UNIVERSITY OF CALIFORNIA SAN DIEGO

Effects of Cigarette Smoke on Antibiotic Resistance and Oxidative Stress Response of *Pseudomonas aeruginosa*

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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2020

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The Thesis of Sedtavut Donald Nilaad is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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(Co-Chair)

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(Chair)

University of California San Diego

2020

DEDICATION

I dedicate this thesis to my family,

who showed me the importance of moving forward, no matter the odds.

And a special thank you to my brother and sister for helping me

become the man I am today.

EPIGRAPH

"All men are created equal, some work harder preseason."

Emmitt Smith

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Abstract of the Thesis

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by

Sedtavut Donald Nilaad

Master of Science in Biology

University of California San Diego, 2020

Professor Laura Crotty Alexander, Committee Chair

Professor James Golden, Committee Co-Chair

In the United States, cigarette smoking causes a significant portion of preventable causes of death and disease. Studies have shown that cigarette smoke can modify the human defense system, which is able to lead the cigarette smoker to have higher risk bacterial infection. However, the field of host-pathogen interactions after exposure to cigarette smoke is still poorly understood. In this study, we focused on the effects of cigarette smoke on the virulent properties of *Pseudomonas aeruginosa* (PSA), an important airway pathogen that is extremely detrimental to cystic fibrosis and immunocompromised patients. We uncovered that PSA exposed to

cigarette smoke extract (CSE) demonstrated increase antibiotic resistance to levofloxacin and gentamicin. In our current paper under review, we have demonstrated that PSA exposed to CSE (CSE-PSA) showed increase resistance to oxidative burst by neutrophil and hydrogen peroxide treatment. To examine the underlying mechanism of these increases in virulence, RT-qPCR was used on control- and CSE-PSA to observe changes in gene expression. Genes that encode parts and regulation of PSA efflux pumps (*mexA*, *mexX*, *mexZ*) showed increased gene expression in CSE-PSA over control-PSA. Of three oxidative stress response genes (*gpx*, *oxyR*, *tpx*), only *tpx,* a gene encoding a thiol peroxidase homolog, demonstrated statistical significance. However, all three genes showed a trend of overall increased expression, suggesting that cigarette smoke is inducing changes in PSA gene expression. We conclude that cigarette smoke increases virulence in PSA virulence by increasing gene expression involved in antibiotic resistance and oxidative stress.

Introduction

Although progress has been made on combating cigarette smoke epidemic, smoking continues to be the leading cause of preventable death causing more than 480,000 deaths annually in the United States[1]. Smoking can lead to a wide range of diseases ranging from respiratory diseases such as chronic obstructive pulmonary disease[2] (COPD) to various cancers[1], making tobacco smoking a prominent driver of chronic illnesses worldwide[1].

The Effects of Cigarette Smoking on Human Health

Cigarette smoking alters the human's ability to clear mucus out of the airways, rendering the individual to become more susceptible to infections[3]. The exact processes underlying the increased susceptibility are not completely understood, however many studies have shown changes in the respiratory tract structure and immune response when exposed to both direct and secondhand cigarette smoke[4].

The respiratory tract structure undergoes changes when stimulated by cigarette smoke due to the wide array of constituents in cigarette smoke, such as [3]. The mixture of particulate matter is dispersed throughout the respiratory tract, with larger particles resting on membranous tissue of the larger, upper airways; and smaller particles infiltrating the alveolar spaces[3]. While these constituents, which include many reactive oxygen species (ROS), rest on the tissue lining the airways, they can trigger cellular responses altering the tissue functions[4]. Examples of changes can be seen in forms of reduced mucociliary clearance, mucus hypersecretion, loss of lung elasticity, damages to epithelial junctions, or even cell death[2,5,7]. These structural altercations are consequential to host immunity, as they are involved in airway pathogen clearance and provide a higher risk of bacterial colonization[6].

Cigarette smoke changes the dynamic of the host innate and adaptive immune system[6,8]. Cigarette smoke induces release of specific chemokines, which can lead to an increased count of neutrophils, a type of highly abundant granulocyte, and other inflammatory cells recruited in the bronchioles[6]. Neutrophils are one of the first responders during early stages of infection and plays a major role in the innate immune system[9]. However, when exposed to cigarette smoke, neutrophils demonstrate reduced antimicrobial defense functions, compromising the integrity of the innate immune system[10,11]. Although there is increased neutrophil recruitment to the respiratory tract epithelium, the neutrophils exposed to cigarette smoke has decreased effectiveness clearing a bacterial infection. This combination of ineffective neutrophil activity and altered respiratory tract structure leads to chronic inflammation that can further exacerbate bacterial infections[10].

