PHYSICAL AND CHEMICAL TREATMENTS FOR BACTERIAL BIOFILMS

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Physical and Chemical Treatments of Bacterial Biofilms

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Physical and chemical treatments have been investigated for the treatment to remove biofilms. This thesis examines the problem of the removal and prevention of biofilms by: (i) using a water jet to determine biofilm stability and (ii) testing the effect of β-phenylethylamine (PEA) on growth and biofilm amounts.

Three dimensional structures of biofilms vary in different genetic backgrounds of *E. coli*, we wanted to see whether changes in structures were paralleled by differences in stability of the biofilm. The water jet apparatus was used to test biofilm stability of *E. coli* mutants. Alteration of the cell surface structures was detrimental to biofilm stability, while alterations in metabolism had less effect on stability. PEA (0 to 50 mg/mL) was applied to bacterial strains to see the effects on growth and biofilm amounts. PEA had an inhibitory effect on growth and biofilm amounts of some bacterial strains tested.

ACKNOWLEDGEMENTS

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CHAPTER 1. LITERATURE REVIEW[1, 2]

Biofilms

Biofilms are defined as communities of bacteria that form on a variety of surfaces and can occur in many natural, environmental, clinical, and industrial settings [1]. Bacterial biofilms are tougher to treat than planktonic bacteria and can end up being detrimental to human health. For example, biofilms have been shown to form on implants, which can result in chronic bacterial infections that can't be treated with standard antibiotics [2, 3]. Because of the negative effects bacterial biofilms can have to human health, a lot of research has been done over the years to better understand biofilms and develop novel treatments.

Formation of biofilms occur in several steps and has been postulated that each step is characterized by a specific cell surface organelle [4, 5]. Reversible attachment is the first step of biofilm formation, where the bacterium may loosely attach to a surface and detach again. Flagella, fimbriae, and pili aid in the initial contact with the surface. Irreversible attachment occurs when the bacteria start to produce adhesions and some extracellular polymers. During the maturation phase the attached cells produce an extracellular polymeric substance matrix which helps define the three-dimensional structure of the biofilm [4, 6-8].

Signal transduction pathways have recently become a mechanism to develop novel biofilm prevention and treatment techniques. Two examples of this are quorum sensing and two

[1] ß-phenylethylamine, a small molecule with a large impact. **Meredith Irsfeld**, Matthew Spadafore, and Dr. Birgit M. Prüß. 2013. WMC Microbiology. WMC004409.

[2] The Literature Review is a combination of the above paper and additional information to address the entirety of the thesis. Paragraphs that are taken from the paper are indicated as [Irsfeld, Spadafore, Prüß, 2013]. References are provided at the end.

component signaling, which permit the regulation of biofilm amounts by introducing or modulating external signals [9]. Quorum sensing allows bacteria to communicate amongst one another within the biofilm and two-component signaling allows the bacteria to elicit a response when there are changes in their environment [10].

Cell to cell communication within the biofilm, quorum sensing, results in a coordinated community response due to shifts in gene expression [11]. Responses elicited from the biofilm due to quorum sensing include competence, conjugation motility, sporulation, biofilm formation, and many other virulence factors [12]. One example of this is that in *Escherichia coli*, production of virulence factors and toxins are facilitated through quorum sensing [13].

Each two-component system requires a sensor kinase, which is activated by a signal from the environment. This results in autophosphorylation of a histidine, which then transfers the phosphate to the response regulator [14]. In *E. coli*, there are 37 two-component systems that are triggered by a variety of different environmental changes [15]. One example of a two-component system *E. coli* has is the EnvZ/OmpR whose response regulator is OmpR and responds to changes in external osmolarity. This causes increased expression of *csgBAC*, resulting in increased biofilm formation [16].

One example of utilizing two-component signaling as a mechanism to control biofilm amounts is the regulation of the FlhD/FlhC complex by the phosphorylated forms of OmpR and RcsB in *E. coli K-12* [17]. It was first suggested by Silverman et al. that an operon, consisting of multiple genes, controlled flagellar assembly and function in *E. coli* [18]. The two regulatory subunits of the FlhD/FlhC complex were later sequenced [19]. It was later shown that FlhD/FlhC was a global regulator involved in many cellular processes [20], such as cell divison [21]. Acetyl-phosphate was shown to be a global signal, which regulated the synthesis of cell surfaces

structures, like flagella and type I fimbriae, and physically altered biofilms formed by *E. coli K-12* mutants [22].

Water Jet

As described above, biofilms are ubiquitous and can have very detrimental effects on human health. The physical characteristics of the surfaces can play a role in the initial attachment of a biofilm or it can affect how easily that biofilm can be removed. Surface roughness was shown to promote increases in biofilm formation and promote bacterial settlement of *Geobacter sulferreducens* grown on steel cathodes [23]. Removal of biofilms has also been shown to be dependent on the chemical characteristics of the material which surfaces are made of. An example of this is when *Bacillus subtilis* and *Pseudomonas fluorescens* biofilms were grown on stainless steel and polymethyl methacrylate (PMMA); increased removal of biofilms was seen on stainless steel in comparison to PMMA [24]. Stainless steel and plastics, like PMMA, are used in many industrial settings, where conditions can promote biofilm formation [25-27].

Adhesion strength of biofilm bound bacteria that is dependent on the physical and chemical characteristics of the surface, as well as the biology of the bacteria can be determined with the water jet apparatus [28]. The water jet apparatus consists of three plate stacking hotels which can house a combined 39 multiwell plates, a five axis robotic arm from Thermo Corporation to bring the plates over to the water jet, and the actual water jet. The water jet is off-center spinning jet to illicit a water stream into the bottom of the wells of a 24-well plate. The water jet is set off-center because this allows the water to cover more surface area than if a stationary, impinging jet was used. A coupling gear motor to a rotating hydraulic shaft allows the nozzle to spin. The nozzle spins at 120 rpm, which can make ten rotations in only 5 s. The nozzle is off-set 3.5 mm and rotates in a circle with a diameter of 7 mm, which covers the 15 mm

diameter well. Pressurized tanks are highly regulated to control the pressure of the water jet released from the nozzle [28, 29].

Originally, the water jet apparatus was developed as a way to test adhesion strength of marine microorganisms to antifouling coatings with the ultimate goal to prevent marine biofouling of ship hulls [30]. *Cellulophaga lytica* and *Halomonas pacifica,* both bacteria, and *Navicula incerta,* a diatom, were used to investigate the effect of antifouling (AF) and fouling-release (FR) surface coatings. A high throughput study was used to test 24 unique coating compositions and identify the AF/FR of the surface coatings. Surface energy, water contact angle hysteresis, and atomic force microscopy (AFM) were used to determine AF/FR of the surface coatings. Composition of the coatings were found to influence AF/FR interaction with the bacteria and diatom when analysis was performed using ANOVA [31].

Other studies using *Halomonas pacifica* have been done to develop the technique and determine the parameters to effectively quantify biofilm removal from coatings on ship hulls [28]. For this thesis, the water jet technique has been modified as a screen to determine the stability of bacterial biofilms of *E. coli K-12* mutants [32]. Future experiments using the water jet will be focused on screening biofilm adhesion strength of *E. coli* strains on different coatings. The coatings can be integrated with antimicrobial chemicals, such as PEA, which could be developed into biofilm prevention materials in the future.

ß-phenylethylamine, a small molecule with a large impact [Irsfeld, Spadafore, Prüß, 2013]

Chemical properties of PEA

PEA is known under a variety of names including β-phenylethylamine, β-phenethylamine, and phenylethylamine. According to the International Union of Pure and

Applied Chemistry (IUPAC), the proper name of PEA is 2-phenylethylamine. Its molecular formula is denoted by $C_8H_{11}N$. The general information on and the chemical properties of PEA are summarized in Table 1.

Table 1. General information on and chemical properties of PEA. Information was taken from the Compound database from the NIH (http://pubchem.ncbi.nlm. nih.gov) and the Material Safety Data Sheets (MSDS) from TCI America.

	Solvent independent properties	**Solvent dependent properties**			**Reference**
		In ddH_2O	In lipid	In Plasma	
Alternative names	phenylethylamine, β-phenylethylamine, 2-phenylethylamine, benzeneethanamine, phenethylamine, β-phenethylamine, 2-phenethylamine	NA	NA	NA	http://pubchem.ncbi.nlm.nih.gov
Molecular Formula	$C_8H_{11}N$	NA	NA	NA	http://pubchem.ncbi.nlm.nih.gov
Molecular weight	121.17964 g/mol	NA	NA	NA	http://pubchem.ncbi.nlm.nih.gov
Companies that sell PEA	Forest Health, Vitacost, Amazon, Walmart	NA	NA	NA	NA
Toxicity	Mouse LD_{50} (oral) 400 mg/kg	NA	NA	NA	MSDS, TCI America
Solubility	NA	High solubility	Low solubility	High solubility	MSDS, TCI [33, 34]
Half life	NA	NA	NA	~5-10min	[34]

Natural occurrence and biological synthesis of PEA

The occurrence of PEA and its derivatives has previously been reviewed [35]. PEA can be found in many algae [36], fungi and bacteria [37] as well as a variety of different plant species [38]. PEA is the decarboxylation product of phenylalanine.

In several bacterial species, the decarboxylation is catalyzed by the enzyme tyrosine decarboxylase, which also converts tyrosine to another trace amine, tyramine [39, 40]. Intriguingly, PEA synthesized by fungi and bacteria can also be found in food products [41], where it serves as an indicator of food quality and freshness. Such foods include the Korean natto [37] and commercial eggs [42]. Another food that contains PEA is chocolate, where it is not produced by bacteria, but during the thermal processing of cocoa [43]. PEA can also be found in members of the family *Leguminosae*, which is the second-largest family of seed plants and is comprised of trees, shrubs, vines, herbs (such as clover), and vegetables (such as beans and peas).

PEA has also been found in the brains of humans and other mammals [44, 45]. This is attributed to the high solubility of PEA in plasma and its ability to cross the blood-brain barrier [46]. Like its α-methylated derivative, amphetamine, PEA has stimulant effects which lead to the release of so called biogenic amines, including dopamine and serotonin [47, 48]. Unlike amphetamine, PEA is rarely found in high concentrations in the human body, due to its oxidative `deamination to phenylacetic acid by the enzyme B monoamine oxidase (MAO) [49]. Phenylacetic acid has an effect that is similar to the activity of the natural endorphins, an effect that is known as a "runner's high".

Due to its impact on the levels of several 'feel good hormones' (see above), PEA has recently gained popularity as a nutritional supplement that is sold by numerous health stores to improve mood. Since it also decreases the amount of water intake, it aids weight loss efforts [50]. Altogether, PEA appears to have a number of positive effects on human health without the risks of its structural relatives.

Chemical synthesis of PEA

Two different pathways that lead to the chemical synthesis of PEA have been established in the 1940s and 1950s. First, PEA is produced by reduction of a nitrile into an amine [51]. A second, simpler way of producing PEA is to reduce ω-nitrostyrene with lithium aluminum hydride in ether [52]. The experimental procedure that employs the use of lithium aluminum in reduction reactions follows the mechanism used in a Grignard synthesis.

Recent literature focuses on the biological synthesis of PEA, rather than the chemical one. 1-phenylethylamine can be synthesized by *Escherichia coli* overexpressing ω-transaminase [53]. Likewise, the PEA biosynthetic enzyme from *Enterococcus faecium* can be expressed in *E. coli*, which leads to large amounts of L-phenylalanine and tyrosine decarboxylase activity [54]. Intriguingly, PEA can serve as a substrate for the synthesis of other drugs, such as sulfonamides that are being used as anti-microbials [55].

