steel discs. Biofilms were grown on steel discs in the MultiRep reactor. *L. lactis* (pJEGFPsp – GFP only) (A) and *L. lactis* (pJE7 – GFP + bindng cassette) (B) were exposed to the biofilms and washed prior to imaging. Images were taken with a fluorescent microscope at 100x magnification.

The fluorescent microscope images qualitatively indicate that the engineered strain of *L. lactis* does have enhanced binding to biofilm structures containing alginate. Next, a quantitative study to analyze the binding of the engineered *L. lactis* strain to the biofilms was designed. Biofilms of both the alginate producing strain (PA NH57388A) and a non-alginate producing strain (PAO1 ΔalgD) were grown on the well surfaces of a 96-well plate. *L. lactis* cells expressing GFP only, or GFP and the binding cassette were then exposed to the biofilms for 3.5 h, washed, and the percentage of fluorescence retained was determined by dividing by the starting fluorescence of each culture. This calculation was performed because the starting fluorescence of the GFP only and the GFP-binding cassette strains had different initial fluorescence expression. The percentage of fluorescence retained after exposure to the alginate-containing biofilms is shown in

Figure 3.6A, while the results after exposure to the non-alginate producing strain are shown in **Figure 3.6B**. The results indicate that there was a significant difference in binding observed between the *L. lactis* strain that contained the binding cassette and the strain that expressed GFP alone. A student's t-test was performed to compare the statistical significance of the means obtained for each comparison. It is interesting to note that as the number of washes increases in **Figure 3.6A**, the statistical significance increases between the percent fluorescence retained for the strain that has the binding cassette and the GFP-only strain. Additionally, when the GFP-only and GFP-binding cassette strains of *L. lactis* are exposed to biofilms that do not contain alginate, the difference between the two strains is lowered as the number of washes increases (**Figure 3.6B).** Therefore, it appears that expression of the binding cassette in *L. lactis* did enable specific binding to *P. aeruginosa* biofilms that contain alginate.

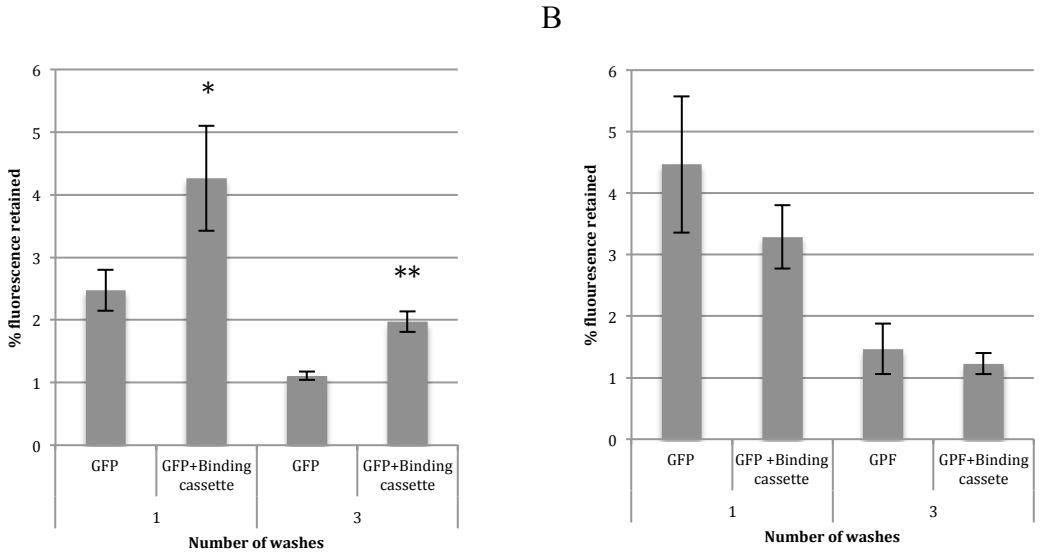

Figure 3.6. Quantitative binding assay – biofilm grown in 96 well plate. (A) *P. aeruginosa* NH57388A - the alginate producing strain and (B) *P. aeruginosa* (PAO1 ΔAlgD) - the alginate knockout strain were grown on the well surfaces of a 96 well plate and exposed to *L. lactis* strains (pJEGFPsp – GFPsp only) or (pJE7 – GFPsp + binding cassette). The cells were washed a number of times and the percentage of fluorescence

retained was calculated. (*) Indicates statistical significance between GFP only and GFP + binding cassette for 1 wash ($p < 0.05$). (**) Indicates statistical significance between GFP only and GFP + binding cassette for 3 washes ($p < 0.01$). Error bars are based on n = 8.

