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Elucidating the Effects of Antibiotics on the Release of Pal from Escherichia coli

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Elucidating the Effects of Antibiotics on the Release of Pal from *Escherichia coli*

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CERTIFICATE OF APPROVAL

M.S. DEGREE THESIS

The M.S. Degree Thesis of Leslie Gallardo
has been examined and approved by the thesis
committee as satisfactory for the thesis required for
the M.S. degree in Chemistry.

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Abstract

Sepsis, a life-threatening clinical condition affecting more than 1.5 million Americans per year, is defined as an over-exuberant immune response to infection. Currently, sepsis is the leading cause of death in U.S. hospitals, and the incidence of sepsis caused by Gram-negative bacteria, such as *Escherichia coli (E. coli)*, has been steadily increasing since the late 1990's. While the detailed mechanism of sepsis is not fully understood, several bacterial components are thought to contribute to the *hyper-inflammatory response* in humans, including lipopolysaccharide endotoxin and several other lipoproteins. Past studies suggest that one of those *E. coli* lipoproteins, peptidoglycan-associated lipoprotein (Pal), might play a significant role in the pathogenesis of sepsis. A patient diagnosed with sepsis will typically be treated with antibiotics. However, the effects of antibiotics on the release of Pal from *E. coli* are currently unknown. This work describes our efforts to elucidate the role of antibiotics in Pal release from *E. coli* using protein detection methods and a mouse model of sepsis. Our preliminary results suggest that β-lactam antibiotics have a significantly higher impact on Pal release compared to other classes of antibiotics.

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Abbreviations

GNS	Gram-negative sepsis
SIRS	systematic immune response Syndrome
E. coli	*Escherichia coli*
LPS	lipopolysaccharide
OMPs	outer membrane proteins
Pal	peptidoglycan associated lipoprotein
Lpp	Braun's lipoprotein or murein lipoprotein
OmpA	outer membrane protein A
CLP	cecal ligation and puncture
TNFs	tumor necrosis factors
IL-6	interleukin-6
IL-1β	interleukin-1β
PBPs	penicillin-binding proteins
OMVs	outer membrane vesicles
LB	Lysogeny broth
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
SDS – PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
APS	ammonium persulfate
TEMED	tetramethylethylenediamine
V	volts
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween-20
PBS	phosphate buffered saline
TEM	transmission electron microscopy
CFU	colony forming units
IP	intraperitoneal
Amp	ampicillin
Gent	gentamicin
Levo	levofloxacin

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1. Introduction

Sepsis Statistics

Sepsis is a condition in which the body exhibits an over-exuberant inflammatory response to a bacterial, viral, or fungal infection.[1] The exaggerated, systemic inflammatory response results in damage to tissues and sometimes organ failure and death.[1] ~1.7 million Americans are affected by sepsis or sepsis-related conditions each year, and an estimated 15-30% of people who get sepsis will die from it.[2] Currently, sepsis is the most common cause of death in U.S. hospitals, and treatments for septic patients are estimated at over $24 billion dollars per year.[2,3] Sepsis also affects more than 30 million people worldwide each year, including 3 million newborns and 1.2 million children.[4] Sepsis can result from infections acquired in the community or in many cases, from the hospital or other health care facilities. Elderly patients and people who suffer from chronic diseases or immune deficiencies are especially susceptible to sepsis. Sepsis can be especially serious and lead to a greater risk of mortality when the initial infection is caused by an antibiotic-resistant strain of bacteria. Two types of Gram-negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli* (*E. coli*), are currently the leading causes of Gram-negative sepsis in the US; approximately 40% of bacterial sepsis cases are caused by Gram-negative bacteria.[3]

Sepsis Diagnostics and Treatments

According to the Centers for Disease Control and Prevention, symptoms of sepsis can be recognized by the acronym S.E.P.S.I.S., where each letter corresponds to a common symptom-

shivering, extreme pain, pale skin, sleepiness, “I feel like I might die,” and shortness of breath. However, these symptoms overlap with common symptoms from other diseases, and thus are not reliable for a definitive diagnosis of sepsis. Sepsis diagnosis is challenging, due in large part to a lack of definitive and effective diagnostic tools.

Patients often experience several clinical stages of sepsis. The first stage is systematic immune response syndrome (SIRS), where the patient’s body temperature is greater than or equal to 38 °C or less than 36 °C, with a heart rate of less than 90 min^{-1}, a respiration of less than 20 min^{-1}, and a white blood cell count less than 12.0 x 10^9 L^{-1} or greater than 4.0 x 10^9 L^{-1}.[5] When a patient meets two of the above criteria, then the patient is diagnosed with SIRS.[5] If the SIRS patient’s condition worsens or if there is an identified infection, then the patient is diagnosed with sepsis.[5] The final stage of sepsis is septic shock. Septic shock occurs when the body responds to the exaggerated inflammation, which can lead to organ failure and arterial hypotension.[6] When a patient reaches the stage of septic shock, it is often very difficult for the patient to recover and unfortunately, in many cases, leads to death.

Currently in the US, most sepsis patients are treated with antibiotics, kidney dialysis, and corticosteroids.[7] However, these treatments often do not help to stop the inflammation, but instead help to suppress/remedy the symptoms of sepsis.[8] In many cases, the course of action is to get the infection under control, while waiting for the patient’s body to self-correct the hyper-inflammation.

Antibiotic treatment within the first 6 hours of diagnosis is typically crucial for a positive patient outcome.[8] In most cases, the patient is initially prescribed broad spectrum antibiotics, while samples are collected to identify the specific type of bacterial infection, which can take

several hours to overnight.[9] The bacteria can also be analyzed for their susceptibility to drugs/antibiotics, and blood cell counts are quantified to monitor the infection.[10] Sometimes, the blood is also checked for the presence of bacterial endotoxins, which are typically not found in the blood of healthy humans.[11] Procalcitonin levels may also be monitored, since these protein levels are known to increase with increased bacterial loads.[11] Currently, one of the biggest challenges to sepsis treatment is the emergence of antibiotic-resistant bacteria.[12] According to the CDC, at least 2 million people are infected with antibiotic-resistant bacteria each year, and ~20,000 people die from the resistant infection.[12]

In addition to antibiotics, there are a few new and developing treatments for sepsis. For example, fenofibrates are triglyceride-reducing agents that act as an antioxidant and anti-inflammatory medication; these agents have been shown to reduce the sepsis mortality rate by 12.5%.[13] However, this is still a developing treatment and more clinical studies need to be done to ensure there are no negative side effects. Other than the treatments described here, sepsis patients are mostly provided with fluids, vasoconstrictors, and other supportive care to reduce the symptoms and side effects of sepsis while waiting for the patient's body to re-stabilize and correct the hyper-inflammation without going into septic shock.