PEA as a neurotransmitter

PEA is a member of the so-called trace amines (reviewed by Premont et al [56]). The expression "trace amine" is used to refer to a group of amines that occur at much lower intra- and extra-cellular concentrations than the chemically and functionally related biogenic amines and neurotransmitters epinephrine, norepinephrine, serotonin, dopamine, and histamine. The molecular mechanism of the trace amines involves binding to a novel G protein-coupled receptor, called TAAR (trace amine-associated receptor) [57, 58], the most studied of which, TAAR1, can be activated by the drug amphetamine as well [57]. The downstream events that follow the initial interaction of PEA and TAAR1 are not nearly as well understood as the receptors and their various ligands themselves [59]; it is however believed that binding of PEA to TAAR1 results in an alteration of the monoamine transporter functions, which leads to inhibition

of the re-uptake of dopamine, serotonin, and norepinephrine [60]. Eventually, this will cause an increased concentration of these neurotransmitters at the synapses. A similar increase in the synaptic concentrations of dopamine can be accomplished by blocking the dopamine transporter directly. Methylphenidate is an example of a class of drugs that can perform this blockage [61]. Figure 1 is a graphic representation of the regulatory pathway from the trace amine PEA to the increased concentration of the biogenic amines and neurotransmitters dopamine and serotonin.

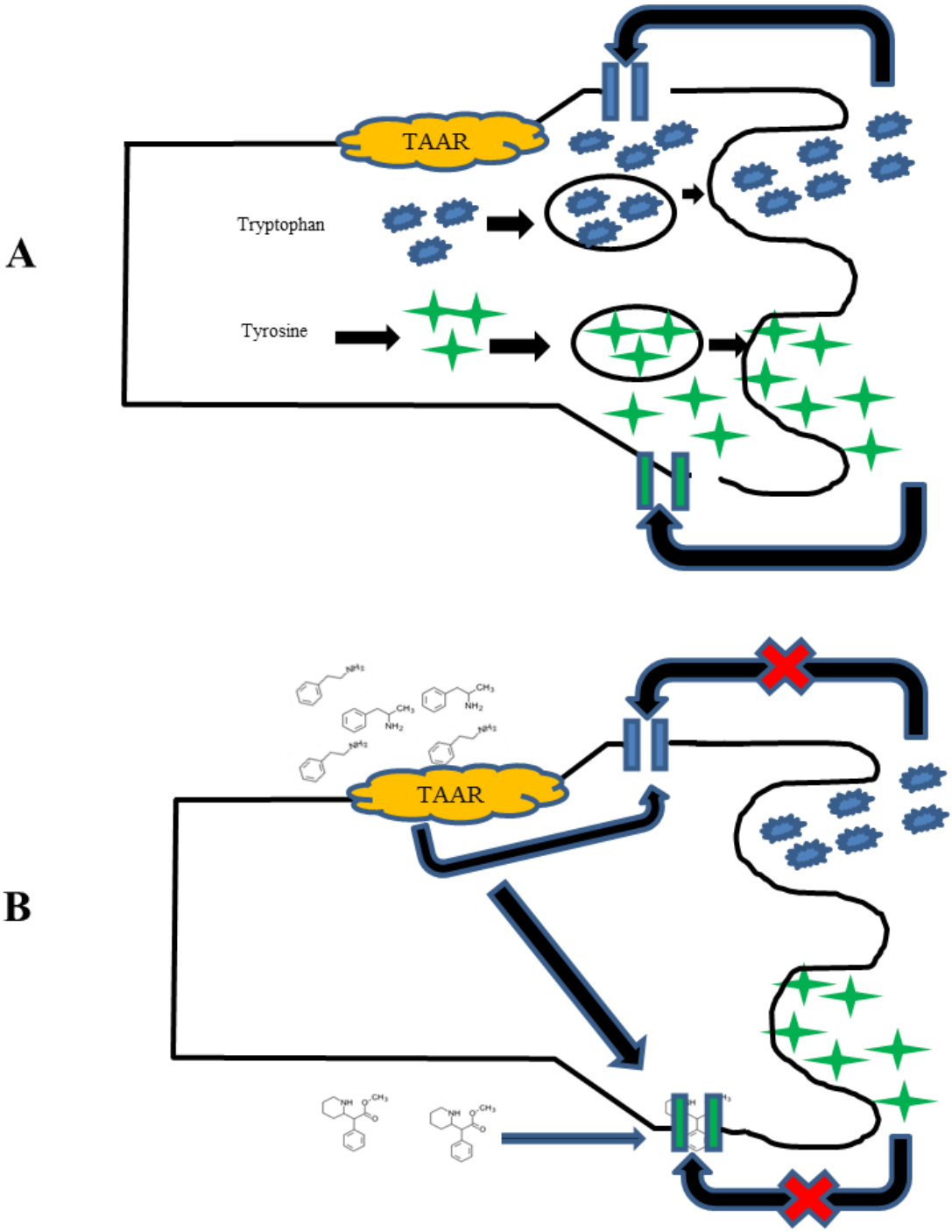

Figure 1: Schematic of the inhibition of the dopamine and serotonin receptors by PEA, amphetamine, and methylphenidate. Panel A shows the normal action of release and re-uptake of the biogenic amines dopamine and serotonin. Panel B shows the inhibition of the re-uptake transporters by PEA, amphetamine, and methylphenidate.
Serotonin , dopamine , serotonin receptor , dopamine receptor ,
PEA , amphetamine , methylphenidate

PEA and other amines in food as a result of microbial contamination

Some foods that contain microorganisms can release high levels of amines, including the trace amines PEA, tyramine, and tryptamine, the biogenic amine histamine, and the polyamines putrescine and cadaverine [62]. Such amines are formed by bacteria of the genera *Lactobacillus*, *Clostridium*, *Pseudomonas*, and *Enterobacteriaceae* that contain amino acid decarboxylases, which remove an α-carboxyl group from the respective amino acid [62, 63].

This increased concentration of amines is due to bacterial metabolic processes and is commonly associated with foods and food products made through the process of fermentation [64]. A good example of this is the "cheese reaction", which refers to high levels of tyramine as a result of elevated levels of tyrosine in cheese that has had increased storage times at temperatures higher than recommended by the producer. As mentioned earlier, PEA can be a by-product of the tyrosine decarboxylase reaction because the same enzyme that is capable of converting tyrosine to tyramine can also metabolize phenylalanine to PEA [39]. In individuals taking monoamine oxidase inhibiting drugs, the 'cheese reaction' can result in a hypertensive crisis [65-67].

A second group of food or food product that contains elevated levels of amines is meat and/or fish [68-70], where amine production is part of the food spoilage reaction and can be used as an indicator of food freshness and quality. Specifically, bacteria from the families *Enterobacteriaceae* and *Pseudomonadaceae* can produce cadaverine and putrescine in spoiled turkey meat. Fraqueza and coworkers suggested to use tyramine, putrescine, and cadaverin to quantify meat freshness [71]. In vegetables, high levels of tyramine were only seen in brine, unless the vegetables were contaminated prior to processing or the temperature and storage time were extreme [72].

Since elevated levels of amines are usually an indicator of food spoilage, it is possible to use detection of amines as an indicator of food freshness. One such technique is thin-layer chromatography to separate and identify amounts of tyramine and PEA [73].

PEA as a modulator of bacterial gene expression

Above we mention contamination and food spoilage by bacteria. One such initially contaminated food can cross-contaminate additional food products through the food processing chain because of the bacteria's ability to attach to food contact surfaces and form biofilm [74].

Recent research advances that are aimed at the prevention of biofilms include the manipulation of the bacteria's signal transduction pathways, including quorum sensing [75] and two-component signaling [76]. One such environmental signal that bacteria can respond to is PEA, but the mechanism through which this is occurring needs more investigation. PEA was found to have the greatest inhibitory effect of 95 carbon and 95 nitrogen sources screened for their effect on *E. coli* O157:H7 growth, bacterial cell counts, and biofilm amounts. In liquid beef broth medium, PEA reduced biofilm amounts, bacterial cell counts, and planktonic growth of *E. coli* O157:H7. In a final experiment, bacterial cell counts of *E. coli* O157:H7 were determined from beef meat pieces that were treated with different dilutions of PEA and subsequently inoculated with the bacteria; this resulted in a 90% reduction of bacterial cell counts when the beef was treated with a concentration of PEA at 150 mg/ml. We were unable to determine biofilm formation on the beef meat pieces [77]. The question arises whether it could be possible to integrate PEA into novel biomaterials that can then be used to coat food processing equipment to prevent biofilm formation on such equipment and cross contamination during the food processing chain.

Objectives for this thesis

Objective I constitutes the physical component of treatment of biofilms. This aim investigates the effect of *E. coli K-12* isogenic mutants on biofilms mechanical stability, which was tested with the automated water jet. This Aim is addressed in Chapter I.

Objective II constitutes the chemical component of treatment of biofilms, investigating the effect of PEA on growth and biofilm amounts of bacterial pathogens. This Aim is addressed in Chapter II.

A general Discussion is included at the end of this thesis which combines important observations and future perspectives for both Specific Aims.

CHAPTER 2. WATER JET EXPERIMENT[3]

Introduction

Biofilm research has grown dramatically over the past two decades due to an ever increasing appreciation of the immense impact that these microbial communities have in so many different settings and aspects of our daily lives. Accordingly, numerous studies have been undertaken during this time to unravel the complexities and intricacies associated with the biofilm mode of life, including those that have probed the general processes that regulate and control biofilm growth and development [4] to detailed studies that have focused on the characterization of the biofilms three dimensional architecture [78, 79], the monitoring of gene expression profiles [80-82], the measurement of antibiotic susceptibility/resistance [83] and the examination of horizontal plasmid transfer [84], just to name a few.

Another important facet of the biofilm phenotype that has gained attention in recent years is the study of its physical and rheological properties, where factors such as surface roughness, porosity, density, elastic modulus, tensile strength and yield stress work in concert with one another to dictate the mechanical integrity and cohesive strength of the biofilm [85]. The mechanical integrity or stability of biofilms plays a pivotal role in the establishment of microbial communities in industrial settings and engineered systems where high hydrodynamic shearing conditions typically prevail, such as wastewater treatment plants [86], drinking water distribution systems [87], and heat exchangers [88]. Several studies have been published in recent years that have investigated various mechanical properties of microbial biofilms, employing sophisticated tools such as atomic force microscopy, micro-cantilever devices, centrifugation and collision

[3] Screening the mechanical stability of *Escherichia coli* biofilms through exposure to external, hydrodynamic shear forces. **Meredith Irsfeld**, Birgit M. Prüß, and Shane J. Stafslien. 2014. J. Basic Microbiol. 54:1-7.

devices and annular reactors [85, 89]. Although these methods are highly effective and provide robust and reliable information, they are often limited in regards to the number of samples, treatments, strains or environmental conditions that can be evaluated at any one time.

This experiment was designed to develop a laboratory screening methodology that would overcome these aforementioned limitations and facilitate quick assessments of the mechanical stability of biofilms formed by isogenic mutants of *E. coli* K-12. The water jetting technique that was employed for this purpose had previously been developed to rapidly characterize the adhesion profile of microbial biofilms cultured on the surface of antifouling marine coatings [28]. One objective for this experiment was to identify an optimal combination of water jetting parameters that would yield the largest difference in biofilm removal between one selected mutant and its isogenic parent strain. A second objective, the water jetting technique was used to assess the mechanical stability of a small number of mutants that were selected based upon previous observations of biofilm growth [17].