An additional quantitative study was performed with the *P. aeruginosa* biofilms grown on steel discs in the MultiRep reactor. For this study, both the percent fluorescence retained and the CFU/disc recovered was determined for each group studied (**Figure 3.7**). The results from this study again indicated that there was a statistically significant difference between the GFP-only and GFP-binding cassette cells recovered. It is important to note that for the CFU/disc determination, the *L. lactis* cells recovered were diluted and plated onto MRS agar. Normally, *L. lactis* is grown on M17 + glucose media, but MRS was chosen because *P. aeruginosa* does not grow on this media. The *L. lactis* did not grow as quickly on the MRS media, and the colonies were smaller.

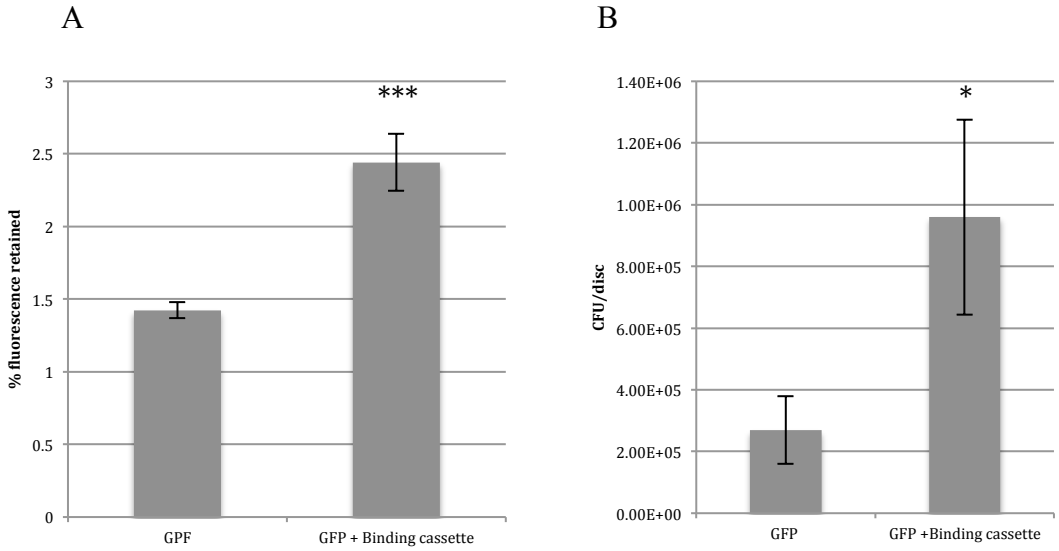

Figure 3.7. Quatitative binding assay – biofilm grown in MultiRep reactor. *P. aeruginosa* NH57388A was grown on steel discs in the MultiRep reactor and exposed to *L. lactis* (pJEGFPsp – GFP only) or *L. lactis* (pJE7 – GFP + binding cassette). Left graph (A) shows the percentage of fluorescence retained by the *L. lactis* cells. Right graph (B) shows CFU of *L. lactis*/disc recovered. (*) Indicates statistical significance between GFP

only and GFP + binding cassette ($p < 0.05$). (***) Indicates statistical significance between GFP only and GFP + binding cassette for 3 washes ($p < 0.001$). Error bars are based on n = 6 for (A), and n = 4 for (B).