Toxic Components of Gram-Negative Bacteria

Gram-negative bacteria contain two lipid bilayer membranes: the outer and inner (cytoplasmic) membranes.[14,15] Between the two membranes is the periplasmic space, which contains peptidoglycan and other periplasmic components (Figure 1). The outer leaflet of the outer membrane consists mostly of lipopolysaccharide (LPS), also known as endotoxin, while, the

inner leaflet of the outer membrane and the inner membrane largely consist of phospholipids.[16] The Gram-negative envelope performs several important biological functions for the bacterium, such as nutrient acquisition, adherence, secretion, signaling, and protection from the environment.[16]

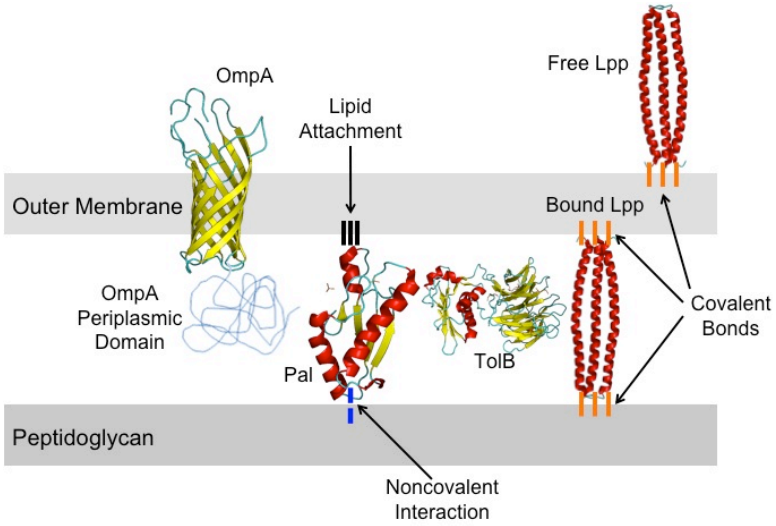

Figure 1. A schematic of a few of the components of the outer membrane, periplasmic space, and peptidoglycan layer in Gram-negative bacteria. The outer leaflet of the outer membrane consists mostly of LPS; OmpA, Pal, and Lpp are other bacterial components that are proposed to play important roles in Gram-negative sepsis.

The structure of LPS consists of three parts: Lipid A, core oligosaccharide, and O-antigen (Figure 2).[17] Lipid A is contained in the inner most layer of LPS and is thought to be the most toxic component of LPS, contributing to inflammation in sepsis patients.[18]

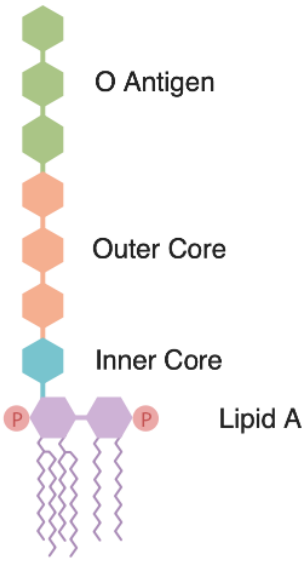

Figure 2. LPS, located in the outer leaflet of the outer membrane, is known as a bacterial endotoxin and contains lipid and sugar structural components. Lipid A is thought to be the most toxic component of LPS, inducing inflammation when exposed to human cells. (Created with BioRender)

Although the mechanism of Gram-negative sepsis (GNS) is not well understood, a few components of Gram-negative bacteria are thought to play the most important roles in inducing the over-exuberant inflammation that is characteristic of sepsis. One of those components is LPS, which, under certain conditions, is released from the Gram-negative bacteria into the bloodstream. The released LPS toxin then leads to a chain of reactions.

First, LPS interacts with bacterial immune cells, causing the activation of pro-inflammatory cytokines and endothelial activation and dysfunction.[19] More specifically, LPS causes an inflammatory response by activating the coagulation system and inhibiting the anticoagulant system and fibrinolysis.[19] By inhibiting the anticoagulant system, LPS prevents the mediation of the release of proinflammatory cytokines, thus allowing for excess release of proinflammatory cytokines into the bloodstream.[19] The body reacts by releasing anti-inflammatory cytokines; however, the continued release of proinflammatory cytokines

overwhelms the anti-inflammatory cytokines.[3] A tug-of-war-like effect occurs when the body tries to stop the inflammation, but as a result, it overexerts itself, leading to hyper-inflammation and a sudden decrease in blood pressure, tissue damage, and organ failure.[3] In addition, the activation and dysfunction of endothelial cells caused by LPS and other bacterial toxins further enhances inflammation in the body. The endothelial cells respond with structural changes like cytoplasmic swelling and functional changes like expression of adhesion molecules.[19] The endothelium typically functions to prevent coagulation, but during an infection, it undergoes physiological changes that trigger the coagulation cascade.[19] *In vitro* and *in vivo* studies have shown that LPS induces endothelial apoptosis, which causes the apoptotic endothelial cells to circulate in the body, which is one of the symptoms of septic shock .[19–21]

Since many studies have showed that bacterial LPS contributes to sepsis-related inflammation, it was proposed that GNS immunotherapy should be targeted against the LPS endotoxin.[22] In one study, scientists attempted to "neutralize" LPS using antibodies produced in rabbits, which were inoculated with a genetically altered strain of *E. coli*, containing no O-antigen and only the core elements of LPS (including Lipid A).[23] Known as the J5 mutant strain of *E. coli*, Ziegler and coworkers assumed J5 antisera would contain a large amount of antibodies against the toxic Lipid A component of LPS. The J5 antiserum was used in a clinical study to treat sepsis patients, who were administered either J5 antiserum or a negative control serum. Patients injected with the J5 antiserum had an increased survival rate compared to the negative control patients.[23] Specifically, J5 antisera patients had a mortality rate of 44%, while the negative control group mortality rate was 77%. The statistically significant differences in mortality between the two groups suggested that something within the J5 antisera was

protective and/or therapeutic in these sepsis patients. Ziegler *et al*. proposed that anti-LPS antibodies were the major protective component within the antisera.

In a more recent study, whole *E.coli* bacteria and purified LPS were incubated with the J5 antisera; authors of this study were unable to identify any anti-LPS antibodies in the sera.[24] However, the authors did identify antibodies to three other *E. coli* outer membrane proteins (OMPs): outer membrane protein A (OmpA), Braun's lipoprotein or murein lipoprotein (Lpp), and peptidoglycan associated lipoprotein (Pal).[24] OmpA, a 35-kDa transmembrane protein contained in the outer membrane of *E. coli*, is thought to play a role in maintaining the structural integrity of the bacterial cell and also undergoes a dramatic conformational change that creates a large porin in the membrane.[14,25–27] Lpp is a small, ubiquitous 9kDa protein, which has been shown to be tethered to the OM via a lipid moiety and to also covalently bond to the peptidoglycan layer of the cell. Lpp also has a "free" subpopulation that does not interact with peptidoglycan and is thought to be surface exposed (Figure 1).[24,28] Lpp has been shown to cause the release of proinflammatory cytokines and to contribute to *E. coli*'s toxicity in humans.[29] Antibodies to Pal, a 19 kDa lipoprotein in *E. coli*, were also identified in the J5 antisera, suggesting that Pal may also be an important target for sepsis therapy and/or a contributor to the pathogenesis of *E. coli* sepsis.[24]

Peptidoglycan Associated Lipoprotein (Pal)

Pal is a Gram-negative bacterial lipoprotein that is tethered to the inner leaflet of the outer membrane via an N-terminal lipid moiety and is non-covalently bound to the peptidoglycan layer (similar to Lpp).[24] Like Lpp, Pal has been shown to have a second

subpopulation that is not bound to peptidoglycan and is also surface exposed.[30] Pal and several other "Tol" proteins (ex. TolB, TolA, etc.) make up the Tol-Pal complex. These proteins interact with each other, the outer and inner membranes, and peptidoglycan, forming a complex web of interactions that contribute to the structural integrity of the bacterial cell and are thought to play a role in cell division.[31] *E. coli* strains with mutant or deleted Pal are still viable, but are morphologically challenged and more fragile compared to their wild-type counterparts.[32]

Due to the presence of anti-Pal antibodies in protective J5 antisera, Pal was thought to be an important player in *E. coli* sepsis.[33] Subsequent studies showed that Pal was released from *E. coli* in an experimental infected burned rat model of GNS.[34] Pal was detected in 81% of the plasma samples from the septic rats.[34] Pal was also shown to be released *in vitro* in the presence of human sera and in a cecal ligation and puncture (CLP) mouse model of sepsis.[24,34] In another study, mice were injected with 10 μg of Pal or 50 mM sodium phosphate pH 7.4 (negative control group); blood samples were collected every hour for 16 hours.[32] Blood serum samples were analyzed for proinflammatory cytokine levels, specifically for tumor necrosis factors (TNFs), Interleukin-6 (IL-6), and Interleukin-1β (IL-1β).[32] The mice that were injected with Pal showed significantly higher levels of all the tested proinflammatory cytokines compared to control mice, suggesting that Pal was responsible for inducing inflammation in septic mice.[32]