Materials and methods

Bacterial strains

Bacterial strains were derivatives of the *E. coli* K-12 strain AJW678 [90]. As a 'housekeeping' mutation which would not be expected to affect biofilm amounts and/or stability, we used the *argD*::*gm* (*argD*, acetylornithine aminotransferase) mutation from strain UU1246 which was moved into AJW678 by P1 transduction to yield strain BP1548. The *fliA* mutant strain was AJW2145, containing a *fliA*::Tn*5* [22]. The final two mutations affected acetate metabolism and were previously associated with biofilm formation [17, 22]. The *ackA pta* mutant was AJW2013 that contains a Δ(*ackA pta hisJ hisP dhu*) zej*223*-Tn*10* deletion. The *ackA* mutation was an *ackA*::Tn*phoA*'-2 that was moved from CP891 [91] into AJW678 by P1

transduction to form strain BP1330. Complementation of BP1330 was performed by transforming BP1330 with *packA* expressing *ackA.* This plasmid is part of the ASKA collection of cloned open reading frames in pCA24 [92]. To serve as a control vector, pHP45 [93]was also transformed into BP1330. Bacterial strains were maintained as freezer stocks at -80ºC and plated onto Luria Bertani (LB) plates prior to each experiment.

Formation of the biofilms

Overnight cultures in tryptone soy broth (TSB) were pelleted by centrifuging at 4,500 *g* for 10 min. Bacteria were resuspended in 10 ml of 1 x phosphate buffered saline (PBS). Cultures were adjusted with PBS until their optical density values at 600 nm (OD_{600}) was 0.7 ± 0.05. The final inocula were prepared by diluting the PBS cultures 1:100 in TSB and used to inoculate individual wells of a 24-well polystyrene plate (1 ml/well). The plates were then incubated statically for 24 h at 37°C.

Water-jetting

Water jet treatments were carried out using an automated apparatus as described previously [28]. Briefly, liquid culture was removed from the wells by inverting the plates over a waste container, the remaining biofilms were rinsed twice with 1 ml of PBS. The plates were inserted into one of three plate stacking hotels and subjected to water jet treatments. The plates were sequentially removed from the hotel and inverted over the water jet nozzle with the aid of a five axis, robotic arm. Water jetting pressures ranged from 3 psi to 20 psi at 5 s of jetting duration. The first column of each plate was left untreated (i.e., 0 psi) to quantify the initial amount of biofilm growth. In a second experiment, the water jetting duration was varied from 1 to 10 seconds and held at a constant pressure of 5 psi. The retained biofilms from both experiments were allowed to air dry for at least 1 hour.

Quantification of biofilms

To quantify the amount of biofilm retained in the wells after water jetting, the crystal violet (CV) staining method was used [94, 95]. 1.0 ml of 0.35 % CV in ddH_2O was added to each well of the 24-well plates and incubated at room temperature for 15 min, then rinsed three times with 1.0 ml of ddH_2O. The plates were then inverted and tapped against an absorbent pad and allowed to dry at ambient laboratory conditions for 1 h. Images of the 24-well plates were captured [96] and the CV was extracted by adding 500 µl of 33% acetic acid to each well, followed by 15 minutes of incubation. 0.15 ml of each extract was then transferred into a 96 well plate and the OD_{600} measurements were determined with a Safire^2 plate reader (Tecan, USA).

Analysis of the biofilm removal data

Each mutant strain or complemented strain was processed three times together with the parent strain and compared to the parent strain that was processed on the same day. Biofilm removal was determined as follows: % biofilm removal = (1-(OD_{600}J / OD_{600}NJ)) x 100, where OD_{600}J is the OD_{600} value of the jetted wells and OD_{600}NJ the OD_{600} value for the wells that did not get water jetted. Averages and standard deviations were determined for the triplicate experiments and plotted as a function of the water jetting pressure or water jetting duration. Statistical analysis was carried out using a one-way ANOVA and a Tukey's range comparison test ($\alpha = 0.05$) to determine the statistical significance of the differences in the mean percent biofilm removal values between each mutant and its isogenic AJW678 parent strain.

Results and discussion

Water jet pressure has a larger effect on biofilm removal than duration of water jetting

In the first experiment of this study, the amount of biofilm removal as a function of water jetting duration was characterized. In this regard, biofilms cultured in polystyrene plates for 24 h for the *E. coli* K-12 strain AJW678 and its isogenic *ackA pta* mutant AJW2013 were subjected to varying durations of water jetting (1 to 10 s) at a fixed water jet pressure (5 psi) (Fig. 2).

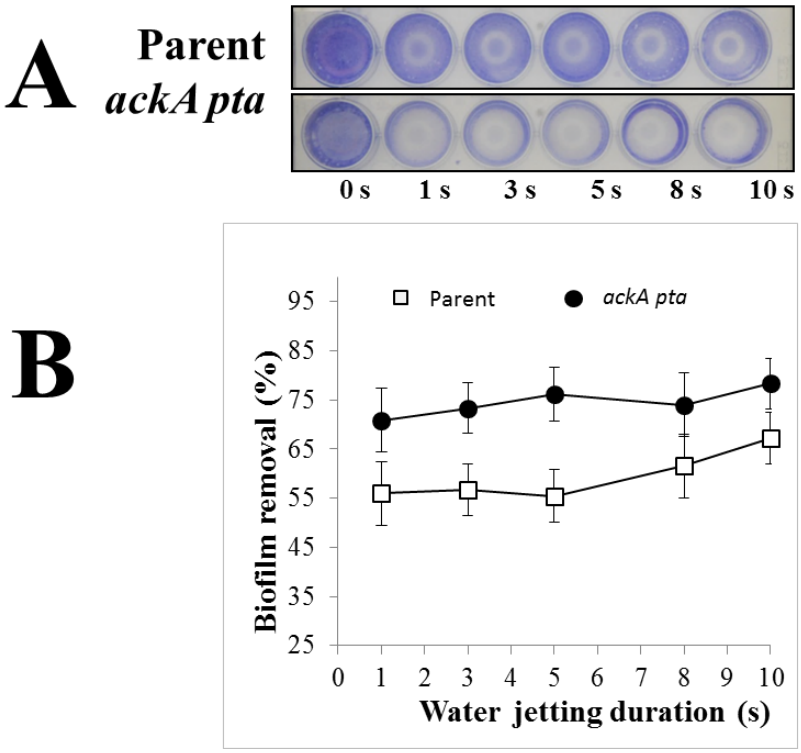

Figure 2. Biofilm removal as a function of water jetting duration (Panels A and B). Panel A shows two rows of a 24-well plate representing AJW678 and AJW2013 (*ackA pta*) after water jetting at different duration and CV staining. The first well is the untreated control (0 s) and the subsequent wells (2-6) show increases in duration of water jetting (1-10 s) held at a constant 5 psi. Panel B is the quantitative data of the same data in Panel A, averaged across all replicates.

Panel A displays a representative set of images of biofilm removal after CV staining of the biofilms in the 24 well plates. A cursory inspection of these images reveals an obvious difference in the mechanical stability of the biofilms between these two strains, where

considerably more biofilm removal was achieved for the *ackA pta* mutant than its parent strain. It is important to note here that the circular-shaped rings/zones of clearing that are visible in the center of the wells for the parent strain in Panel A indicate areas where the pressurized stream of water directly impacted the well bottoms as the nozzle rotated during the water jetting treatments; thus removing the biofilm at those points of impingement. Panel B in Fig. 2 provides the corresponding quantitative analysis of the data obtained from absorbance measurements (600 nm) of the acetic acid extractions of the CV dye. For both strains, more than half of the biofilm was removed after 1 s of water jet duration, with only a marginal increase (8-12%) in removal observed across the subsequent 9 s of water jetting. The largest difference between the two strains (~20% biofilm removal) was observed after 5 s of water jetting.

Using a fixed water jet duration of 5 s, the effect of varying the water jet pressure (3 psi to 20 psi) on the difference in biofilm stability between the *ackA pta* mutant and parent strain was subsequently examined. As shown in Fig. 3A and B, the amount of biofilm removal increased steadily with increasing water jet pressure for both strains. It is evident that substantially more biofilm was removed for the *ackA pta* mutant than for the parent strain at the lower range of pressures. In this regard, the two lowest water jetting pressures evaluated, namely 3 psi and 5 psi, resulted in approximately 20% more biofilm removal for the *ackA pta* mutant. The difference in removal between the two strains became less pronounced as the water jet pressure reached 15 and 20 psi, with both strains approaching 90% removal of the biofilm. Even though these differences were not as pronounced at the higher water jet pressures, a larger amount of biofilm was still removed for the *ackA pta* mutant at each water jetting pressure examined, providing compelling evidence that the mechanical integrity of the biofilms produced by the mutant strain were markedly less stable or weaker than that of the parent strain. Based on

the results from these experiments, a fixed water jet duration of 5 s and a pressure range of 3 psi to 10 psi was determined to be an optimal set of parameters for elucidating differences in biofilm stability among isogenic strains of *E. coli* K-12.

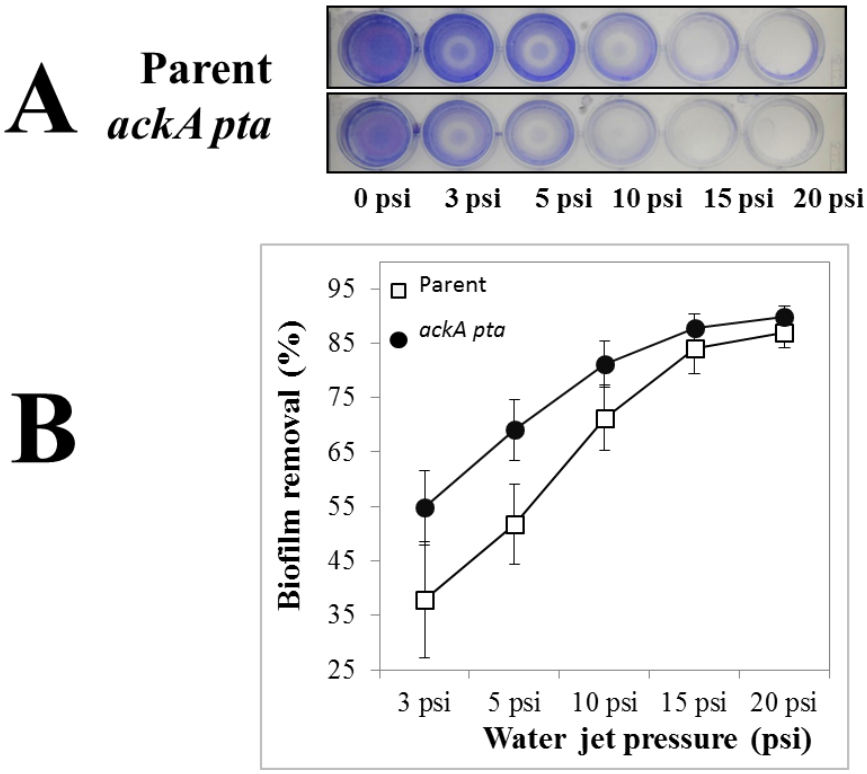

Figure 3. Biofilm removal as a function of water jetting pressure (Panels A and B). Panel A shows two rows of a 24-well plate representing AJW678 and AJW2013 (*ackA pta*) after water jetting at different water pressures and CV staining. The first well is the untreated control (0 s) and the subsequent wells (2-6) shows increases in water pressure (3-20 psi) held for 5 s. Panel B is the quantitative data of the same data in Panel A, averaged across all replicates.

Mutations in flagella biosynthesis and acetate metabolism result in mechanically less stable biofilms

Biofilm amounts of AJW678, the *argD* housekeeping strain, the *fliA* flagella mutant and both acetate mutant strains (*ackA* and *ackA pta*) were determined prior to water jetting, which is displayed in Fig. 4. The biofilm amounts prior to water jetting of AJW678, *argD*, *fliA*, and *ackA* were very similar, whereas the mutant lacking fimbrae (*fimA*) formed very little biofilm.

However, the acetate double mutant (*ackA pta*) formed twice as much biofilm prior to water jetting as the parent strain. The *fimA* isogenic mutant formed no biofilm prior to water jetting, which may be an indication that fimbrae plays important role in establishing an initial attachment required for biofilm formation.