Growth rate of induced L. lactis strains

Next, a study was performed to determine the effect that expression of these proteins may have on *L. lactis* growth over time. Three strains of *L. lactis* were compared: *L. lactis* Wt, *L. lactis* pJEGFPsp (GFP), and *L. lactis* pJE7 (GFP-binding cassette) over a total time of 7 h. This time period was chosen because this was the normal time of culture prior to harvest for binding studies. After 2 hours of growth, protein expression was induced with nisin (10 ng/mL). The expression of these recombinant proteins in *L. lactis* does appear to have a slight effect on growth rate initially (**Figure 3.8**). However, there does not appear to be a significant difference between the growth rate of the GFP-only and the GFP-binding cassette strain.

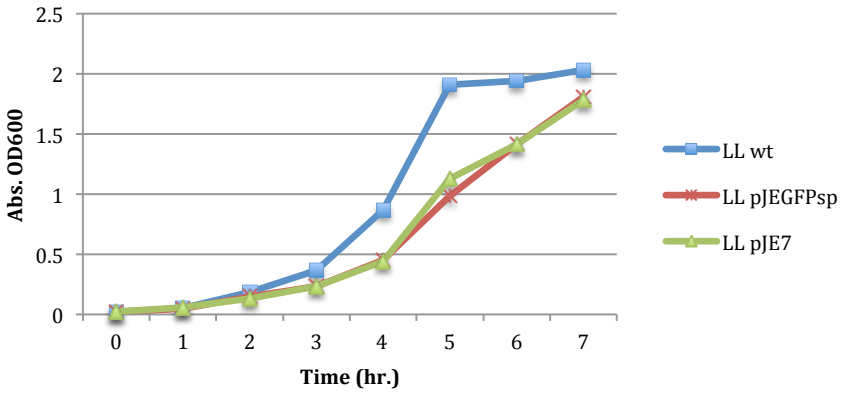

Figure 3.8. Growth rate of *L. lactis* strains over time. LL = *L. lactis,* pJEGFPsp (GFP expression only), pJE7 (GFP + binding cassette). Each strain was induced with nisin (10 ng/mL) at the 2 h time point.

Preliminary testing of LAB strains against *P. aeruginosa* biofilms

The feasibility of engineering a LAB strain to attach to biofilm structures of *P. aeruginosa* was demonstrated. The next step for this project is to develop the anti-biofilm component. The biofilm attachment studies were done in *L. lactis* due to the ease of genetic manipulation, and because the nisin inducible system is well characterized in this organism **(**Mierau and Kleerebezem, 2005**)**. However, the next phase of this project may involve the implementation of the binding components into a different LAB strain with useful enzymatic, inhibitory or immune-modulation activities such as an *L. plantarum* spp. Native *L. plantarum* strains have been found previously to have anti-biofilm properties (Ramos *et al*., 2012). Preliminary testing with a number of wild-type *L. plantarum* strains was performed to determine which strain, if any, has a natural inhibitory effect on *P. aeruginosa* biofilms. In this study, both the supernatants (**Figure 3.9**) and the cell cultures (**Figure 3.10**) of four *L. plantarum* strains (WCFS1, NC8, 14917, and 10241) were tested against *P. aeruginosa* biofilms. Overnight cultures of each of the *L. plantarum* strains were prepared, and either the supernatants (untreated or pH neutralized), or the cell cultures were added to pre-formed *P. aeruginosa* PAO26 biofilms and the amount of biofilm removed was analyzed using the crystal violet assay. For the supernatant study, it was hypothesized that the low pH of *L. plantarum* cultures (pH ~ 3.7) may lead to biofilm removal because low pH has been found to decrease *P. aeruginosa* biofilm production (Hostacká *et al*., 2010). To test this hypothesis, neutralized or un-neutralized supernatants were exposed to preformed biofilms and analyzed. In each of these studies in this section, *P. aeruginosa* PAO26 (A mucoid isolate from the lungs of a cystic fibrosis patient) was used instead of *P. aeruginosa*

NH57388A because this strain formed robust biofilms more quickly than the NH57388A strain.