In a toxicity study, mice were injected with Pal-nonsense, Pal-deficient, or wild-type *E. coli* cells.[32] The Pal-nonsense gene contained a pre-mature stop codon, resulting in truncated Pal protein, while the Pal-deficient cells expressed reduced amounts of Pal.[32] Mice infected with

wild-type *E. coli* had an average survival rate of 7%, while mice injected with Pal-deficient or Pal-nonsense *E. coli* had average survival rates of 33% and 100%, respectively.[32]

In summary, results from past studies suggest that Pal is released from *E. coli in vitro* in human sera and *in vivo* in several animal models of sepsis; Pal is toxic, and Pal induces the release of proinflammatory cytokines (*i.e.*, Pal contributes to inflammation). All of these findings point to Pal's pathological role in *E. coli* sepsis.

Thesis Project

The goals of this project were to: A) determine the effect of specific antibiotics on the release of Pal from *E. coli*, B) develop a monomicrobial sepsis model in mice, and C) determine whether or not Pal was detectable in biological samples of septic mice. Previous members of the Michel lab were able to detect Pal in the urine of several human sepsis patients, so we proposed to detect Pal in the urine of septic mice.

When sepsis patients are first admitted to the hospital, it is likely that they will be immediately prescribed a regimen of broad-spectrum antibiotics. However, not much has been done to show the effect of those antibiotics on the release of Pal, or other toxic molecules like LPS, from *E. coli*, which have been shown to contribute to inflammation. Therefore, while antibiotics are critical for controlling bacterial levels, we propose that they may also be enhancing Pal's release and thereby contributing to the induction of sepsis.

In our *in vitro* studies, we used several antibiotics that are commonly prescribed at hospitals and other health care facilities. These antibiotics can be categorized into three broad groups of antibiotics: beta-lactams, quinolones, and aminoglycosides. Antibiotics have different mechanisms of action to inhibit bacterial growth (bacteriostatic) or to cause bacterial cell death (bactericidal). Quinolone antibiotics target the A subunit of DNA gyrase and block DNA synthesis, which is crucial for cell growth and division.[35,36] Aminoglycoside antibiotics bind to rRNA at the 30S subunit, causing misreading of the genetic code and inhibiting protein translation.[37] Beta-lactam antibiotics are known to form covalent complexes with the penicillin-binding proteins (PBPs) and inhibit the synthesis of peptidoglycan.[38]

Since beta-lactam antibiotics target and inhibit the peptidoglycan layer of the bacterial cell, we proposed that Pal, which binds tightly to peptidoglycan, may be more readily released in the presence of beta-lactam antibiotics compared to other types of antibiotics. As described above, the release of Pal into the patient's bloodstream could enhance/worsen a septic patient's condition. We also proposed that the release of other toxic bacterial components (like LPS) may be enhanced by beta-lactam antibiotics, due to the inhibition or degradation of peptidoglycan. In the discussion section of this thesis, we describe our hypothesis that Pal, as well as several other toxic bacterial components, are likely released in outer membrane vesicles (OMVs), which are small non-living membrane-bound vesicles released from *E. coli* under stressful conditions.

Our collaborator, Dr. Judith Hellman, showed that Pal is released from *E. coli* in both CLP and burn models of sepsis. She was able to detect Pal in the blood of those septic animals using a polymicrobial sepsis model. We pursued a similar study, but with the following modifications. First, we sought to develop a monomicrobial sepsis model in mice, using the same *E. coli* strain that we employed for our *in vitro* antibiotic experiments. Second, we sought to determine whether or not we could detect Pal in the urine of our septic mice instead of detecting Pal in the sera, as was done by Hellman. We theorized that when Pal was released from *E. coli* into the mouse's bloodstream, Pal and other toxic molecules might be filtered by the kidney, liver, or spleen, and excreted into urine. Since urine contains far fewer proteins than blood, we hypothesized that we would be able to detect Pal in urine more readily than in blood. We proposed to use this method of tracking Pal in septic mouse urine in order to A) corroborate our pilot study, in which we detected Pal in the urine of human patients' with sepsis, B)

determine whether or not Pal levels in urine correlate with sepsis severity, and C) determine whether or not antibiotics affected the release/detection of Pal *in vivo*.

2. *E. coli* Pal Release (*In Vitro* Studies)

The overall goal of this part of my thesis project was to determine the effect of specific antibiotics on the release of Pal from *E. coli*. First, we had to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for all of the antibiotics to be used in our experiment. These antibiotic concentrations were considered equally effective at inhibiting growth (MICs) or killing the bacteria (MBCs) for our specific *E. coli* strain. Under these equally "potent" antibiotic conditions, we determined whether or not certain antibiotics enhanced the release of Pal to a greater extent than others.

Materials and Methods

E. coli Cultures

A clinical strain of *E. coli* K1 RS218 was gifted to us by Dr. Kwang Sik Kim (Johns Hopkins Children's Center). Cells were cultured in Lysogeny broth (LB) at 37 ˚C in a shaker at 200 rpm until log phase (optical density at 600 nm ~0.6).

Determining Minimum Inhibitory and Minimum Bactericidal Concentrations

Minimum inhibitory concentrations (MICs) were determined for each antibiotic, using our *E. coli* strain (K1 RS218). Briefly, 2-fold dilutions of each antibiotic were prepared in LB. A starter *E. coli* culture was grown to log phase and then used to inoculate the antibiotic LB media. Cultures were incubated overnight (~12 hours) at 37 ˚C, shaking at 200 rpm. The MIC of each antibiotic was visually determined as the lowest antibiotic concentration for which there was no visible growth. Cultures with no visible growth were plated (25 μL) on LB agar and incubated overnight (~12 hours) at 37 ˚C. The minimum bactericidal concentration (MBC) of each antibiotic was determined as the minimum antibiotic concentration, which prevented growth on LB agar (*ie*, plates with no *E. coli* colonies).

Antibiotic Preparations

Ampicillin

A stock solution was prepared by adding 1 g of ampicillin (GoldBio, St Louis, MO) to 10mL of ultrapure water to make a 100 mg/mL stock. The stock solution was diluted in ultrapure water

to a concentration of 10 mg/mL and syringe filtered using a 0.45 μm syringe filter. For the MIC/MBC experiments, 2-fold dilutions were prepared from the 10 mg/mL stock in sterile LB.

Gentamicin

A sterile, liquid solution of gentamicin (Fresenius Kabi USA, Lake Zurich, IL) was purchased at 40 mg/mL and stored at 4 ˚C. A stock solution was prepared by diluting to a concentration of 2.5 mg/mL in ultrapure water and then used to prepared 2-fold dilutions in LB.

Levofloxacin

Levofloxacin (Auromedic Pharma LLC, Dayton, NJ) was purchased as a sterile liquid at 25 mg/mL and stored at room temperature. A stock solution was prepared by diluting to 100 μg/mL in ultrapure water, which was then used to prepare the 2-fold dilutions in LB.