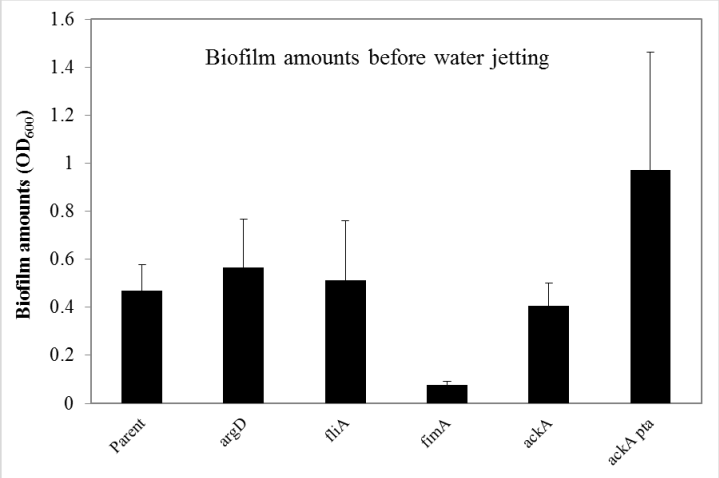

Figure 4. Biofilm amounts prior to water jetting. Quantitative data prior to water jetting of biofilm amounts of the parent and mutant strains were determined, averaged across all replicates.

Fig. 5 summarizes the biofilm removal profiles for the *argD* housekeeping strain, the *fliA* flagella mutant, both acetate mutant strains (*ackA* and *ackA pta*), and the *ackA* mutant that was complemented with the p*ackA* plasmid. The experiment was done using the optimal water jetting parameters identified in the experiments discussed above (the 15 psi and 20 psi water jet pressures were also included to clearly illustrate the difference in removal profiles among the isogenic strains).

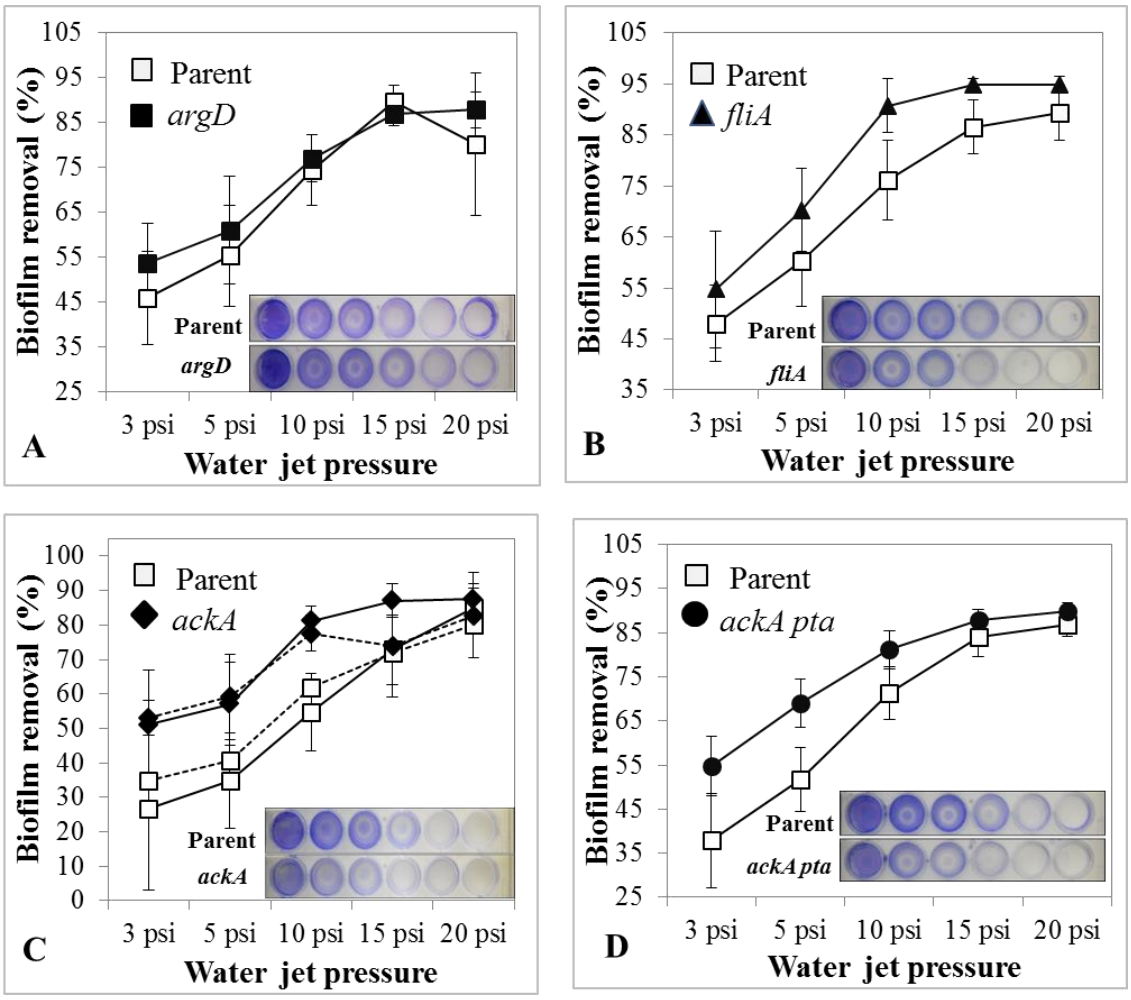

Figure 5. Biofilm removal data. Panel A-D show quantitative removal data and qualitative CV staining after water jetting. Panel A compares biofilm removal between the parent and housekeeping mutant *argD*, Panel B compares the parent to the *fliA* mutant, Panel C to the *ackA* mutant, and Panel D to the *ackA pta* mutant. The complementation experiment data is shown in Panel C, represented by the two additional dotted lines. The closed diamond with dotted line represents the positive control vector, while the open square with dotted line represents *ackA* expressing plasmid. Averages and standard deviations of the quantitative data were calculated across all replicates. In the bottom right corner of each graph are CV images after water jetting; the first well represents the untreated control (0 s) and the subsequent wells (2-6) shows increases in water pressure (3-20 psi) held for 5 s.

The removal profile for the *argD* housekeeping strain was very similar to that of the AJW678 parent strain (Panel A) and the *p*-values from the Tukey's test (Table 2) indicated a lack of statistical significance for these differences. This demonstrates that the mutagenesis process itself (*i.e.*, P1 transduction), does not render biofilm from the isogenic mutants less mechanically

stable than that from the parent strain. In contrast, the *fliA*, *ackA*, and *ackA pta* mutants exhibited more biofilm removal than the AJW678 parent strain at each water jet pressure evaluated (Panels B, C and D, respectively), and in most instances, these observed differences were determined to be statistically significant (Table 2).

Table 2. Statistical analysis of the biofilm removal data. Differences in biofilm removal are considered statistically significant with a *p*-value less than 0.05. These are indicated with the bold print.

Strains	**Water jet pressure**				
	3 psi	5 psi	10 psi	15 psi	20 psi
BP1548 *argD*	0.0592	0.2430	0.3372	**0.0462**	0.1237
AJW2145 *fliA*	0.0944	**0.0085**	**<0.0001**	**<0.0001**	**0.0024**
BP1330 *ackA*	**0.0061**	**0.0003**	**<0.0001**	**0.0003**	0.2239
BP1330 p*ackA*	0.3424	0.2669	0.2253	0.8003	0.1662
BP1330 pHP45	**0.0006**	**0.0002**	**<0.0001**	0.8646	0.6159
AJW2013 *ackA pta*	**0.0002**	**<0.0001**	**<0.0001**	**0.0294**	**0.005**

For the *fliA* mutant, the largest difference in biofilm removal (15%) from the parent was observed at 10 psi (Panel B). Significantly more removal was also observed at the 5 psi (10%; $p = 0.0085$), 15 psi (8%; $p<0.0001$) and 20 psi (6%; $p = 0.0024$) water jet pressures, while the removal exhibited at 3 psi was statistically equivalent ($p = 0.0944$) to the parent strain. Thus, flagella appears to play an important role in establishing the cohesiveness and mechanical stability of *E. coli* biofilms even after 24 h of incubation, which is a considerable length of time after the reversible attachment phase (typically characterized by the production of flagella) would have been completed. These results support the findings of a recently published study

which showed that flagella are used by *E. coli* as key architectural elements to construct and reinforce the biofilm superstructure [97].

When considering the acetate mutants, both the *ackA* and *ackA pta* strain exhibited the largest difference in biofilm removal (17%) at lowest water jet pressure evaluated (3 psi) when compared to the parent strain (Panels C and D). The *ackA pta* double mutant also showed 17% more removal at 5psi, with 10% more removal being attained at 10 psi and approximately 3% more biofilm removed at 15 psi and 20 psi. The differences in biofilm removal at all five pressures were determined to be statistically significant for this strain (Table 2.). For the *ackA* single mutant, the difference in biofilm removal dropped to 10% (down from 17%) for both the 5 psi and 10 psi pressures, with only the 10 psi pressure determined to be statistically significant ($p = 0.0039$). It is apparent from this data set that mutations introduced into acetate metabolism have an adverse effect on the overall integrity and stability of *E. coli* biofilms. This may be due, at least in part, to the impairment of the resulting mutants' ability to produce either flagella (*ackA*) or type-1 fimbriae (*ackA pta*), which have both been shown to play a significant role in the biofilm developmental process [22]. Panel C includes the biofilm removal profiles of the *ackA* expressing plasmid (open square dotted line) and the pHP45 control vector (closed diamond dotted line). The *ackA* expressing plasmid has a biofilm removal profile that is statistically not significant from the parent strain which is displayed in Table 2. The similarity in the biofilm removal profiles between the parent strain and BP1330 with the *ackA* expressing plasmid shows restoration of the *ackA* mutation. This indicates that the difference in stability between the parent strain and the mutant is really due to the lack of the *ackA* gene and not some other chromosomal defect. To further ensure that the plasmid itself wasn't causing the dramatic biofilm removal,

pHP45 was transduced into BP1330 as a control vector. Similar biofilm removal profiles between the *ackA* mutant and BP1330 with pHP45 were seen.

Concluding remarks

A water jetting technique has been used to quickly probe the mechanical stability of biofilms produced by isogenic strains of *E. coli* K-12. Mutations introduced into flagella biosynthesis and acetate metabolism were shown to significantly impair the integrity of biofilms cultured for 24 hours, which resulted in more biofilm removal for the mutants when compared to their parent strain. It is envisioned that this technique will be employed as a beneficial tool in future experiments to screen large sets of isogenic mutants under a wide range of environmental conditions to identify critical factors that contribute to the biomechanical stability of biofilms. Such information may be used to develop new and effective strategies for the control of biofilm formation in environments where biofilms are exposed to high shearing forces.

CHAPTER 3. β-PHENYLETHYLAMINE EXPERIMENT

Introduction

Biofilms are defined as complex communities of bacteria that form on a variety of surfaces and can occur in many natural, environmental, clinical, and food processing settings [1]. Environmental biofilms can be found in natural waters, water-treatment plants, and chlorinated distribution networks, as well as in the soil, and on plants [98]. In the case of food processing, *Escherichia coli* O157:H7 and other microbes associated with food-related illnesses can be spread to beef, poultry, and vegetables from the equipment, surfaces, and containers used in different stages of food processing [99-101]. It is estimated that 48 million people annually ingest microbe contaminated food products in the United States alone, which costs billions a year in medical and workers compensation expenses [102]. In clinical settings, bacterial biofilms can form on implants, heart valves, joint prostheses and urinary catheters implanted into the human body. *Pseudomonas aeruginosa* and Methicillin Resistant *Staphylococcus aureus* (MRSA) are examples of pathogens linked with chronic biofilm associated infections [103, 104].