The Effects of Cigarette Smoke on Bacterial Pathogenicity

Cigarette smoking have previously been shown to increase an individual's susceptibility to bacterial infections and tend to lead to more severe respiratory infection presentations[3]. This phenomenon has been studied in-depth, revealing the major structural changes in the smoker as mentioned earlier. Other studies have also shown that direct and second-hand smoke exposure changes the normal composition of nasopharyngeal microflora, suggesting that cigarette smoke exposure may change major gene expression within microbial species[2,5].

Our laboratory has shown that methicillin-resistant *Staphylococcus aureus* (MRSA) demonstrated increased resistance to human antimicrobial peptide LL-37, increased hydrophobicity to bind to epithelial cells, and virulence in mice models when subjected to cigarette smoke[12]. Changes in bacterial adherence has also been seen in other bacterial models,

such as *Bordetella pertussis*, *Neisseria meningitides*, and *Streptococcus pneumonia*; revealing that these bacteria show higher binding to epithelial cells in smokers compared to non-smokers[13]. These previous studies provide basic understanding that cigarette smoke is able to change an opportunistic pathogen's virulence.

Another relevant opportunistic pathogen is the Gram-negative bacterium, *Pseudomonas aeruginosa* (PSA). This relevant pathogen has proven to be extremely detrimental and even fatal to immunocompromised and cystic fibrosis patients[12]. An even growing concern is its innate resistance to many antibiotics and its rapid acquisition of further resistance mechanisms over the years[14]. Although there have been studies of the effects of cigarette smoking on potential opportunistic pathogens that may reside within the nasopharynx, the field overall is still lacking in literature[2]. To further explore the relationship between cigarette smoke and changes to this opportunistic pathogen, we present this study. This thesis is a continuation of a paper being reviewed, confirming the design of an experiment and defining the effects of cigarette smoke on PSA virulence.

Results

Residual cigarette smoke extract has no effect on neutrophil death rate

Neutrophils, a key component of the innate immune system, serve as first responders to defend against various microorganisms foreign to the body[6,9]. In a paper by our laboratory under review, Chein et al. demonstrated that cigarette smoke exposure significantly enhances PSA survival against neutrophils. While this result seems interesting, cigarette smoke has been shown to disrupt functions of neutrophils, monocytes, macrophages, and dendritic cells in previous studies[15,16]. To check if the neutrophils may have been hindered due to the presence of residual cigarette smoke extract (CSE), neutrophils activated by phorbol myristate acetate (PMA) were incubated in the control-PSA and CSE-PSA supernatants (excluding the PSA for each group) after washes and measured for cell viability using propidium iodide at selected time points. If the CSE did have influence on neutrophil death rate, then this evidence would serve as a confounding variable to our earlier result of increase CSE-PSA survival rate when treated with neutrophils.

After incubating the neutrophils with the CSE-PSA supernatant, neutrophils were stained with proprium iodide and underwent flow cytometry to measure neutrophil death over time. Timepoints were taken at 0 minutes, 30 minutes, and 60 minutes after the addition of the control- and CSE-PSA supernatants to the neutrophils. All timepoints demonstrated no difference in neutrophil death rate between the two groups (Figure 1). This confirms that the increase survival of PSA after cigarette smoke exposure is not due to the less live neutrophil present in the treatment over time, but rather that the CSE-PSA is more resistant to neutrophil killing compared to the control.

Cigarette smoke exposure increases PSA resistance to levofloxacin and gentamicin

Chein et al. showed that cigarette smoke exposure led to a dose-dependent inhibition of PSA growth rate. We hypothesized that because the cigarette smoke acted as a stressor on the PSA, it is possible that this stress may trigger phenotypic changes as a result of PSA compensating the stress as has been shown in other bacteria exposed to smoke[18]. Two antibiotics were selected to access CSE-PSA antibiotic tolerance. Levofloxacin, a quinolone inhibits bacterial topoisomerase IV and DNA gyrase. Gentamicin, an aminoglycoside inhibits bacterial protein synthesis though binding of the 30S ribosome. Control-PSA and CSE-PSA were treated at various concentration of levofloxacin and gentamicin for 24 hrs. The results showed that CSE-PSA had a significant increase in minimum inhibitory concentration (MIC) for levofloxacin by one-fold compared to control PSA (Table 1). CSE-PSA treated with gentamicin had a half-fold increase in MIC compared to control PSA, however this result was not statistically different (Table 1).