The Pal Release Experiment

E. coli was cultured as described above. For each sample, 1 mL of culture was placed in a microfuge tube and pelleted at 5,000 *xg* for 2 minutes. Supernatants were discarded, and the pellets were resuspended in LB (control), the MBC of ampicillin, gentamicin, or levofloxacin, or twice the MIC of ampicillin, gentamicin, or levofloxacin. Each sample was transferred to a 15 mL Falcon tube and incubated for 4 hours at 37 ˚C, shaking at 200 rpm. Cells were pelleted at 5000 *xg* for 2 minutes, and supernatants were filtered using a 0.45 μm syringe filter. Filtered supernatants were combined with 2X Sample Buffer (recipe below) for further analysis. Cell pellet samples were prepared by resuspending each pellet in 1 mL of LB and then further diluting the solution (10 μL of cell slurry + 900 μL of LB). The diluted pellet sample was then combined with 2X Sample Buffer for further analysis.

Outer Membrane Vesicle Preparation

Large cultures (250 mL) of *E. coli* K1 RS218 were grown overnight in LB or LB + 10 μg/mL ampicillin. Overnight cultures were pelleted at 5000 *xg* for 20 minutes. The culture supernatants were filtered (0.45 μm) to remove any whole cells and then concentrated using 10 kDa molecular weight cut-off Amicon concentrators (ThermoFisher). The concentrated supernatants were then pelleted in the ultracentrifuge at 100,000 *xg* (33,000 rpm) for 1 hour at 4 ˚C. The ultracentrifuged pellets were expected to be enriched with *E. coli* OMVs. OMV pellets were resuspended in 1 mL of phosphate buffered saline (PBS).

Protein Detection and Analysis

Protein Gel Electrophoresis

Released Pal was analyzed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE) gel. The resolving gel contained 3.27 mL 30% acrylamide/bis-acrylamide, 3.33 mL Tris/SDS (182g Tris, 1.5 g SDS, pH 8.0), 1.28 mL ultrapure water, 2.12 mL 50% glycerol, 100 μL of 10% ammonium persulfate (APS), 10 μL tetramethylethylenediamine (TEMED). The stacking gel contained 405 μL 30% acrylamide/bis-acrylamide, 775 μL Tris/SDS, 1.95 mL of ultrapure water, 20 μL 10% APS, 5 μL TEMED. The protein samples were run in 1X SDS Running Buffer (10X SDS Running Buffer: 30 g Tris base, 144 g Glycine, 10 g SDS in 1 L ultrapure water). All protein samples were prepared 1:1 in 2X Sample Buffer (0.12 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue) and boiled for 10 minutes. Fourteen microliters of samples were loaded alongside 5 μL of Multicolor Broad Range Protein Ladder (Thermo Scientific, Waltham, MA). The protein samples were typically separated on the gel for 45 – 60 minutes (120 – 180 V).

Semi Dry Transfer

Separated proteins were transferred onto a 0.45-micron nitrocellulose membrane (Thermo Scientific) using a semi-dry transfer cell (BioRad). The nitrocellulose membrane, 2 filter papers (8 cm x 10.5 cm), and the SDS – PAGE gel was rinsed in in 1X transfer buffer (10X transfer buffer: 58.2 g Tris base, 29.3 g Glycine in 1 L ultrapure water; diluted in 1:10 ratio with ultrapure water for 1X) for 15 minutes. The following items were placed on the bottom electrode of the transfer cell, in order: filter paper, nitrocellulose membrane, gel, filter paper. The top electrode was secured in place and the proteins were allowed to transfer for ~20 minutes at 15 volts (V).

Western Blot

After the proteins were transferred to the nitrocellulose membrane, the membrane was blocked in 25 mL of 5% milk (1.25 g powdered milk) in 1X Tris-buffered saline (TBS) (10X TBS: 80 g NaCl, 2 g KCl, 30g Tris Base in 1 L ultrapure water) for 30 minutes. The membrane was then incubated overnight with primary antibody at 4 °C [1:4,000 dilution of mouse monoclonal anti-Pal of 1% milk in TBST (1% milk TBST: 100 mL 1X TBS, 50 µL Tween-20, 1 g powdered milk)]. The following day, the membrane was washed twice in 1X TBST (1X TBS with Tween-200) for 10 minutes each and then incubated in secondary antibody [1:12,000 dilution of goat anti-mouse IgG-H+L HRP conjugate (Bethyl Laboratories Inc., Montgomery, TX)] in 1% milk for an hour. The membrane was washed three times in 1X TBST and two additional times in 1X TBS for 10 minutes each wash. Pal was detected using the LumiGlo Chemiluminescent Reserve kit (SeraCare, Milford, MA) and the BioRad ChemiDoc XRS+ system. The ImageLab software was used to calculate band volumes, and all data were further analyzed using Microsoft Excel.

Electron Microscopy

OMVs were prepared as described above. A 10 µL drop of the OMV solution was placed on a carbon-film-covered 400 mesh Cu TEM grid. After 10 seconds, the solution was wicked off. Images at 30,000x magnification were captured on a JEOL 2010 TEM operating at 200 kV. The mean diameters of OMVs were measured using Image J software.

Results and Discussion

Minimum Inhibitory and Bactericidal Concentration of Antibiotics

Antibiotics are usually the first form of treatment for patients diagnosed with a bacterial infection, including sepsis patients. Doctors will initially prescribe broad spectrum antibiotics since, often, the exact type of bacterial infection is unknown. Since antibiotics are so commonly used to "treat" sepsis patients, we sought to determine whether or not antibiotics had an effect on the release of Pal from *E. coli*. There are many different types of antibiotics and several different broad categories of antibiotics, which kill bacteria or prevent their growth using different mechanisms. In addition, different bacteria may have different susceptibilities to each antibiotic. Thus, we decided to determine the effect of three commonly prescribed antibiotics on Pal release.

The minimum inhibitory concentration (MIC) is the lowest concentration of a drug or antibiotic that will inhibit the growth of an organism.[39] The minimum bactericidal concentration (MBC) is the lowest concentration of a drug or antibiotic that is required to kill an organism.[40] Most doctors will prescribe greater than the MIC or MBC dose in order to ensure that the patient is able to clear the infection. Therefore, in our study, we employed 2X the MIC values of antibiotics as well as 1X the MBC values of antibiotics. Experiments were performed multiple times to ensure reproducibility. Using *E. coli* strain K12 RS218, we found the following MIC and MBC values (Table 1).

Table 1. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined for each antibiotic employed in our study. These values were specific for our *E. coli* strain (K12-RS218).

Antibiotic	Minimum Inhibitory Concentration (MIC)	Minimum Bactericidal Concentration (MBC)
Ampicillin	4 μg/mL	512 μg/mL
Gentamicin	8 μg/mL	32 μg/mL
Levofloxacin	0.125 μg/mL	2 μg/mL

The Effect of Antibiotics on Pal Release

Pal has been shown to be released from *E. coli* under certain conditions and *in vivo* in two different animal models of sepsis.[24,25,32–34] In most of these studies, Pal was readily released in the presence of beta-lactam antibiotics, including ampicillin, ceftazidime, and imipenem/cilastatin.[25] Hellman and coworkers did not, however, test other types of antibiotics in their Pal studies. Interestingly, western blots of released Pal from *E. coli* (*in vitro*) showed far less released Pal when *E. coli* was incubated in human sera alone compared to when *E. coli* was incubated with human sera and one of the beta-lactam antibiotics.[25] Therefore, we hypothesized that beta-lactam antibiotics ***enhance*** Pal's release from *E. coli*. Specifically, we proposed that beta-lactam antibiotics inhibit the formation of peptidoglycan in *E. coli*, and thus prevent the production of the major bacterial component to which Pal is tethered, allowing for its efficient release from *E. coli*.

We performed the Pal release experiment in LB with three different types of antibiotics: ampicillin, gentamicin, and levofloxacin at twice their MIC values (2XMIC). Our results suggest that, at these equally inhibitory concentrations of antibiotic, more Pal is released from *E. coli* in the presence of ampicillin compared to gentamicin and levofloxacin (Figure 3). The Pal bands on the western blots were quantified using ImageLab Software (Bio Rad). Volumes of each band

were calculated from the band size and intensities (Table 2). For easy comparison, we normalized the antibiotic Pal band volumes to the LB-only Pal band volume (Table 3, Figure 4). Here, we showed that under equally inhibitory concentrations of antibiotics, ~8-fold more Pal is released in the presence of ampicillin compared to gentamicin and levofloxacin.