Unfortunately, eradication of biofilms with conventional methods in these settings is becoming harder with the influx of antibiotic resistant bacteria [105, 106]. Biofilm preventing technologies, such as silver coatings or embedded silver nanoparticles, have advanced as our technology has [107, 108]. Other strategies have focused on new treatment options for biofilms, like combinations of antibiotics with antimicrobial peptides and superheated steam [109-111]. However, any strategies that are aimed at killing the bacteria encourage microbial resistances; while silver and other coatings have high production costs. Some researcher are trying to address both of these problem by focusing research on signal transduction pathways, which aims to regulate bacterial phenotypes by introducing or modulating external signals [9].

One such signal transduction pathway is two-component signaling, which allows bacteria to respond to signals in their environment and was reviewed by Lynnes et al [76]. One example of utilizing a two-component system as a mechanism for prevention of biofilms is the FlhD/FlhC complex in *E. coli K-12* [17]. FlhC, a flagella regulator, was found to reduce cell division, biofilm amount, and virulence of *E. coli* O157:H7 grown on meat [112]. Another study screened 190 chemicals for their effect on *E. coli* O157:H7 growth, planktonic bacterial counts, and biofilm amounts. Among these chemicals, PEA had the greatest inhibitory effect. This effect of PEA was also seen when pieces of beef were treated with PEA prior to inoculation with *E. coli* O157:H7 [77].

During this study, an experiment was developed to demonstrate the effectiveness of PEA on bacterial biofilms and growth of different strains and determine IC_{50} values. IC_{50} values determine at what concentration bacterial growth and biofilm amounts are reduced to 50%. One objective for this experiment was to examine the effect of PEA on biofilm amounts, growth, and biofilm stability on the nonpathogenic strain of *E. coli*, AJW678. The second objective was to investigate the effect of PEA on biofilm amounts and growth of pathogenic strains of *P. aeruginosa* and *S. aureus*.

Materials and methods

Bacterial strains

Bacterial strains used include *E. coli* K-12 strain AJW678, which is the wild type for acetate metabolism and biosynthesis of flagella, type I fimbriae, and colanic acid [90]. *P. aeruginosa* (American Type Culture Collection, ATCC 15442) and *S. aureus* (American Type Culture Collection, ATCC 25923) were kindly provided by Shane J. Stafslien (Center for Nanoscale Science and Engineering Fargo, ND). Bacterial strains were maintained as freezer

stocks at -80ºC in 8% dimethyl sulfoxide (DMSO). Prior to each experiment, AJW678 was plated onto Luria Bertani (LB) plates. *P. aeruginosa* and *S. aureus* were plated onto tryptone soy agar (TSA) and the plates were incubated over night at 37°C.

Formation of the biofilms

Liquid overnight cultures in tryptone soy broth (TSB) were pelleted by centrifuging at 4,500 *g* for 10 min. Bacteria were resuspended in 10 ml of 1 x phosphate buffered saline (PBS). Cultures were adjusted with PBS until their optical density values at 600 nm (OD_{600}) was 0.7 $\pm$ 0.05. The final inocula were prepared by diluting the PBS cultures 1:100 in 2 x TSB. Dilutions of PEA were prepared in PBS; PEA concentrations ranged from 0 mg/ml to 100 mg/ml. A 1:1 dilution of final inocula and PEA solutions were used to inoculate individual wells of a 24-well or 96-well polystyrene plate (1 ml/well). This yields an inoculation OD_{600} of 0.035 in 1 x TSB/0.5 x PBS and a PEA concentration range from 0 to 50 mg/ml. The plates were then incubated statically at 37°C. The experiments were done on three replicated plates. For the 24 plates, each bacterial strain was processed twice per plate. For the 96 well plates, each plate contained eight replicates of the same strain.

Determination of bacterial growth

Growth was monitored by using a Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT), which recorded OD_{600} every 2 h for 48 h at 37°C. Growth curves were plotted using the OD_{600} readings every 2 h for 48 hrs. Two analyses were performed. First, the maximum velocity was calculated. Maximum velocity is the slope of the line on the growth curves, where the bacteria is growing at the fastest rate. Averages and standard deviations of maximum velocity were determined over 18 replicates when the bacteria was grown on 96-well plates and over 6 replicates when the bacteria was grown on 24-well plates. These data are

presented as bar plots, where each bar represents one concentration. Statistical analysis software (SAS) was used to analyze this data. Per the recommendation of our statistician we used Fisher's Least Squared Difference (LSD) to determine the statistical significance of the difference between the growth means for each concentration when compared to the mean growth of the concentration 0 mg/ml. Second, for a subset of the data, the time it took the bacteria to grow to stationary phase was compared. For this analysis, averages and standard deviations were determined across the 18 (or 6) replicates at each time point. These data are presented as line plot, where each line represents one concentration.

Determination of biofilm amounts

To quantify the amount of biofilm retained in the wells after 48 h, the crystal violet (CV) staining method was used [94, 95]. 1.0 ml of 0.35 % CV in ddH_2O was added to each well of the 24-well plates and incubated at room temperature for 15 min, then rinsed three times with 1.0 ml of ddH_2O. The plates were then inverted and tapped against an absorbent pad and allowed to dry at ambient laboratory conditions for 1 h. The CV was extracted by adding 500 µl of 33% acetic acid to each well, followed by 15 minutes of incubation. 0.15 ml of each extract was then transferred into a 96 well plate and the OD_{600} measurements were determined with a Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT). Averages and standard deviations were determined across the three independent experiments for biofilm amounts and growth. SAS was used to analyze this data using LSD to determine the statistical significance of the difference between the growth (and biofilm amounts) means for each concentration when compared to the mean growth of the concentration 0 mg/ml.

Effect of β-phenylethylamine on water-jetting AJW678

PEA was added to cultures of AJW678 at concentrations of 0 mg/ml and 2 mg/ml. Biofilms were allowed to form on 24 well polystyrene plates for 24 h at 37ºC. Water jet treatments were carried out on the biofilms, using an automated apparatus as described previously [28]. Briefly, liquid culture was removed from the wells by inverting the plates over a waste container, the remaining biofilms were rinsed twice with 1 ml of PBS. The plates were inserted into one of three plate stacking hotels and subjected to water jet treatments. The plates were sequentially removed from the hotel and inverted over the water jet nozzle with the aid of a five axis, robotic arm. Water jetting pressures ranged from 3 psi to 20 psi at 5 s of jetting duration. The first column of each plate was left untreated (i.e., 0 psi) to quantify the initial amount of biofilm growth. The retained biofilms from both experiments were allowed to air dry for at least 1 hour. CV extraction was used to quantify biofilm amounts and biofilm % removal was determined as described [94, 95].

Biofilm removal was determined as follows: % biofilm removal = (1-(OD_{600}J / OD_{600}NJ)) x 100, where OD_{600}J is the OD_{600} value of the jetted wells and OD_{600}NJ the OD_{600} value for the wells that did not get water jetted. Averages were determined for 12 replicates. Biofilm removal in percent was plotted as a function of the water jetting pressure.

Determination of IC_{50} values for β-phenylethylamine

IC_{50} values for PEA was determined for growth and biofilm amounts of each bacterial strain, using curve fitting as previously described [113, 114]. Master Plex® Reader Fit analysis software (Hitachi Solutions American, Ltd., San Francisco, CA) was used to find the best curve fit for both assays; the software utilizes a four or five parameter logistics curve fitting model [115]. IC_{50} values are used to determine the effectiveness of PEA because it tells us the concentration at

which the bacterial growth and biofilm amounts are reduced to 50%. Coefficient of determination values (R^2) are calculated with the software as a quantitative way to show how well the curve actually fits. Higher R^2 values indicate confidence that the curve fits and the IC_{50} values are accurate.

Results and discussion

β-phenylethylamine reduces growth and biofilm amounts of AJW678

The *E. coli* K-12 strain AJW678 was grown on 96-well polystyrene plates in the presence of a range of concentrations of PEA to test the inhibitory effect of this nutrient on growth and biofilm amounts. Final concentrations of PEA ranged from 0 mg/ml to 50 mg/ml.

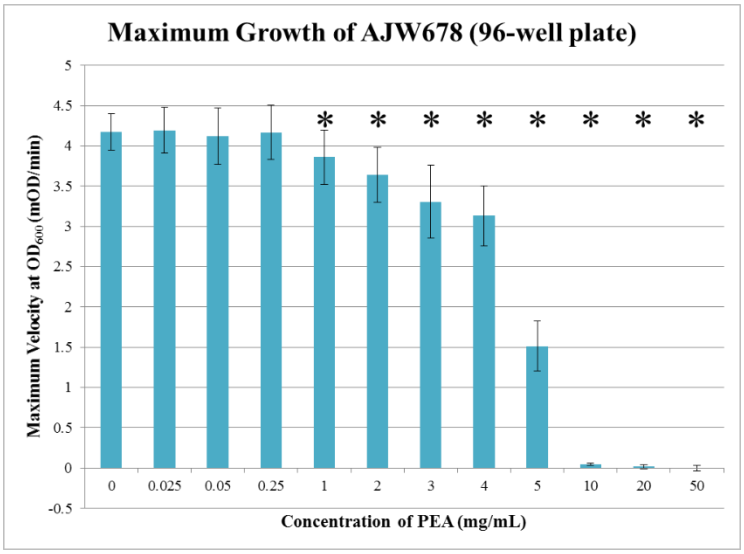

Figure 6. Effectiveness of PEA on AJW678 growth in a 96-well polystyrene plate. AJW678 was grown in the presence of varying concentrations of PEA, shown on the x-axis. Averages and standard deviations of maximum velocities (y-axis) were computed across all 18 replicates. Asterisks indicate a statistically significant difference between the mean growth at the respective PEA concentration in comparison to the mean growth at a PEA concentration of 0 mg/ml.

Fig. 6 represents the growth of AJW678 as maximum growth velocity in mOD/min, calculated across the time frame where the bacteria doubled at the fastest rate. This data gives us information about growth at an early phase of biofilm development in comparison to using final

growth readings. Fig. 7 demonstrates the biofilm amounts as quantified by the CV assay after 48 h. In this experiment, it was demonstrated that PEA had inhibitory effects on both growth and biofilm amount of AJW678. Overall, the inhibitory effect of PEA on biofilm amounts was more gradual, and was not as steep as was seen for growth. Statistically, 1 mg/ml was the lowest concentration at which inhibition was seen for both growth and biofilm amounts.

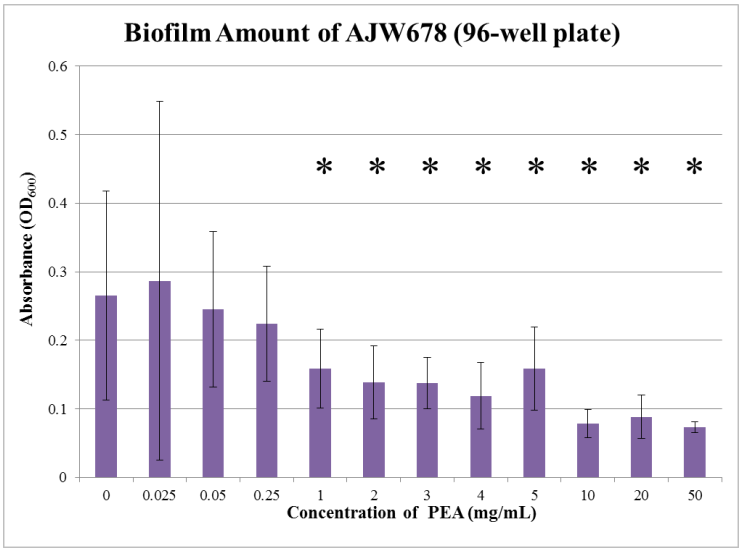

Figure 7. Effectiveness of PEA on AJW678 biofilm amounts in a 96-well polystyrene plate. AJW678 was grown in the presence of varying concentrations of PEA, shown on the x-axis. Averages and standard deviations of OD_{600} values (y-axis) from the CV assay were computed across all 18 replicates. Asterisks indicate a statistically significant difference between the mean biofilm amounts at the respective PEA concentration in comparison to the mean biofilm amount at a PEA concentration of 0 mg/ml.