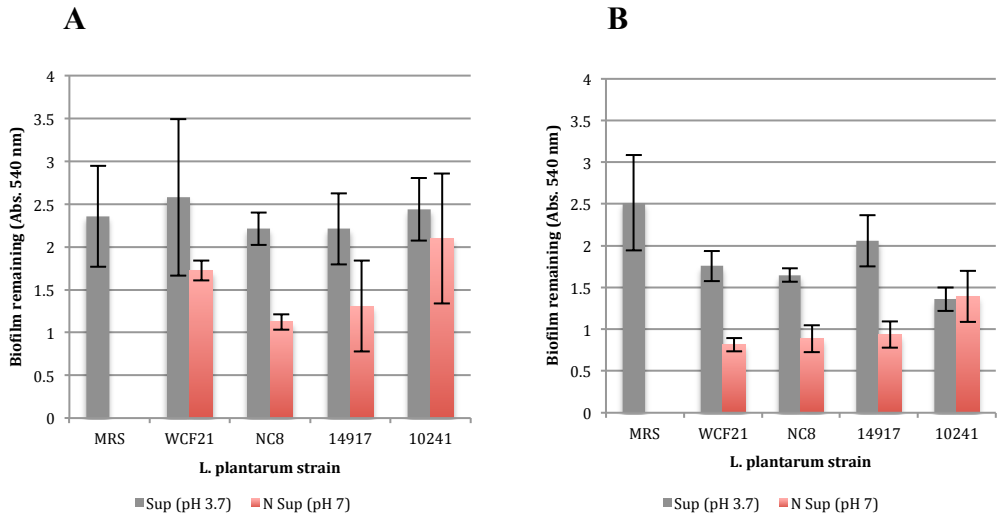

Figure 3.9. *L. plantarum* supernatant effect on *P. aeruginosa* PAO26 biofilms. Biofilms were grown for 24 h, then treated with raw, or neutralized supernatants from *L. plantarum* cultures for an additional 24 h. Following treatment, the crystal violet assay was performed to quantify the biofilm remaining. (A) is 5% supernatant, and (B) is 25% supernatant concentration in the final test volume. MRS (pH 6.5) is the broth that the *L. plantarum* strains were grown in. Error bars are based on n = 3.

The results from these studies indicate that some of the *L. plantarum* cells may have the ability to degrade or remove *P. aeruginosa* biofilm structures. Interestingly, the neutralized supernatants, for some of the strains, appeared to have a greater ability to remove *P. aeruginosa* biofilm than the acidic supernatants. This indicates that the low pH was not the primary mechanism of biofilm removal, and there may be products that these organisms make that have a *P. aeruginosa* biofilm removal effect under neutral pH conditions. The results from the *L. plantarum* cell culture study (**Figure 3.10**) indicate that some of the *L. plantarum* cells, when grown in the presence of *P. aeruginosa* biofilms, may also have a biofilm removal effect. It was important to perform these initial

tests, because the addition of the *L. plantarum* cells to the biofilm cultures could have enhanced biofilm growth, which would have been less than ideal for our purposes. However, it appears that this is not the case, and that some of these strains may be good candidates to choose for further development.