RT-qPCR reveals increased expression efflux pumps and oxidative stress response genes after cigarette exposure

To further confirm the changes in PSA due to CSE, RT-qPCR was used to characterize gene expression levels involved in oxidative stress response or antibiotic resistance at mid-log phase of control-PSA and CSE-PSA growth. To examine for gene expression change involved in oxidative stress responses, *gpx*, *tpx*, and *oxyR* primers were used to observe the expression of PSA glutathione peroxidase[25], thiol peroxidase[23], and a transcriptional activator[24], respectively

through RT-qPCR. Glutathione peroxidase and thiol peroxidase both reduces hydrogen peroxides and hydroperoxides to water and alcohols[25]. RT-qPCR showed that CSE-PSA has a significant increase in *gpx* expression compared to the control-PSA (Figure 2). CSE-PSA also had increased expression in *oxyR* and *tpx*, however the changes were not statistically significant (Figure 2).

The genes *mexA*, *mexX*, and *mexZ* were selected to measure the expression of the multidrug efflux pump complexes, mexAB-OprM[26], mexXY-OprM[27], and a mexXY-OprM repressor, mexZ[27]. These genes have been shown to be involved in antibiotic resistance mechanisms for different antibiotics by promoted efflux of antibiotics out of the bacterial cell[26,27]. All genes showed significant increases in the CSE-PSA compared to the control-PSA group (Figure 3). *rspL,* a gene encoding 30S ribosomal S12 in PSA[27], was used as a housekeeping gene to analyze relative abundance.

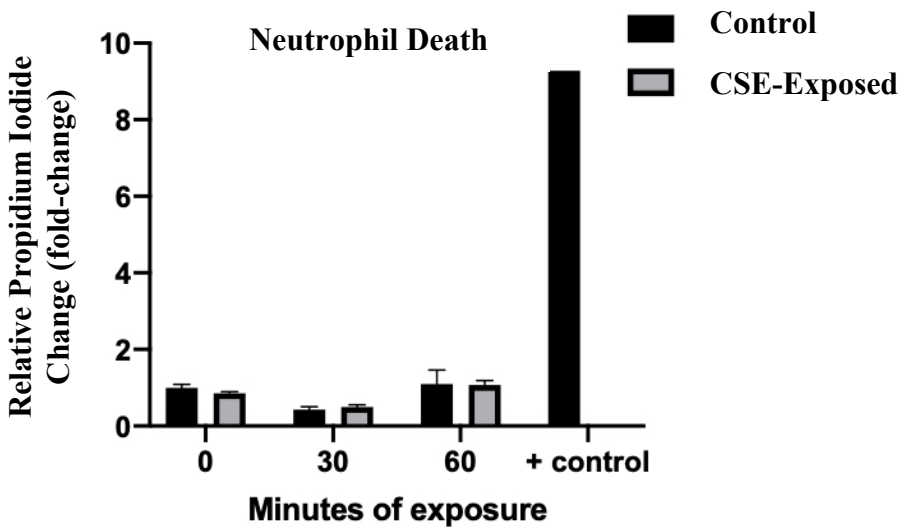

Figure 1: **Neutrophil Death when incubated in Control- and CSE-PSA supernatant showed no difference at all time points.**
Neutrophils were incubated in control-PSA supernatant ("control neutrophils") or CSE-PSA supernatant ("CSE-exposed neutrophils") after being activated with phorbol myristate acetate (PMA) and measured for cell viability using propidium iodide staining and flow cytometry. Time points 0 minutes, 30 minutes, and 60 minutes were taken, and cell unviability was normalized to the control neutrophils. Heat-killed neutrophils were used as a positive control. There were no observable differences between control neutrophils and CSE-exposed neutrophils.