Fig. 6 shows a 50% reduction in AJW678 growth at approximately 5 mg/ml of PEA. A 50% reduction in biofilm amount can be seen already at 1 mg/ml of PEA. Almost no, or very little, bacterial growth or biofilm amount can then be seen after the concentration of PEA was increased to 10 mg/ml and above. PEA has an inhibitory effect on both growth and biofilm amounts of AJW678 at low concentration, which makes it a potential candidate to use in materials for prevention of biofilms.

An explanation of how this reduction in growth and biofilm amounts could be occurring is through FlhD/FlhC. In a recent study by other researchers, PEA was shown to mimic overexpression of DisA, phenylalanine decarboxylase, from *Proteus mirabilis*, which was transformed into *E. coli.* Over expression of DisA in *E. coli*, as well as growing the bacteria with PEA resulted in decreased motility, flagellar gene expression, and biofilm amounts. It was hypothesized that PEA or biproducts of DisA decarboxylation affect the FlhD/FlhC complex [116]. Other research has identified other small molecules, like citrus limonoids, have the ability to reduce biofilm formation through cell surface organelle genes, which have been shown to play a role in biofilm formation. These include type 1 pili, flagella, and extracellular polymeric substance [117-120]. Essential oils of thyme, oregano, and cinnamon have also been shown to inhibit biofilm amounts, but little is known about the mechanism [121]. Further research needs to be done to determine the exact mechanism by which PEA is acting to reduce growth and biofilm amounts. However, in the future we could use PEA in industrial, environmental, and clinical settings to reduce bacterial growth and biofilm amounts.

The surface area impacts the PEA effect on biofilm amounts, but not growth of AJW678

We next investigated the effect surface area has on differences in growth and biofilm amounts of AJW678 grown in the presence of a range of concentrations of PEA. 24-well polystyrene plates were used, providing a larger surface area than 96-well plates. Fig. 8 represents the growth of AJW678 as maximum growth velocity in mOD/min and biofilm amounts on a 24-well plates. Comparison of Fig. 6, 7, and 8 demonstrates the effect surface area has on the growth and biofilm amounts after 48 h on 96-well and 24-well plates.

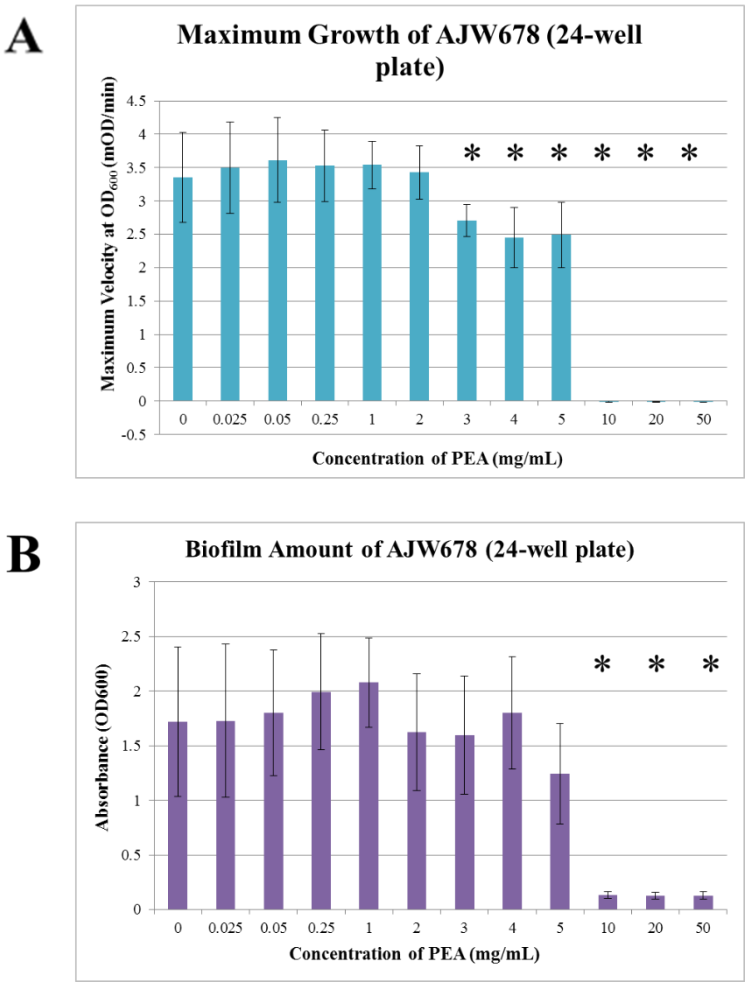

Figure 8. Effect of surface area on differences in growth and biofilm amounts. Panel A shows the maximum growth velocities (y-axis) in the presence of varying concentrations of PEA (x-axis). Panel B shows the biofilm amounts after 48 h. Asterisks indicate a statistically significant difference between the mean growth (Panel A) and biofilm amounts (Panel B) at the respective PEA concentration in comparison to the mean growth or biofilm amount at a PEA concentration of 0 mg/ml.

In this experiment, it was demonstrated that surface area has an effect on differences in PEA's inhibition of growth and on biofilm amounts. We see that larger surface areas increase differences in the inhibitory effect of PEA. The inhibitory effect of PEA on growth of AJW678 can be seen at 1 mg/ml on the 96-well and 3 mg/ml on the 24-well plates. With respect to biofilm amounts, differences in biofilm amounts between 96-well and 24-well plates were more dramatic. When comparing the initial amount of biofilm, six times more biofilm was seen on the

24-well plates than on the 96-well plates. Higher amounts of biofilm were expected to be seen on the 24-well plates because of the greater surface area. The 96-well plate yields gradual decrease in biofilm amounts of AJW678 as concentrations of PEA are increased, compared to the abrupt decrease in biofilm amounts seen on the 24-well plate. An inhibitory effect on biofilm amounts on the 96-well plate can be seen at 1 mg/ml of PEA. On the 24-well plate, between 0 mg/ml and 4 mg/ml of PEA there is little change as the concentrations of PEA increase. It takes until 10 mg/ml of PEA to see the inhibitory effect on biofilm amounts on the 24-well plate. After 5 mg/ml of PEA, there is little biofilm amount seen on both the 96-well plate and 24-well plate. The explanation for differences in inhibitory effect of PEA on 96-well and 24-well plates could be due to the differences in surface area between the two; there is actually a higher concentration of PEA in the 96 well plate versus the 24 well plate due to total surface area differences of the wells. Another explanation could be due to oxygen availability, which has been shown to impact susceptibility of *P. aeruginosa* when grown as planktonic bacteria and biofilms [122].

In conclusion, PEA does have an inhibitory effect on growth and biofilm amounts of AJW678 regardless of the surface area. However, using the 24-well plate provides a greater surface area, which may resemble more closely how PEA would behave in a clinical environment (*e.g.* knee replacement). Therefore, it may be advantageous to use a plate with a larger surface area when examining differences in growth and biofilm amounts and determining IC_{50} values of treatment solutions. Other physical properties, such as smooth or rough surfaces and material type, like steel versus plastics, have been shown to effect the ability of bacteria to form biofilms, which could also play a role in determining the inhibitory effect of PEA [23, 24, 123, 124].

β-phenylethylamine increases the time it takes AJW678 to reach stationary phase

Performing the above described experiments, it was noticed that PEA did not just decrease the maximal growth velocity, but also appeared to have an impact on the time it took AJW678 to reach stationary phase. The 24 well data set was subjected to a second analysis, where entire growth curves (up to 35 h) were compared between the lower PEA concentrations (1 to 5 mg/ml) (Fig. 9).

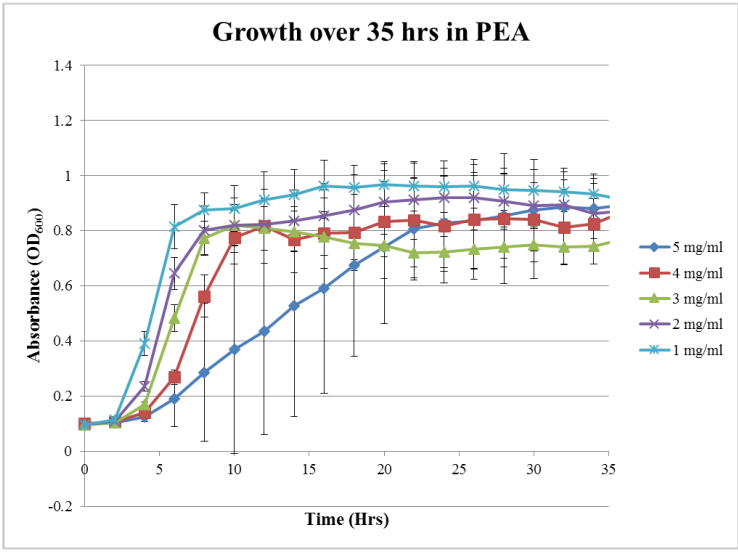

Figure 9. The effect PEA has on the time it takes AJW678 to reach stationary phase. AJW678 was grown in varying concentrations of PEA, shown on the x-axis. Averages and standard deviations of OD_{600} values (y-axis) were computed across all 6 replicates.

When the concentration of PEA was increased from 1 mg/ml to 5 mg/ml it takes the bacteria longer to reach stationary phase. At 5 mg/ml, it takes more than 20 h for AJW678 to reach stationary phase and it takes 15 h to reach the rate where the bacteria are doubling the fastest. In comparison, at 1 mg/ml, stationary phase is reached at 7 h and 4 h is where the doubling rate of the bacteria is the fastest. This should be taken into consideration when interpreting maximum growth velocities, because even though the doubling rate may appear to be similar, it may take more time for the bacteria to reach this doubling rate. This indicates that

PEA inhibits during the early stages of biofilm formation and that the use of PEA in conjunction with standard operating procedures to prevent biofilms, can aid in the prevention of biofilms at the initial stage of biofilm formation.

ß-phenylethylamine affects biofilm stability of AJW678

The water jet was used to determine differences in the stability of the biofilms that AJW678 had formed under different concentrations of PEA. PEA concentrations were chosen, at which the bacteria were still able to form a biofilm. Using a fixed water jet duration of 5 s, the water jet pressure was varied (3 psi to 20 psi). Biofilm amounts were quantified following water jetting using CV extraction and are expressed as biofilm removal in percent (Fig. 10). A low biofilm removal of 20% (orange line, first data point) is indicative of a high stability of the biofilm.

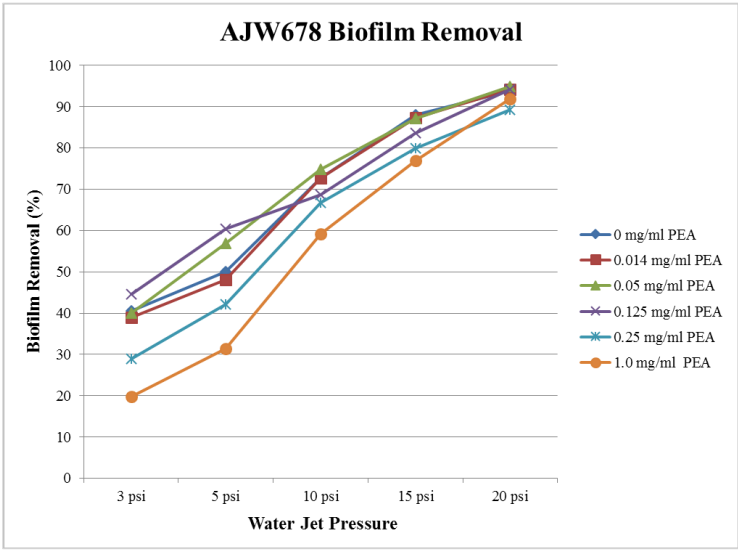

Figure 10. Biofilm removal as a function of water jetting pressure when AJW678 was grown with PEA. Quantitative removal data is shown above, averages of the quantitative data were calculated across all 12 replicates. The key to the right shows the concentrations of PEA in which AJW678 was grown.