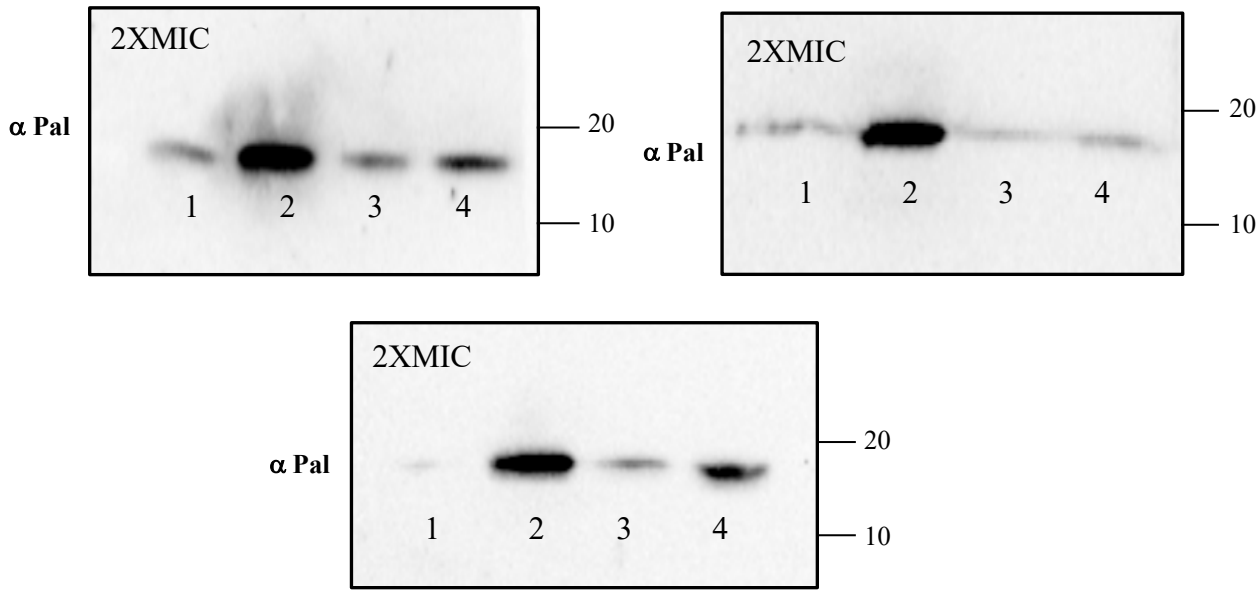

Figure 3. Three replicate western blots showing released Pal in the supernatant of *E. coli* cultured were in 1. LB alone, 2. 2XMIC of ampicillin, 3. 2XMIC of gentamicin, and 4. 2XMIC of levofloxacin. Blots were detected using mouse monoclonal anti-Pal, goat anti-mouse-HRP, and the Lumiglo Reserve kit. Pal (18kDa) bands were quantified using the Biorad ImageLab software.

Table 2. Pal band volumes (obtained from ImageLab Software) for three trials of the Pal release experiment using 2XMIC values of each antibiotic.

Pal Band Volumes	Trial #1	Trial #2	Trial #3	Average
LB only	704	384	1124	737.3
Ampicillin	4248	3146	12482	6625.3
Gentamicin	736	213	1459	802.67
Levofloxacin	1012	363	409	594.67

Table 3. Pal band volumes for three trials of the Pal release experiment using 2XMIC values of each antibiotic, normalized to the Pal band volume for LB alone.

Pal Band Volumes	Trial #1	Trial #2	Trial #3	Average
LB only	1.0	1.0	1.0	1.0
Ampicillin	6.0	8.2	11.1	8.4
Gentamicin	1.0	0.6	1.3	1.0
Levofloxacin	1.4	0.9	0.4	0.9

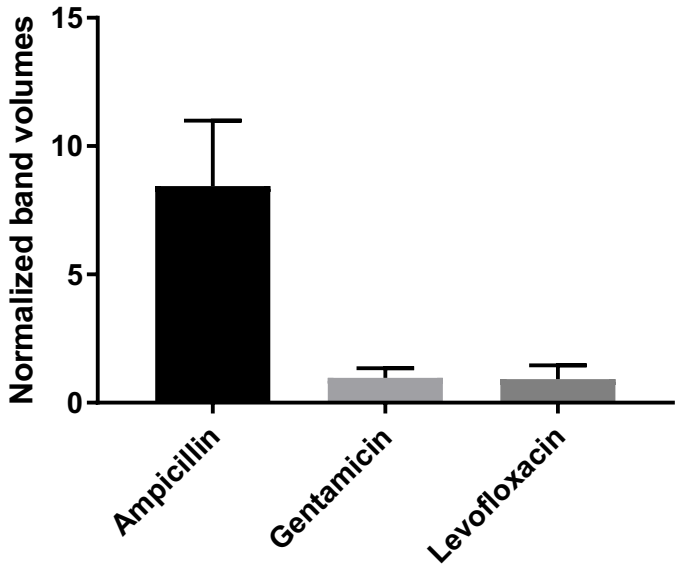

Figure 4. Pal band volumes were calculated using ImageLab software; 2XMIC data were normalized to the band volume for the LB only sample from the corresponding trial. A total of three trials were included in the analysis. The bar graph shows the mean values for each antibiotic, with standard deviation error bars.

The same Pal release experiment performed using MBC values of antibiotics yielded similar results. We showed that under equally bactericidal concentrations of antibiotics, ~25-fold more Pal is released in the presence of ampicillin compared to gentamicin and ~8-fold more compared to levofloxacin (Figure 5, Table 4, Table 5, Figure 6).

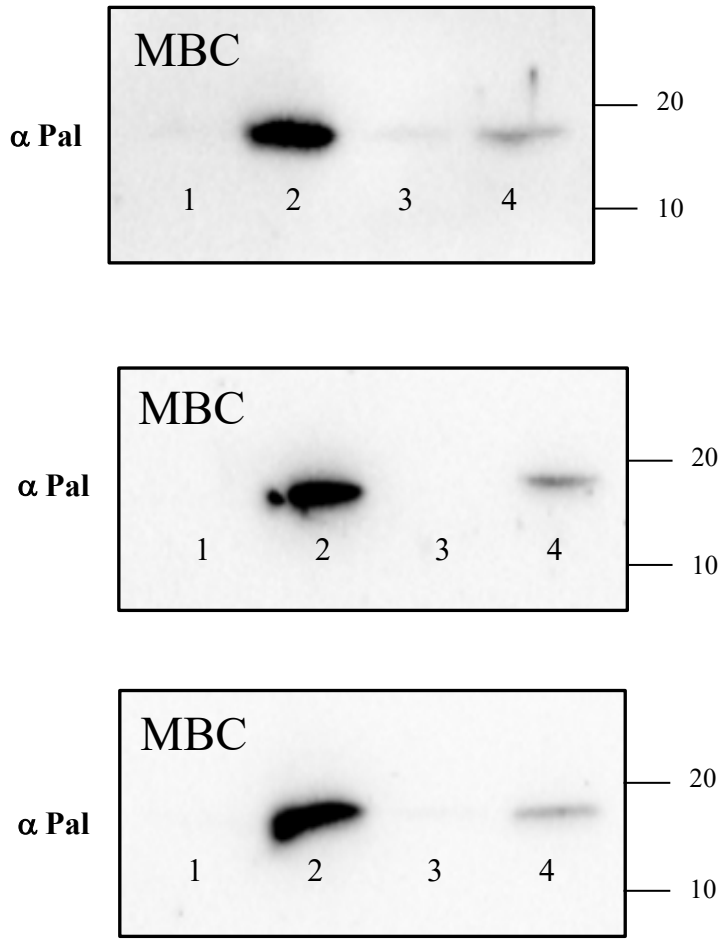

Figure 5. Three replicate western blots showing released Pal in the supernatant of *E. coli* cultured in 1. LB alone, 2. the MBC of ampicillin, 3. the MBC of gentamicin, and 4. the MBC of levofloxacin; detected using mouse monoclonal anti-Pal, goat anti-mouse-HRP, and the Lumiglo Reserve kit. Pal (18kDa) bands were quantified using the Biorad ImageLab software.