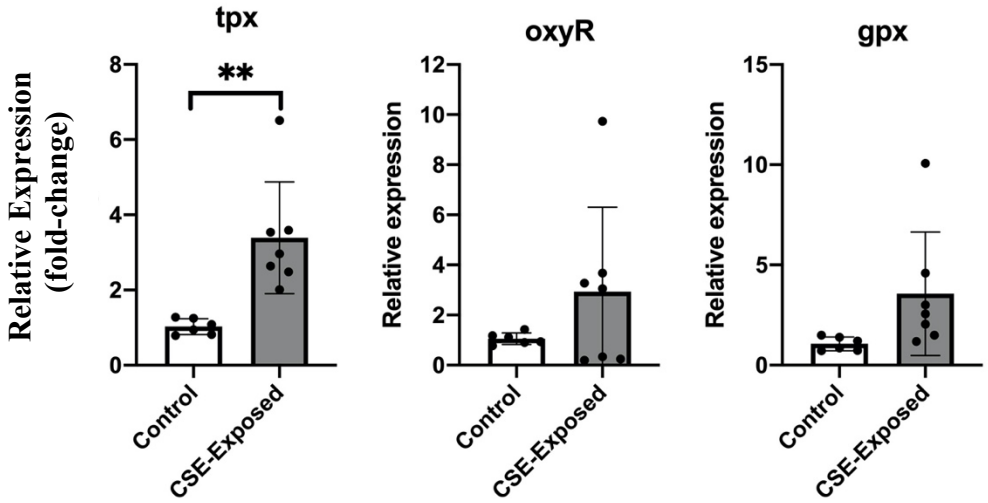

Figure 2: **Influence of CSE on PSA oxidative stress gene expression: *tpx, oxyR*, *gpx.***
tpx, *oxyR*, and *gpx* genes encodes thiol peroxidase, a transcriptional activator, and glutathione peroxidase, respectively. Fold-change was normalized to *rspL* from control PSA. Only *tpx* displayed a significant difference.

Table 1: **CSE-PSA shows increased MIC against both levofloxacin and gentamicin, but only levofloxacin was statistically significant.**
Control- and CSE-PSA were grown to mid-long then treated with a different concentration of levofloxacin or gentamicin for 24 hours. The results showed CSE-PSA on average had an increased MIC for both levofloxacin and gentamicin, but only levofloxacin showed a statistically significant difference ($p < 0.05$).

	Average Minimum Inhibitory Concentration (μg/mL)	
	Levofloxacin (*)	**Gentamicin**
Control-PSA	2.8	1.3
CSE-PSA	3.2	1.8

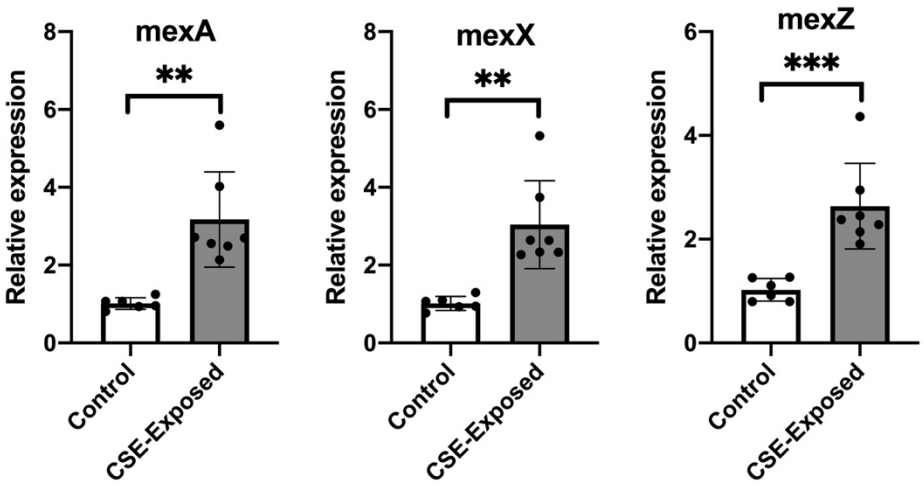

Figure 3: **Influence of CSE on PSA efflux pump expression: *mexA, mexX, mexZ***
mexA and *mexX* encodes components for the MexAB-OprM and MexXY efflux pumps respectively. *mexZ* encodes a MexXY operon repressor. Fold-change was normalized to *rspL* from control PSA. All three genes displayed statistically significant increases in the CSE-PSA.