The hypothesis was that dramatic decreases in biofilm stability of AJW678 would be seen when we increased the concentration of PEA. However, only slight differences in the

removal percentage of the biofilms were seen as PEA concentrations increased. Furthermore, the biofilm appeared to be more stable as the PEA concentrations increased to 1 %. One explanation of this could be that at this low concentration of PEA, bacteria are breaking the chemical down and utilizing it as a nutrient, which could contribute to more growth and stronger biofilms. Investigation of higher concentrations of PEA may show a more dramatic effect on biofilm stability [39]. Current research has investigated the affect shearing forces have on biofilms [125, 126]. However, to my knowledge, nothing is known about the effect PEA in conjunction with shearing forces has on biofilm stability.

β-phenylethylamine reduces growth and biofilm amounts of pathogens

Two pathogens, *P. aeruginosa* and *S. aureus*, were grown in the presence of increasing concentrations of PEA to investigate the inhibitory effect on growth and biofilm amounts. Fig. 11 represents the growth of *P. aeruginosa* as maximum growth velocity in mOD/min, calculated across the time frame where the bacteria doubled at the fastest rate. Fig. 12 demonstrates the biofilm amounts as quantified by the CV assay after 16 h.

As seen with AJW678, PEA has an inhibitory effect on the bacterial growth rate of *P. aeruginosa*. Small reductions of bacterial growth rate were seen between 0 mg/ml and 3 mg/ml of PEA, but at 4 mg/ml a 40% reduction in bacterial growth rate was seen. It took until 5 mg/ml of PEA to see a 90% reduction in bacterial growth rate. At higher concentrations beyond 5 mg/ml little or no bacterial growth was seen. In comparison, we did not see a significant reduction in bacterial growth rate of AJW678 until the concentration of PEA is 5 mg/ml.

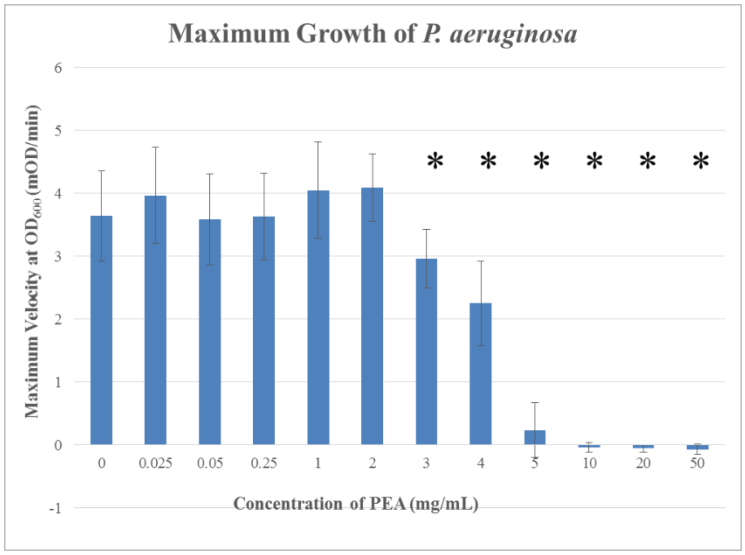

Figure 11. Effectiveness of PEA on *P. aeruginosa* growth. *P. aeruginosa* was grown in varying concentrations of PEA, shown on the x-axis. Averages and standard deviations of maximum velocities (y-axis) were computed across all 6 replicates. Asterisks indicate a statistically significant difference between the mean growth at the respective PEA concentration in comparison to the mean growth at a PEA concentration of 0 mg/ml.

A significant reduction in biofilm amounts (~40%) was seen at 2 mg/ml of PEA. As concentrations of PEA increased from 2 mg/ml to 50 mg/ml, only minor additional reductions in biofilm amounts were seen. *P. aeruginosa* biofilm amounts come down at lower concentrations of PEA (2 mg/ml) in comparison to AJW678, where significant reduction in biofilm amounts weren't observed until the concentration of PEA was 10 mg/ml. However, unlike AJW678 where the biofilm was eradicated when concentrations of PEA were greater than 5 mg/ml (Fig. 9), *P. aeruginosa* still formed close to 50% of the original biofilm at these higher concentrations of PEA (10 mg/ml to 50 mg/ml). It is also interesting to note that even though there was very little bacterial growth of *P. aeruginosa* at concentrations of PEA above 10 mg/ml, the bacteria was still able to form some biofilm at these concentrations. One explanation of this could be that the few cells that grew may have formed biofilms that were then resistant to PEA.

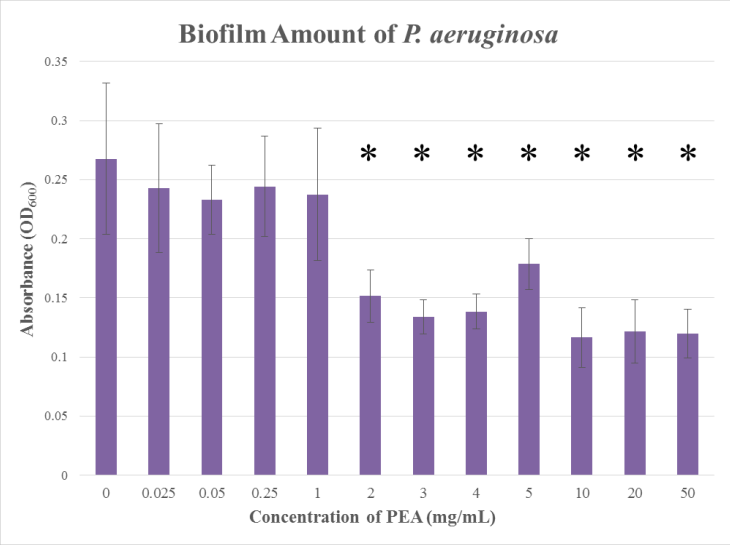

Figure 12. Effectiveness of PEA on *P. aeruginosa* biofilm amounts. *P. aeruginosa* was grown in varying concentrations of PEA, shown on the x-axis. Averages and standard deviations of OD_{600} values (y-axis) were computed across all 6 replicates. Aste**r**isks indicate a statistically significant difference between the mean biofilm amounts at the respective PEA concentration in comparison to the mean biofilm amount at a PEA concentration of 0 mg/ml.

Next, the inhibitory effect of PEA was tested against the gram-positive pathogen *S. aureus.* Fig. 13 represents the growth of *S. aureus* as maximum growth velocity in mOD/min, calculated across the time frame where the bacteria doubled at the fastest rate. Fig. 14 demonstrates the biofilm amounts as quantified by the CV assay after 16 h.

As observed with AJW678 and *P. aeruginosa*, PEA has an inhibitory effect on the bacterial growth rate of *S. aureus*. Small reductions in *S. aureus* growth rate were seen between 0 mg/ml and 10 mg/ml of PEA. To even get a 20% reduction in bacterial growth rate of *S. aureus*, the concentration of PEA had to be increased to 10 mg/ml. In comparison, little or no bacterial growth was seen for AJW678 and *P. aeruginosa* at this same concentration. Growth of *S. aureus* could still be seen at 20 mg/ml of PEA, but finally at 50 mg/ml little bacterial growth was seen.

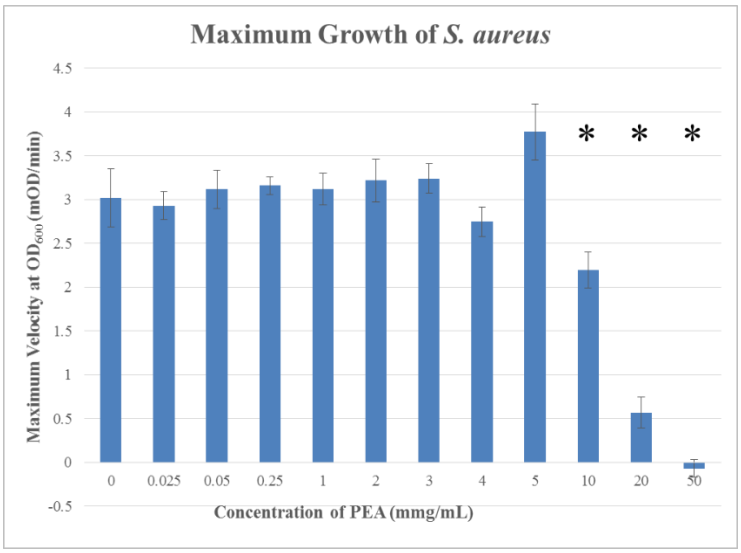

Figure 13. Effectiveness of PEA on *S. aureus* growth. *S. aureus* was grown in the presence of varying concentrations of PEA, shown on the x-axis. Averages and standard deviations of maximum velocities (y-axis) were computed across all 6 replicates. Aste**r**isks indicate a statistically significant difference between the mean growth at the respective PEA concentration in comparison to the mean growth at a PEA concentration of 0 mg/ml.

Despite the high bacterial growth of *S. aureus* we saw in Fig. 13, there was very little biofilm even in the absence of PEA (Fig. 14). Recent research has suggested that more pathogenic strains of *S. aureus* (ie. MRSA) may actually form better biofilms [111]. Another interesting observation, was that in each replication of this experiment at least one well of the 24-well plate at the concentration of 10 mg/ml of PEA, had massive amounts of biofilm. This could just be an anomaly or more investigation needs to be done to explain this phenomenon.

In conclusion, the gram-negative strains of bacteria (AJW678 and *P. aeruginosa*) weren't able to grow very well at the higher concentrations of PEA, but the gram positive bacteria (*S. aureus*) was still able to grow at these high concentrations . This could be due to environmental factors such as the pH of PEA in the nutrient medium. *S. aureus* biofilm production has been shown to be influenced by pH [127]. Another reason could be this strain has acquired some resistance genes or physical characteristics, such as capsular polysaccharides, which are impacting PEA effectiveness [128, 129]. The hypothesis that PEA is transduced through

FlhD/FlhC, which *S. aureus* lacks could explain the growth of *S. aureus* at high concentrations of PEA [130].

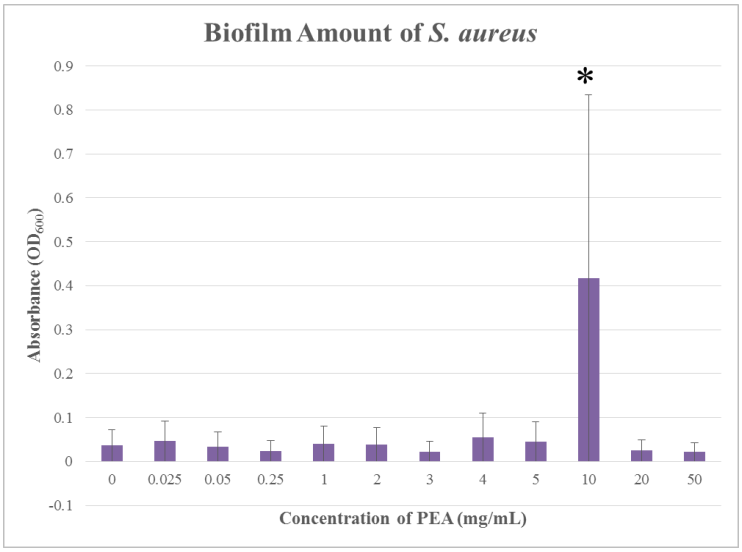

Figure 14. Effectiveness of PEA on *S. aureus* biofilm amounts in a 24-well polystyrene plate. *S. aureus* was grown in varying concentrations of PEA, shown on the x-axis. Averages and standard deviations of OD_{600} values (y-axis) were computed across all 6 replicates. Aste**r**isks indicate a statistically significant difference between the mean biofilm amounts at the respective PEA concentration in comparison to the mean biofilm amount at a PEA concentration of 0 mg/ml.