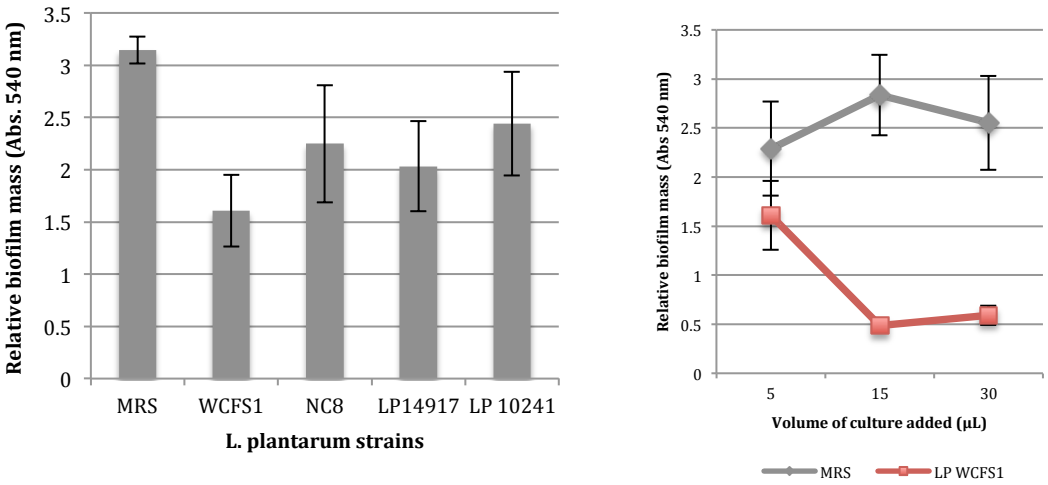

Figure 3.10. Removal of *P. aeruginosa* PAO26 biofilms with *L. plantarum* cell cultures. (A) Biofilms were grown for 24 h, and treated with 2 µL of *L. plantarum* cell culture for an additional 24 h (Error bars based on n = 3). (B) Additional testing of biofilm removal with *L. plantarum* WCFS1 using different starting volumes of *L. plantarum* culture, final volume in each well was 160 uL (Error bars based on n = 4).

Based on the results of the study comparing the biofilm removal ability of multiple *L. plantarum* strains, strain WCFS1 was chosen for further testing. One future application of our engineered LAB strain may be to enhance the efficacy of antibiotic treatments against biofilms. A study was designed to gain an initial understanding of the impact *L. plantarum* WCFS1 cell cultures may have on the antimicrobial activity of tobramycin against *P. aeruginosa* biofilms. Tobramycin is a commonly used antibiotic for *P. aeruginosa* infections, but along with many of the treatment options for this pathogen, is much less effective against the biofilm-associated cells (Hill *et al*., 2005).

For this study, *P. aeruginosa* PAO26 was grown on the well surfaces of a 96-well plate. The biofilms were then treated with *L. plantarum* WCFS1 cell cultures overnight, followed by treatment with tobramycin (120 or 360 μg/mL). The results in **Figure 3.11A** indicate that the pre-treatment of *P. aeruginosa* biofilms with *L. plantarum* cell cultures may slightly increase the activity of tobramycin against *P. aeruginosa* biofilms. Although this difference was small in this study, further testing should be completed with higher replication.

Next, a study was performed to determine the effect that the biofilm growth method has on the antibiotic resistance of *P. aeruginosa* PAO26. This study was performed against biofilms grown in a 96-well plate and biofilms grown in the MultiRep reactor on steel discs (**Figure 3.11B**). The results indicated that the biofilms grown using the MultiRep reactor were more resistant to the tobramycin treatment; and therefore, the MultiRep reactor may be a better system for biofilm growth for future efficacy testing. Additional testing was performed with alginate lyase, but the results were inconclusive at this point and require further testing.