Table 2: **Primer Sequences for RT-qPCR**

Primer	Oligonucleotide sequence	
tpx 5'	5'-GAAGGATCAACGCAATGG-3'	[23]
tpx 3'	5'-ACCACGGTGTTGGCCAGC-3'	
oxyR 5'	5'-CTCACCGAACTGCGCTACA-3'	[24]
oxyR 3'	5-CGAGTCGGCCAGCACTT-3'	
gpx 5'	5'-TGCGGCTTACCCCGCAGTA-3'	[25]
gpx 3'	5'-ACTTGGTGAAGTTCCACTT-3'	
mexA 5'	5'-ACCTACGAGGCCGACTACCAGA-3'	[26]
mexA 3'	5'-GTTGGTCACCAGGGCGCCTTC-3'	
mexZ 5'	5'-GCATGGGCTTTCTCCGCCAGTGC-3'	[27]
mexZ 3'	5'-GCGTCCGCCAGCAACAGGTAGGG-3'	
mexX 5'	5' -TGAAGGCGGCCCTGGACATCAGC - 3'	[27]
mexX 3'	5'-GATCTGCTCGACGCGGGTCAGCG-3'	
rspL 5'	5'- GCAAGCGCATGGTCGACAAGA - 3'	[27]
rspL 3'	5'- CGCTGTGCTCTTGCAGGTTGTGA -3'	

Discussion

This study shows that cigarette smoke increases resistance to neutrophil killing and antibiotic treatment. Our laboratory has shown that CSE-PSA has increased survival rate when treated with neutrophils activated by PMA. Cigarette smoke has been known to hinder neutrophil effectiveness[16], so a follow-up experiment was conducted to observe if any residual CSE were harming neutrophil function, thus skewing the observation that PSA had higher survival rate when subjected to neutrophil treatment. Figure 1 demonstrates that neutrophil death rates were not different when neutrophils were incubated in control-supernatant or CSE-PSA supernatant, therefore we strengthen our hypothesis that CSE is directly increasing PSA resistance to neutrophil killing. This is a significant observation, as the combination of increased PSA resistance to activated neutrophils and the decrease neutrophil antibacterial killing suggests that the innate immune system of a cigarette smoker may have more difficulty in handling a PSA infection[15,16].

Our lab has also observed increase H_2O_2 and neutrophil extracellular traps (NETs) resistance post exposure to CSE, two mechanisms neutrophils use to kill bacteria[17]. We selected three genes known to be involved with in oxidative stress responses, *gpx*, *tpx*, and *oxyR* encoding PSA glutathione peroxidase[25], thiol peroxidase[23], and a transcriptional activator[24], respectively. While only *gpx* showed a difference in RNA expression, both *tpx* and *oxyR* displayed an average increase in RNA expression of our selected genes in the CSE-PSA group (Figure 2). These results may explain the resistance to neutrophil killing by oxidative burst observed in our paper under review[17].

Antibiotics have commonly been used to treat PSA infections, however PSA has an arsenal of tools to combat both the immune system and treatment, such as enhancing antibiotic resistance[19]. The two antibiotics selected, levofloxacin and gentamicin, have demonstrated effectiveness in treating PSA infections[20] and were used to examine if PSA would demonstrate increased antibiotic resistance after CSE exposure. The results showed that levofloxacin had significant MIC increase ($p < 0.05$) while gentamicin had a higher MIC average but did not have significance (Table 1). To uncover the mechanism behind the increase MIC, we selected genes involved in antibiotic resistance, namely genes involved in regulation and synthesis of PSA efflux pumps. PSA has been known to use multiple efflux pumps to excrete out antibiotics from the cell, hindering the antibiotic effectiveness[21]. We hypothesize that if there is an increased gene expression in an efflux pump involved with antibiotic resistance due to exposure to cigarette smoke, then the increase expression of efflux pump may contribute to the increased MIC.

The genes, *mexA* and *mexX* were selected because these genes encode components of the MexAB-OprM[26] and MexXY[27] pumps, respectively; both known to contribute the increase tolerance to antibiotics levofloxacin or gentamicin[21]. Using RT-qPCR, we observed an increase expression of in both of these genes in the CSE-PSA compared to the control PSA, which may contribute to the increase MIC. *mexZ*, a gene encoding a repressor that regulates the expression of MexXY-OprM[19,21,22,27], was used to examine another gene involved in antibiotic resistance. We expected that since there were increased MICs for both antibiotics after CSE exposure, then there should be a decrease in *mexZ* expression due to its repressive nature. Interestingly, *mexZ* showed the change with most significance in expression after exposure to cigarette smoke. One explanation can be drawn from a study by Hay, et al., where they discover an anti-repressor, ArmZ, that is inducible in the presence of the antibiotic, spectinomycin[19]. This anti-repressor

modulates MexZ and its functions, allowing *mexX* to be expressed at high levels in presence of spectinomycin[19]. It may be possible that CSE is inhibiting mexZ function, therefore hindering its ability to repress *mexX* function properly. While this may be an interesting consideration, more research may be conducted to examine the underlying reason why both *mexX* and *mexZ* are high in expression due to CSE as this relation between cigarette smoke and PSA gene expression has not been extensively researched.