Calculation of IC_{50} for biofilm amounts and growth of AJW678, Ps. aeruginosa, and S. aureus

IC_{50} values were calculated for the inhibitory effect of PEA on biofilms amounts and growth of AJW678, *Ps. aeruginosa,* and *S. aureus*. These were calculated using Master Plex® Reader Fit analysis software (Hitachi Solutions American, Ltd., San Francisco, CA), which was used to find the best curve fit for both assays; the software utilizes a five or four parameter logistics curve fitting model [115]. The output from the Master Plex® Reader Fit analysis shows the best fit curve for biofilm amounts and growth, which helps to determine the IC_{50} and R^2 values (Fig. 15).

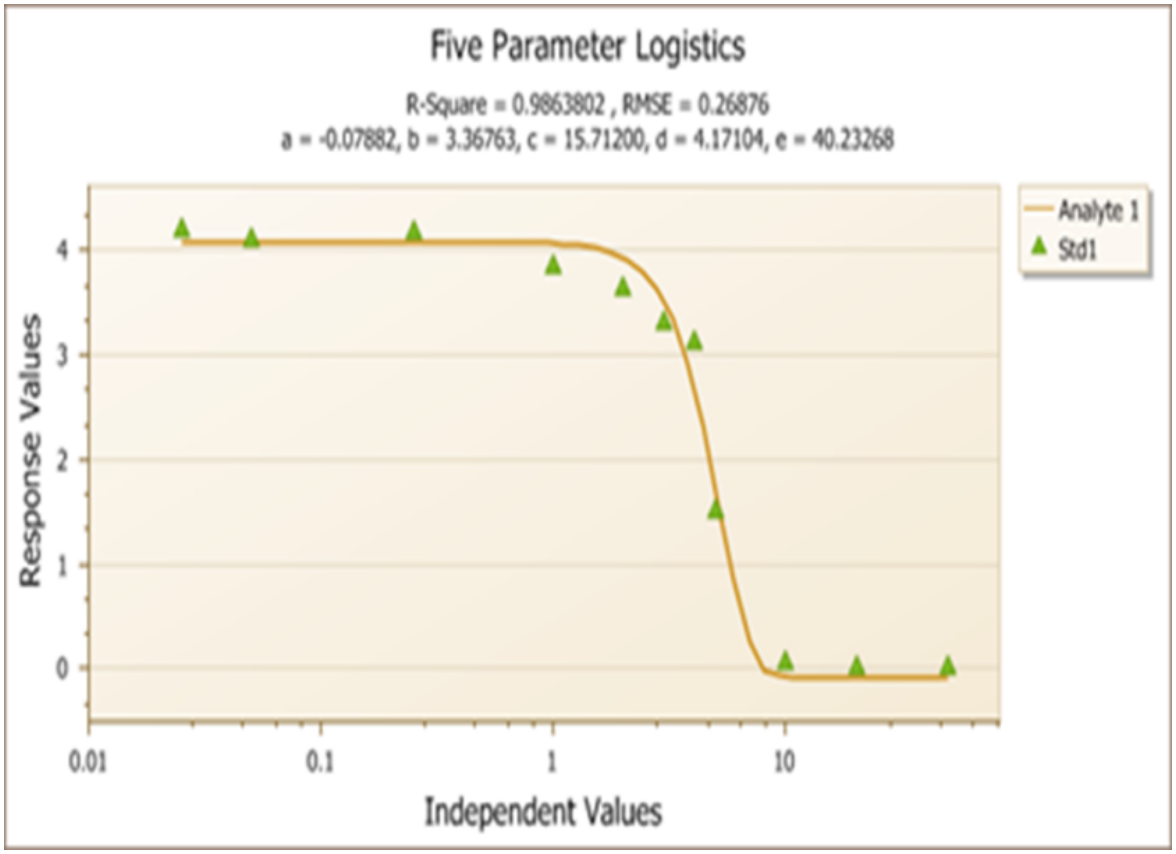

Figure 15. Example of an output from Master Plex® Reader Fit analysis software (Hitachi Solutions American, Ltd., San Francisco, CA). The half maximal inhibitory concentration (IC_{50}) and coefficient of determination (R^2) values are displayed at the top of the graph. The graph displays the average growth of AJW678 on a 96-well plate and the best curve was calculated to best fit the average.

The curve fit with the five parameter logistic was calculated as F(x) = A + (D/(1+(X/C)^B)^E), where A is the value for the minimum asymptote, B is the slope, C is the concentration at inflection point, D is the value for the maximum asymptote, and E is the asymmetry factor. The equation for the four parameter logistic was calculated as F(x) = A + (D/(1+(X/C)^B)), where the variables are the same as in the five parameter logistics equation minus E, the asymmetry factor. The IC_{50} values are calculated as x = C((2^(1/E) – 1)^(1/B)) and are given in mg/ml. The coefficient of determination (R^2) is the ratio of the explained variance to the total variance. The IC_{50}, R^2 values, and one representative piece of data from the SAS analysis are presented in Table 3.

Table 3. IC_{50}, R^2 values, and SAS analysis for the inhibitory effect of PEA on growth and biofilm amounts.

Microorganisms	Growth		Biofilm		ANOVA[4]	
	R^2	IC_{50}	R^2	IC_{50}	Growth	Biofilm
E. coli (AJW678 96 well)	0.986	4.68	0.943	1.32	1 mg/ml	1 mg/ml
E. coli (AJW678 24 well)	0.982	5.79	0.967	5.36	3 mg/ml	10 mg/ml
P. aeruginosa (24 well)	0.981	4.10	0.831	1.33	4 mg/ml	2 mg/ml
S. aureus (24 well)	0.939	13.52	NA	NA	10 mg/ml	NA

PEA was found to have IC_{50} values below 10 mg/ml at R^2 values > 0.9 for AJW678 growth and biofilm amounts when the bacteria were grown in 96-well plates and 24-well plates. The IC_{50} values are indicative of an inhibitory effect of PEA on growth and biofilm amounts of AJW678. Large R^2 values indicate that the curves fit, which gives us more confidence in the IC_{50} value. PEA also has an IC_{50} values below 10 mg/ml at high R^2 values for *P. aeruginosa* growth and biofilm amounts. For *S. aureus*, the IC_{50} value was higher than for AJW678, also at a high R^2 value. The IC_{50} value couldn't be computed for this bacterium, due to little biofilm production.

[4] Concentration at which the first statistically significant difference was observed between the means of growth or biofilm amounts at the respective PEA concentration and the means of growth or biofilm amounts at 0 mg/ml PEA.

CHAPTER 4. DISCUSSION AND FUTURE OUTLOOK

The overall goal of this thesis was to investigate biofilm removal and prevention through both physical and chemical treatments. This falls under the long term goal of the lab, which aims to prevent and treat biofilm associated bacterial infections. It is important for the future that we find safe, effective ways to treat biofilm associated bacterial infections because they occur in many natural, environmental, clinical, and food processing settings.

We looked at the physical aspect of biofilm removal and prevention in 'Chapter 1. Water Jet Experiment'. Using the automated water jet, we applied shearing forces to biofilms formed by *E. coli* K-12 isogenic mutants to measure the mechanical stability of the biofilms. The isogenic mutants used in this study had genetic deficiencies that affected the formation of the bacterial cell surface structures. Cell surface structures play a role in formation and development of bacterial biofilm, so we hypothesize alteration to the cell surface structures would have an impact on biofilm stability.

It was observed that genetic mutations that directly affected the ability of the bacteria to form flagella or fimbriae was the most detrimental to biofilm stability. The mutant that could form fimbriae weren't able to form biofilm at all. The mutant that couldn't form flagella had a 40% reduction in initial biofilm formation in comparison to the parent and when the water pressure was increased to 3 and 5 psi the biofilm had an addition 20% removal. When the genetic mutations were in metabolism that indirectly impacts the cell surface structures, there was less of an effect on biofilm stability in comparison to the mutations directly impacting fimbriae and flagella. In comparison to the parent, mutants involved in metabolism of cell surface structures formed less stable biofilms. From this experiment we learned about the importance of the bacterial cell surface structures to biofilm stability. It also shows us that these cell surface

structures could be targets for the development of drugs because they can make the biofilms less stable and easier to remove.

In ‘Chapter 2. β-phenylethylamine Experiment’ we looked at the chemical aspect of biofilm removal and prevention. The inhibitory effect of PEA on biofilms of both gram-positive and gram-negative pathogens was investigated. PEA was found to have an inhibitory effect on growth and biofilm amounts of AJW678 and *P. aeruginosa.* On *S. aureus,* it had an inhibitory effect on growth, but it took higher concentrations of PEA to decrease growth in comparison to AJW678 and *P. aeruginosa. S. aureus* was not able to form much biofilm amounts and it couldn’t be determined if PEA had an inhibitory effect. The hypothesis that PEA is transduced through FlhD/FlhC, which *S. aureus* lacks could be an explanation for why growth of *S. aureus* was seen at high concentrations of PEA. Biofilm amounts were quantified using a CV assay, which can’t be fully extracted from gram-positive bacteria due to their thick layer of peptidoglycan. In the future, other alternatives for quantification of biofilm amounts should be investigated because the variation in biofilm formation, which could be due to extra CV on the walls of the wells of the microtiter plate.

PEA was shown to impact the biofilm of AJW678 in multiple ways. Increases in the concentration of PEA resulted in AJW678 taking longer to reach stationary phase of growth. Using the water jet we also tested to see the effect PEA had on biofilm stability. The hypothesis was that decreases in biofilm stability of AJW678 would be seen when we increased the concentration of PEA. However, the biofilm appeared to be more stable as the PEA concentrations increased. The effect surface area plays on the effectiveness of PEA to inhibit biofilm formation was also investigated. An inhibitory effect on biofilm amounts on the 96-well plate were seen at 1 mg/ml of PEA, while on the 24-well plate, at the same concentration of PEA

little effect was observed. It takes until 10 mg/ml of PEA to see the inhibitory effect on biofilm amounts on the 24-well plate. Using the 24-well plate provides a greater surface area, which may be advantageous when examining differences in growth and biofilm amounts and determining IC_{50} values of treatment solutions.

In the future, our lab would like to integrate these two experiments. Previous research has developed a technique to integrate small molecules, like PEA, into different surface coatings, such as polyurethane or steel, which are commonly used in industrial settings. Using the water jet we can screen the effect other physical properties, such as smooth or rough surfaces and material type, like steel versus plastics, have on the ability of bacteria to form biofilms. Other small molecules could also be investigated using the water jet to look at the inhibitory effect on bacterial biofilms in hopes of developing biofilm preventing materials.

We are currently in the process of integrating PEA into polyurethane which are used in industrial settings were biofilms thrive. The development of materials integrated with PEA is aimed to inhibit biofilm formation in conjunction with other prevention methods used in industrial settings. Advantages to using PEA are it has GRAS status and it's considered a safe substance. Other researchers have used biofilm preventing technologies that were based on antibiotics or silver coatings [109, 131]. These strategies encourage microbial resistance and have high production costs. The development PEA containing materials is cost effective and can ultimately be used for a wide range of applications, including medicine and food processing. This is facilitated by the fact that plastics and steel materials are often the material of choice for medical devices and to coat food processing equipment. This work is significant because the development of this PEA infused coatings could be used in prosthetics and catheters which are medically often are associated with biofilm associated bacterial infections.

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