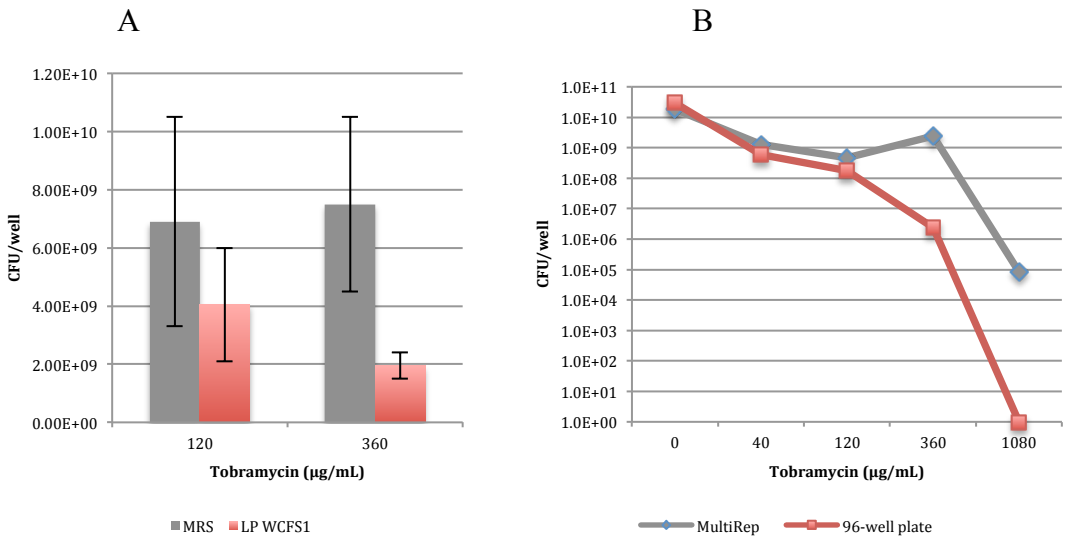

Figure 3.11. Tobramycin and *L. plantarum* cell cultures against *P. aeruginosa* PAO26 biofilms. (A) shows the effect that tobramycin, in combination with *L. plantarum*, has on *P. aeruginosa* biofilm cell viability. (B) Shows a comparison of tobramycin activity against *P. aeruginosa* biofilms grown in the MultiRep reactor or a 96-well plate.

Conclusion

Due to the synergistic effect of antibiotic resistance and a dearth of new antibiotics entering the pipeline, there is a global need for a new paradigm in infection prevention strategies. Biofilms have been highlighted as one of the major contributing factors for chronic bacterial infections that are essentially untreatable in some cases. Here we designed a strain of *L. lactis* that displays a heterologous alginate-binding scFv on its cell wall by anchoring it to a native LAB sortase dependent cell membrane protein. The expression of these proteins was not only directed and anchored to the cell membrane of the *L. lactis* cells, but they also increased the ability of this organism to attach to *P. aeruginosa* biofilms. This is a novel strategy that can be added to the toolbox for development of microbial therapeutics that deliver drugs to the precise location of

pathogenic biofilms. Indeed, this project has demonstrated the feasibility of this technology for future implementation against additional microbial pathogens that form persistent biofilms. Preliminary testing was also performed to characterize the anti-biofilm properties of multiple wild-type LAB strains in order to choose the most effective strains for further development. *L. plantarum* WCFS1 appears to hold promise for further development, not only because it appears to naturally have the ability to remove *P. aeruginosa* biofilms, but it has also been found to modulate the human immune system (Remus *et al*., 2013).

CHAPTER 4

FUTURE DIRECTIONS AND CONCLUSIONS

Before 1673, the world did not know that microorganisms even existed. The field of microbiology was then born through Anton van Leeuwenhoek's discovery of microorganisms. It is interesting to consider the successive advancement from the discovery of microbes, to the realization that they cause infections (Germ theory), to the development of antibiotics for their control. Today, an increasing number of microbial pathogens are resistant to our treatment methods, which has forced us on a global scale to re-evaluate our current infection control strategies. The WHO states, "A post-antibiotic era, in which common infections and minor injuries can kill, far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century." (WHO, 2014).

The significance of biofilms in microbial pathogenesis is now firmly established. The understanding that biofilm-associated microbes are much more resistant to antimicrobial treatment than free-living cells underscores the need for treatments that are effective against these organisms in their biofilm state (Nickel, 1985). To aid in this effort we have developed the MultiRep biofilm reactor that can be used for efficient biofilm testing, and a microbial therapeutic strategy that targets biofilms. Each of these developments holds great promise for future work and applications.

The MultiRep reactor enables the high-throughput growth of biofilms at the air-water interface under laminar flow conditions. This reactor increases biofilm testing efficiency by lowering the time and cost per coupon, and increases the reproducibility of biofilm growth compared to the drip flow reactor that is currently available commercially (Goeres, 2009). Each well in this reactor was designed to accommodate coupons that can

be transferred to a 96-well plate for efficient subsequent testing of anti-biofilm products. Testing with *P. aeruginosa* PAO26 biofilms grown in a 96-well plate or the MultiRep reactor indicated that the biofilms grown in the reactor were more resistant to tobramycin. This highlights the importance of the biofilm growth method used prior to antimicrobial testing. For *in vitro* biofilm testing, the growth method that most closely represents the natural environment must be used in order to have the most accurate forecast of the antimicrobial activity.