During this study, we demonstrated that PSA is affected by cigarette smoking by showing increased antibiotic resistance as well as increased gene expression in oxidative stress and antibiotic resistance. In addition, we confirmed that CSE-PSA supernatant has no influence on neutrophil death.

Materials and Methods

CSE preparation

CSE preparation was based on the most accepted methods of cigarette smoke extract preparation. 10 mL of Luria-Bertani broth or Muller-Hinton broth depending on the assay was drawn into a 60 mL syringe with a sterile needle. A 3R4F research cigarette from the University of Kentucky (now referenced as "1R6F" research cigarette) was attached to a tubing with its filter removed. The syringe was attached to the other end of the tubing via a 3-way stopcock and the cigarette was lit and smoke was drawn into the syringe until full. The syringe filled with smoke was shaken for approximately 15 seconds, infusing the smoke into the medium. After infusion, the smoke was released through the stopcock and this procedure was repeated until less than 1 cm of the cigarette remained. The smoked media was filtered using a 0.2 μm syringe filter tip (now referred as "cigarette smoke extract", or "CSE") and is referred to as 100% CSE. CSE was made fresh before each incubation of PSA for each assay.

Preparing control- and Cigarette-exposed PSA cultures

To create the 75% CSE media, CSE was diluted 3:4 in the selected media for each assay. Subcultures of control- and CSE-PSA were made by inoculating 8 mL of control media and 8 mL of 75% CSE with 1:20 and 1:40 dilutions (controls) and 1:10 dilution (75% CSE) of the overnight PSA-PAO1 culture inoculated the night before. The inoculated tubes were incubated at 37 °C while shaking until mid-log growth phase (OD_{600} 1.2 – 1.4) was reached. The 1:20 and 1:40 control-PSA subcultures with the closest absorbance to the CSE-PSA subculture with the closest absorbances were selected for the assays.

Assessment of Residual Cigarette Smoke Extract Cytopathic Effects on Neutrophils

CSE was prepared as described using mammalian-based medium (MBM) (RPMI + 10% FBS + 20% LB). Subcultures of control and CSE-PSA were created by inoculating 10 mL of control MBM (1:20 and 1:100 dilution of overnight PSA) and 10 mL of 75% CSE-MBM (1:10 dilution of overnight PSA). FBS and LB were included as bacterial growth is stunted in absence of these carbon sources. Tubes were incubated at 37°C shaking until mid-log (absorbance 1.2 – 1.4) growth was reached. Tubes with the closest absorbance between the control-PSA and CSE-PSA subculture were selected and washed twice with PBS in 50 mL conical tubes and spun at 3200 rpm for 8 minutes. Supernatants were discarded, and each pellet was resuspended in 300 µL of PBS to create a PSA "slurry". Two glass tubes were filled with 3mL of PBS and the both slurries were added until OD_{600} = 0.7 was reached in each tube to create diluted slurries. The diluted slurries were diluted in MBM to obtain 5 x 10^6 colony forming units (CFU)/mL, centrifuged at 1,600 rpm for 5 minutes, and resuspended in fresh MBM at 5 x 10^5 CFU/mL to give a multiplicity of infection (MOI) of 0.1. These suspensions were centrifuged at 3,200 rpm for 8 minutes to pellet the control-PSA and CSE-PSA, and 10 mL of each supernatant were collected in 15 mL tubes. The supernatants were stored in a -80°C freezer for future assessment.