Table 4. Pal band volumes (obtained from ImageLab Software) for three trials of the Pal release experiment using the MBC values of each antibiotic.

Pal Band Volumes	Trial #1	Trial #2	Trial #3	Average
LB only	332	189	294	271.67
Ampicillin	5229	3965	5801	4998.3
Gentamicin	274	136	194	201.3
Levofloxacin	736	581	521	612.67

Table 5. Pal band volumes for three trials of the Pal release experiment using the MBC values of each antibiotic, normalized to the Pal band volume for LB alone.

Pal Band Volumes	Trial #1	Trial #2	Trial #3	Average
LB only	1.0	1.0	1.0	1.0
Ampicillin	15.8	21.0	19.7	18.8
Gentamicin	0.8	0.7	0.7	0.7
Levofloxacin	2.2	3.1	1.8	2.4

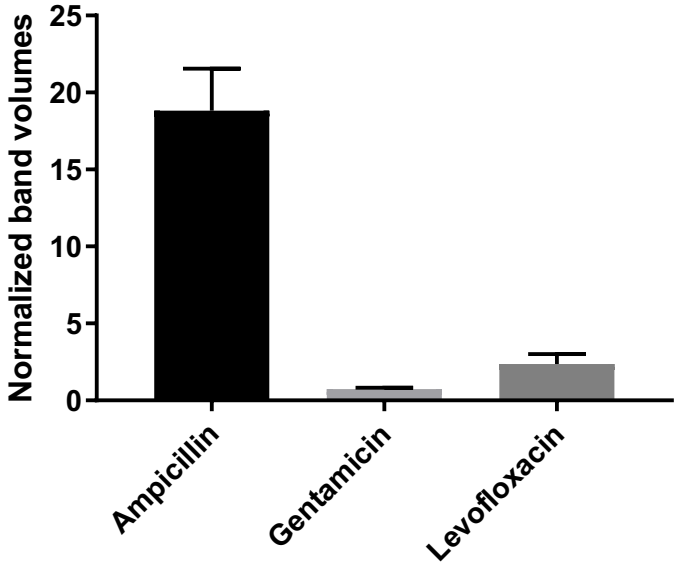

Figure 6. Pal band volumes were calculated using ImageLab software; MBC data were normalized to the band volume for the LB only sample from the corresponding trial. A total of three trials were included in the analysis. The bar graph shows the mean values for each antibiotic, with standard deviation error bars.

In summary, these results suggest that more Pal is released from *E. coli* in the presence of beta-lactam antibiotics compared to quinolones or aminoglycosides. However, before we can verify this claim, we must perform similar experiments on a wider range of these three types of antibiotics, as well as with several other strains of *E. coli*. We believe these findings will be of significant interest to medical doctors who prescribe antibiotics to sepsis patients or patients with bacterial infections who have compromised immune systems or who may be particularly susceptible to developing sepsis.

The Mechanism of Pal Release

Pal is known to bind tightly to peptidoglycan through non-covalent interactions. In our lab, we are studying the specific effect of the Pal-peptidoglycan interaction on its ability to release from *E. coli*. Based on these results and the results of this thesis project, our current hypothesis is that the Pal-peptidoglycan interaction must be altered for Pal to be released from *E. coli*. In other words, we believe that the Pal-peptidoglycan interaction must be broken prior to Pal's release. This hypothesis was partly based on previous work, which identified and described small blebs that emerge from *E. coli* called outer membrane vesicles (OMVs).

The outer membrane of Gram-negative bacteria performs many important functions, including protecting the cell from foreign and potentially hazardous components. If the outer membrane is compromised, it can be lethal to the cell. Thus, Gram-negative bacteria have evolved multiple mechanisms to protect itself and its outer membrane. One of those mechanisms is called blebbing, in which outer membrane vesicles (OMVs) are released from the cell. OMVs are spherical buds varying in size from 20-250 nm; these buds contain LPS in the

outer leaflet of its outer membrane and phospholipids in their inner leaflet.[14,16,41] In an attempt to improve their chances of survival, the bacterium will eliminate misfolded outer membrane proteins and/or toxic materials inside the OMVs.[14,41] OMVs can also act as decoy membranes, such that during the lytic cycle, a phage will inject its DNA into the decoy OMV instead of the bacterium.[16] If the phage is already attached to the bacterium, it will vesiculate to carry away the phage DNA before it has a chance to replicate.[16] OMVs can also act as decoys in the presence of an attacking immune system or antibiotic.[42,43]

In general, OMV budding occurs when there are no protein linkages between the outer membrane and the peptidoglycan layer.[14,16] As seen in Figure 1 of this thesis, both Pal and Lpp tether the outer membrane to the peptidoglycan layer. Therefore, OMV budding can occur in the absence of Pal and Lpp, accomplished in part by decreasing/suppressing the expression of these proteins, ***or*** perhaps by disrupting the Pal/Lpp-peptidoglycan interaction, although the mechanism to accomplish that is unknown.[14,16]

We proposed that antibiotics enhance the release of OMVs from *E. coli*, and in some cases, disrupt the Pal-peptidoglycan interaction, thus allowing for Pal's release inside OMVs. To test this hypothesis, we performed the Pal release experiment and then partially purified the supernatant (containing the released proteins) to enrich for OMVs. OMVs pellet at ultra-high speeds; therefore, we spun the cell culture supernatants in an ultracentrifuge [Beckman Coulter Ultracentrifuge Optima L-90K, Ti50 rotor] at 100,000*xg*. When *E. coli* cells were incubated with 2XMIC of ampicillin, we detected significantly more Pal in our OMV-enriched ultra-pellet (Figure 7). We also detected more LPS when cells were incubated with ampicillin (Figure 7). Since these samples were filtered to remove whole cells, the Pal and LPS detected in these ultra-pellets

were likely contained within OMVs or cellular debris that pelleted along with the OMVs. Approximately **~8-fold** more Pal and **~14-fold** more LPS was detected in the OMV-enriched ultra-pellet when *E. coli* was incubated with ampicillin (compared to LB-only cultures).

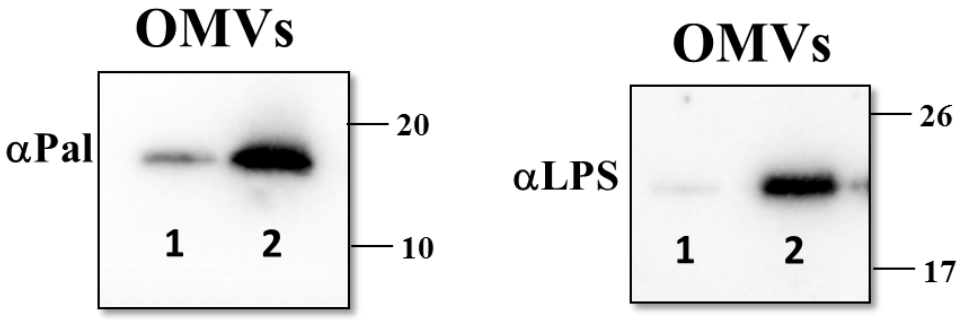

Figure 7. Pal (left) and LPS (right) were detected by western blot in the enriched outer membrane vesicle (OMV) samples. OMV samples were prepared from *E. coli* culture supernatants. *E. coli* were cultured in LB (lane 1) or 2XMIC of ampicillin (lane 2).