One near term direct application of this reactor will involve the growth of *C. albicans* biofilms for further characterization of anti-fungal natural products that were found to have synergist activity with copper against this pathogen. Briefly, a *Streptomyces* sp. bacterium (CES-254) was isolated from the Soudan mine in northern Minnesota, and was found to have anti-fungal activity. Further testing indicated that this organism produced a suite of compounds that are synergistic with copper against *C. albicans* planktonic cells. Preliminary testing against *C. albicans* biofilms grown on steel discs in a 96-well plate indicated that this synergy might also be effective against this organism in the biofilm state (**Figure 4.1**). Nocardomine was identified as one of the primary synergistic components with copper against these biofilms. Additional activity appears to be present; however, the chemical identity(s) is still being characterized.

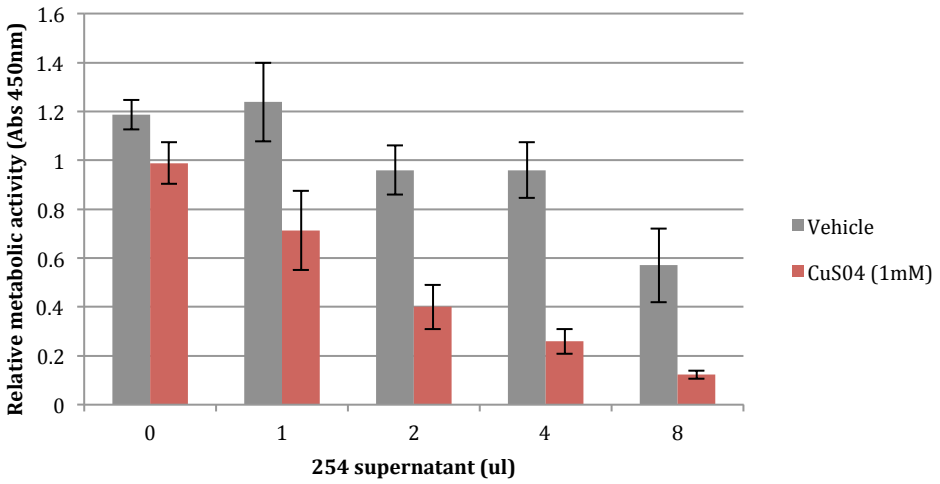

Figure 4.1. CES-254 supernatant and CuSO4 activity against *C. albicans* biofilms. Biofilms were grown in a 96-well plate for 24 hrs on steel discs. The discs were then transferred to clean wells, washed, and treated with CuSO4 (1mM) and CES-254 supernatant overnight. The effect on *C. albicans* cell viability was determined using the XTT assay.

This novel activity against *C. albicans* biofilms is important, because the treatment options against clinically relevant fungi are very limited. Fluconazole is one of the primary treatment options for fungal infections, but many strains are developing resistance (CDC, 2013). Combination therapies such as the one described above, could lead to new treatment options against these resistant pathogens. However, further testing is required, and the MultiRep reactor will be an important tool for further characterization of this activity. This is an example of the potential for this reactor to be used in the development of a wide-range of anti-biofilm therapies.

In addition to the MultiRep reactor, we developed a probiotic bacterial strain that is capable of binding to specific biofilm structures. This was accomplished by engineering a strain of *L. lactis* to display a heterologous alginate-binding scFv on its cell wall by anchoring it to a native LAB sortase dependent cell membrane protein.

Preliminary testing was also performed to characterize the anti-biofilm properties of native LAB strains that may be used for the next phase of this project that focuses on anti-biofilm strategies. Biofilm degrading enzymes such as alginate lyase, DNase, or dispersin B could be heterologously expressed and deployed by our strain once it has attached to the biofilm structures. Expression of antimicrobial peptides could also be employed to aid in killing of the pathogenic cells once the biofilm has been degraded. Yet another strategy could be to deliver quorum quenching enzymes, or signaling peptides that reduce antibiotic-resistance transfer among cells in a particular biofilm.