Under approval from the UCSD IRB, 25 mL of venous blood was collected from healthy donors using a 30 mL heparinized syringe, Blood was transferred to a 50 mL conical tube and layered on top with 20 mL of Polymorphprep™, taking care not to disturb the interface between the two liquids. The blood was centrifuged at 1600 rpm for 35 minutes at room temperature (22°C) with no brake. The plasma and upper mononuclear cell layer were aspirated and the PMNs were transferred to a fresh 50 mL conical tube and rinsed with 50 mL PBS for washing at 1,600 rpm for 10 minutes. The supernatant was discarded, and 5 mL of molecular grade water

was added and mixed via pipetting for 30 seconds to lyse residual red blood cells. Cells were rinsed again with 50 mL of PBS and centrifuged at 1,600 rpm for 10 minutes. The pellet was resuspended in 1 mL of PBS and enumerated using hemocytometry.

Cells were prepared at a concentration of 5 x 10^5 cells/mL using PBS and 166.7 µL was added to 2 mL siliconized tubes. Phorbol myristate acetate (PMA) was prepared at 50 nM (PMA stock was prepared at 16 µM in DMSO) and added to each tube for PMN activation with a final concentration of 25 nM. Siliconized tubes were incubated at 37°C for 20 minutes.

After incubation, 166.7 µL of the frozen supernatant was thawed to room temperature was pipetted into their respective tubes. The supernatant being added indicates time point "0". Cell death was accessed by flow cytometry using proprium iodide stain. Assessment of cell death were taken at time points "0", "30", and "60" in triplicates of control-supernatant-added and CSE-PSA supernatant-added PMN tubes. MBM not exposed to PSA added to activated PMN were used as negative controls. For positive controls, activated PMNs were heat-killed in a hot water bath at 55°C and time points were taken at 5 minutes, 10 minutes, and 15 minutes.

Minimum Inhibitory Concentration Assay

PSA was grown overnight in MHB. On the day of the assay, CSE was made to use to incubate CSE-PSA subcultures. Overnight PSA were diluted 1:20 or 1:100 for control-PSA subcultures and 1:10 for CSE-PSA subcultures (1:10 dilutions were used to compensate for the CSE-induced stunted growth of PSA). The subcultures were incubated in a 37°C incubator shaking at 225 rpm until mid-log growth was reached (absorbance OD_{600} 1.2 – 1.4). Subcultures were washed with 1X PBS and centrifuged at 3200 rpm for 10 minutes twice, then resuspended in 300µL of RPMI+5% MHB now termed "bacterial slurry". The control-PSA and CSE-PSA

slurries were added to glass tubes with 1.5 mL RPMI+5% MHB until the absorbance reached OD_{600} 0.400. The OD_{600} 0.400 control-PSA and CSE-PSA were diluted 1:50 and plated into a flat-bottom 96 well plate at concentrations of the antibiotic used (0 ng/µL, 1 ng/µL, 2 ng/µL, 4 ng/µL, 8 ng/µL, 16 ng/µL, 32 ng/µL, 64 ng/µL). Plates were incubated in a 37°C shaking at 170 rpm for 24 hours cells from and each well was plated onto agar plates to determine minimum inhibitory concentration and minimum bactericidal concentration. Plates were incubated overnight and were checked the next day for PSA survival.

RT-PCR of PSA genes

Control-PSA and CSE-PSA were grown to mid-log phase (absorbance OD_{600} 1.2 – 1.4) in Luria Broth. The two groups were washed with PBS and centrifuged at 3200 rpm for 8 minutes twice. The bacteria were then resuspended in PBS to an OD_{600} 0.800 then diluted 1:4 in PBS. RNA extraction was conducted by using Qiagen RNeasy Protect Bacteria Mini Kit following the kit protocol. The RNA extracts were frozen in a -80 °C freezer until use.

Biorad iTaq Universal One-Step RT-qPCR kit and protocol was used to prepare RNA for RT-qPCR. Applied Biosystems' StepOnePlus Real Time System was used to run RT-qPCR following guidelines from the RT-qPCR kit. Primers were ordered with the sequences in Table 2. Each RNA samples were diluted to equal concentration before undergoing RT-qPCR. Gene expression was normalized to expression of housekeeping gene, *rspL*.

Statistical analysis

All experimental data shown were done with at least three biological replicate experiments, each of which was conducted in duplicate or triplicate technical replicates. All statistical analysis and graphs were made using Prism.

Material presented in this master's thesis contains information currently being prepared for submission for publication. Chien, Jason; Hwang, John H; Nilaad, Sedtavut; Masso-Silva, Jorge; Ahn, Marina; McEachern, Elisa K; Moshensky, Alexander; Byun, Min-Kwang; and Crotty Alexander, Laura E. The thesis author was the contributing author of the paper.

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