Because we did not rigorously purify OMVs, as described in published protocols, we performed several other tests to verify the presence of OMVs. First, we immunoblotted with anti-OmpC/F (Figure 8). OmpC/F is embedded in the outer membrane of Gram-negative bacteria, including *E. coli*, and can be used as an OMV marker. Protein complex OmpC/F has a molecular weight of ~40kDa and functions as a passive diffusion channel for small molecules, like nutrients, toxic salts, and antibiotics.[44]

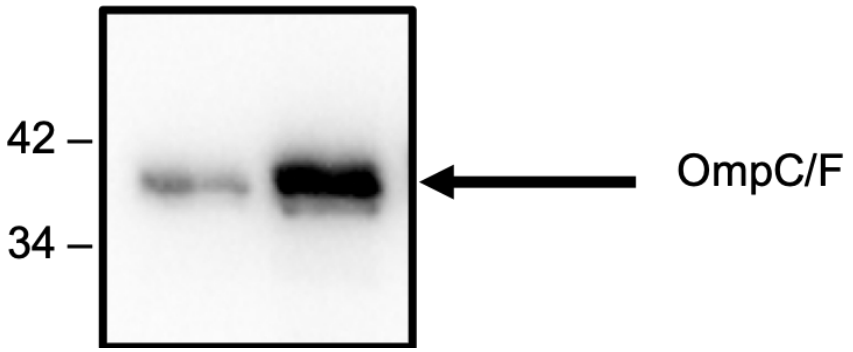

Figure 8. The OmpC/F complex was detected by western blot in the enriched outer membrane vesicle (OMV) samples. OMV samples were prepared from *E. coli* culture supernatants. *E. coli* were cultured in LB (lane 1) or 2XMIC of ampicillin (lane 2).

Using a cocktail of monoclonal antibodies to LamB, OmpA, and RscF (gifted by Dr. Anna Konovalova, University of Texas, Austin), we detected those outer membrane proteins, which are often found in OMVs (Figure 9).

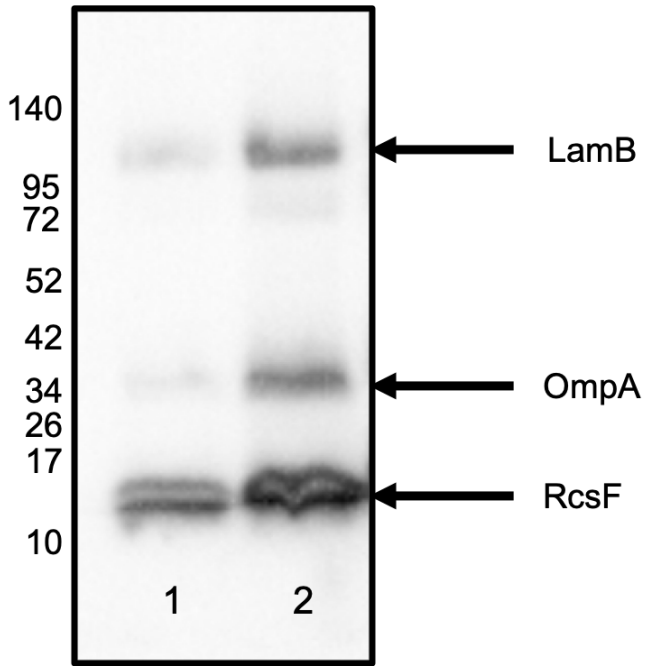

Figure 9. LamB, OmpA, and RcsF proteins were detected by western blot (using a combined antibody solution) in the enriched outer membrane vesicle (OMV) samples. OMV samples were prepared from *E. coli* culture supernatants. *E. coli* were cultured in LB (lane 1) or 2XMIC of ampicillin (lane 2).

Dr. Richard Hailstone (Associate Professor, RIT, Center for Imaging Science) visualized our enriched-OMV samples using electron microscopy. As seen in the electron microscope image, we saw OMVs of expected size and shape (Figure 10, white arrow), as well as some cellular debris (yellow arrow). Therefore, we could not rule out the possibility that Pal was released and contained within the cellular debris of our OMV-enriched sample. More recently, we have collected other images of our OMV samples, which are of enhanced quality, showing a distinct outer membrane on the vesicles (not shown).

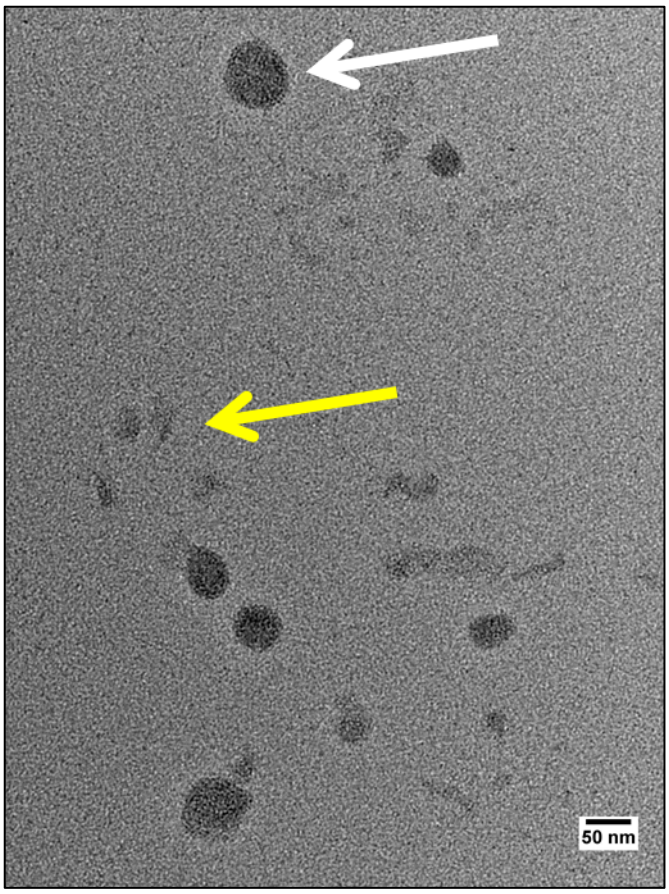

Figure 10. OMVs were purified by ultracentrifugation from overnight *E. coli* cultures and visualized via electron microscopy. OMVs were measured to have a mean diameter of 44.6 +/- 22.5 nm, as expected. Both OMVs (white arrow) and some cellular debris (yellow arrow) were imaged by the microscope.

Negative controls for OMV preps are challenging to assess. In general, OMVs contain an abundance of outer membrane and periplasmic proteins; however, some published OMV preps have also contained DNA/RNA and other inner membrane/cytoplasmic protein contaminants.[45] However, we did run a negative control experiment where we detected for Sigma70 (Figure 11), an RNA polymerase and cytoplasmic protein that should not be contained within a purified OMV sample. We did not detect Sigma70 in the OMV-enriched sample from *E. coli* cultured in LB-only, suggesting this sample contained mostly OMVs. However, we did detect Sigma70 OMV-enriched sample from *E. coli* cultured with ampicillin. We propose that ampicillin is enhancing the amount of cellular debris, thus causing an increase in cellular debris contamination of our OMV sample.