The ability to direct the attachment of a probiotic organism to biofilm structures has a wide-range of potential future applications. First, the most straightforward future work will be to demonstrate the adaptability of this model system. In our system, the binding protein (scFv F429) can easily be substituted with another protein of interest. Therefore, different proteins that bind to other components of biofilms could be utilized. This means that virtually any other organism that forms a biofilm, and has a unique component for attachment, could be targeted. Second, the ability to direct the attachment of a probiotic organism to alginate could be useful in biocatalysis. Polymer matrices composed of agar, polyacrylamide, chitin, or alginate are commonly used as whole-cell entrapment/immobilization agents in biocatalysis (Robinson, 1997). It has been noted that this method is limited due to the transport of substrates through the matrix. If the cells can be immobilized on a monolayer of the substance, the reaction efficacy would theoretically increase because the adsorbed cells would have more direct contact with the substrates. The ability to immobilize cells onto chitin by display of a chitin-binding domain on the cell membrane of *E. coli* (Wang and Chao, 2006) and *L. lactis* (Simsek,

2013) has already been demonstrated. However, immobilization onto alginate is yet to be demonstrated. Third, this system could be used to enhance biofilm growth for application in bioremediation of toxic chemicals from wastewater. And finally, this system could be used for biofilm diagnostic purposes. Biophotonic imaging is used to study bioluminescent bacteria *in vivo*, and allows the researchers to continually monitor biofilm infections in animals without disrupting the biofilm over the course of the disease (Kadurugamuwa and Francis, 2008). Our system could potentially be used in this fashion to diagnose biofilm infections in humans. The fluorescent probiotic cells that bind to specific biofilms could be deployed to identify the pathogen and the location of the biofilms in the host.

In this thesis, a novel reactor for biofilm testing was designed and a new antimicrobial therapeutic strategy that targets microbial biofilms was developed. Each of these developments is important because they hold great potential as valuable tools in the overarching goal of combating resistant microbial biofilms.

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ASTM E2562-12. Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor.

ASTM E2196-07. Standard Test Method for Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with Shear and Continuous Flow Using a Rotating Disk Reactor.

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APPENDIX

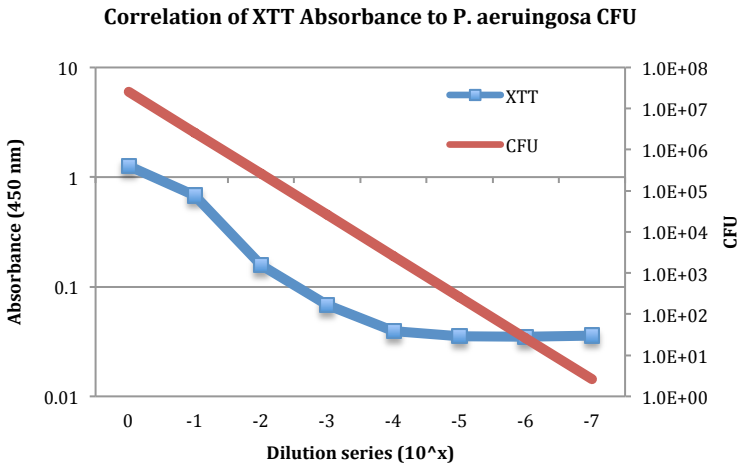

Figure S1. Correlation of XTT absorbance to *P. aeruginosa* CFU enumeration recovered. The XTT absorbance appears to be sensitive down to 100 CFU.

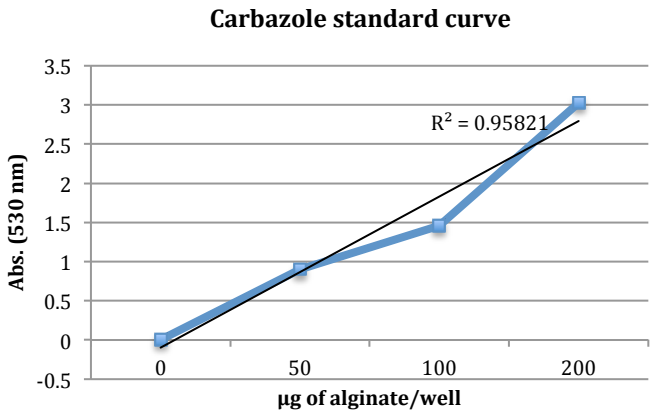

Figure S2. Carbazole standard curve.