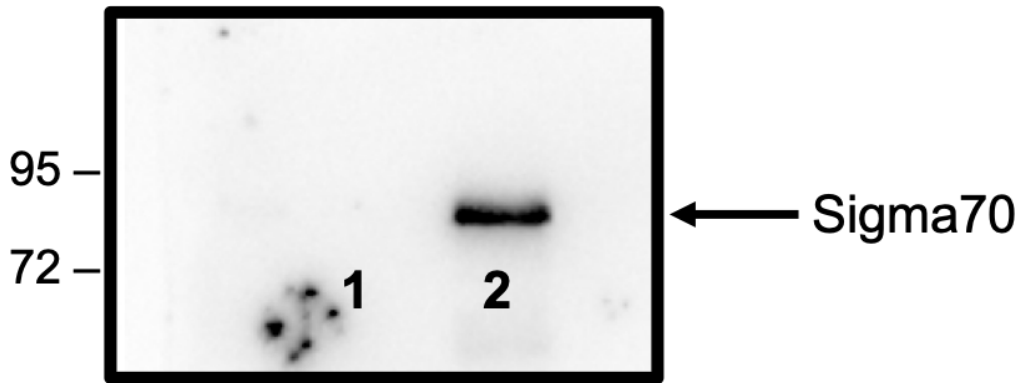

Figure 11. The OmpC/F complex was detected by western blot in the enriched outer membrane vesicle (OMV) samples. OMV samples were prepared from *E. coli* culture supernatants. *E. coli* were cultured in LB (lane 1) or 2XMIC of ampicillin (lane 2).

In conclusion, based on the results of this study, we propose that Pal is released in OMVs ***and*** possibly in cellular debris when ampicillin is present in the cell cultures. We hypothesize that ampicillin is breaking up the *E. coli* cells and possibly the OMVs, thus allowing Pal to be released among the cellular debris.

We also consider the possibility that the *E. coli* cells are releasing more OMVs in the presence of ampicillin. Gram-negative bacterial vesiculation has been shown to increase in the presence of antibiotics; in these cases, the OMVs contain molecules that help protect the organism.[46,47] Our collaborator, Judith Hellman, detected Pal released from *E. coli* as a "complex" with LPS, OmpA, and Lpp; we hypothesize that these complexes were OMVs. Hellman also detected a smaller amount of Pal released separately from those molecules; based on Hellman's studies and the results of our work, we hypothesize that some Pal is released in the cellular debris.

Section Summary and Conclusions

The minimum inhibitory concentrations and minimum bactericidal concentrations of several clinically relevant antibiotics were determined for our clinical strain of *E. coli* K1 RS218. We found that at equally inhibitory or bactericidal concentrations of our antibiotics, Pal release was enhanced to the greatest extent by ampicillin. We had previously hypothesized that ampicillin would cause the greatest amount of Pal release, since its mechanism of action is to inhibit peptidoglycan synthesis, the molecule to which Pal is tethered. We now propose that ampicillin, and perhaps other beta-lactam antibiotics, increase inflammation in sepsis patients by enhancing the release of Pal and other toxic molecules such as LPS, either through increased vesiculation or release of these molecules among cellular debris. Finally, we propose that sepsis patients might benefit from treatment with non-beta-lactam antibiotics that are equally as effective in killing, but do not enhance the release of inflammatory-inducing molecules.

3. Developing a Mouse Model of Sepsis (*In Vivo* Studies)

Based on the results of past studies, performed both in our lab and in our collaborators' labs, we hypothesized that Pal was released from *E. coli* in human patients during sepsis. Our collaborator, Dr. Judith Hellman (University of California at San Francisco), had detected released Pal in the sera of septic mice and rats and *in vitro* in the presence of human sera, but, she had not detected *E. coli* Pal in any human clinical samples. A former graduate student from the Michel lab, Bethany Novick, was able to detect Pal in the urine of several human sepsis patients. We decided to determine whether or not we could detect Pal in the urine of septic mice. We proposed to implement a monomicrobial sepsis model, induced via intraperitoneal (IP) injection of *E. coli*, to test our hypothesis that *E. coli* Pal is detectable in septic mouse urine. Further, we proposed to corroborate our *in vitro* antibiotic studies by detecting Pal in the urine of septic mice treated with different antibiotics.

Materials and Methods

E. coli Stock Culture

A large growth (~50 mL) of clinical strain *E. coli* K1 RS218 was grown to an OD_{600} of 0.7. The culture was split into 1-mL aliquots of bacteria with 8% glycerol and then frozen at -80 ˚C. To determine the concentration of each aliquot of bacteria, the stock was thawed and washed 3 times with phosphate buffered saline (PBS) pH 7.4 (Thermo Scientific, Waltham, MA) at 5000 *xg* for 2 minutes, and then serially diluted in PBS (10x dilutions). Ten μl of each dilution were plated in lines on LB agar and incubated overnight. The next morning, the colonies were counted and used to back-calculate the concentration of the *E. coli* stock solution, which was determined to be 3 x 10^7 colony forming units (CFU) per 100 μL.

Mice

C57BL/6J male mice (~6-8 weeks old) were ordered from Jackson Labs (Ellsworth, ME) and allowed to rest for one week after arrival. Mice were housed in the IACUC approved animal facility at Rochester General Hospital in sterile cages (5 mice/cage). Prior to each experiment, the mice were cleaned (feet, body, tail, etc.) with isopropyl alcohol and placed into a clean cage with sterile Lab Sand (Coastline Global, Inc.). Prior to most manipulations, mice were anesthetized with isoflurane (1%) in 100% oxygen at a delivery rate of 4 liter/min. At the end of each experiment, mice were anesthetized, and then euthanized by cervical dislocation. Our sepsis mouse protocols were approved by the RGH IACUC committee (protocol #2018-002).

Injections

One frozen stock of *E. coli* was thawed and washed three times in PBS before diluting to a final concentration in PBS (see next section for details). Sterile saline was heated in a warm water bath to avoid rapidly lowering the body temperature of the mice. Ampicillin was prepared from stock solutions (see previous section for details) to a final concentration of 20 μg/mL, which was 5 times the determined MIC value. Mice were administered a 1-mL subcutaneous saline bolus just prior to infection. Mice were infected with *E. coli* with an intraperitoneal (IP) injection (100 μl) using a sterile syringe and hypodermic needle. One hour after infection, the mice were administered ampicillin with an IP injection.

Sample Collection

Urine samples were collected using two different techniques. The primary technique involved waiting for the mice to urinate on the sterile Lab Sand, and immediately collecting the urine via pipette into a sterile tube. Extra precautions were taken to ensure that the mice did not contact (*ie*, step in) the sample prior to collection. Even with this precaution, this technique risked fecal contamination of the sample. The second technique involved picking up the mice and collecting the sample in a tube by direct expression of the urine. This technique required the mouse to be firmly grasped by the scruff while applying direct but gentle pressure to the bladder.

Healthy urine samples were collected prior to IP injections of *E. coli*. Mice were observed for signs of distress or sickness throughout the experiment; typically, the mice responded to the infection within one hour of the injections. One hour post-infection, the mice were split into two groups; the first group of mice was administered 5X MIC of ampicillin and

the other group of mice were administered PBS or nothing. During some of the experiments, the ampicillin mice were given a second saline bolus due to signs of dehydration. In retrospect, this may have skewed the data and/or diluted the ampicillin urine samples. Urine samples were collected at various time points post-infection. In most cases, a final urine sample was collected directly from the bladder using a sterile syringe and needle.

Retro-orbital blood samples were collected using a sterile glass Pasteur pipette. Blood was stored in a heparin tube to prevent clotting/coagulation.

Liver and Kidney Collection

During one experiment, liver and kidney organs were harvested from four mice (post-mortem). The tissues were homogenized in PBS and then gently spun at ~1,600 *rpm* for 5-6 minutes. Supernatants of each tissue sample were combined with 2X Sample Buffer and boiled for 10 minutes.

Sample Analysis

Urine samples were either filtered using a 0.45 µm syringe filter or gently pelleted (5000 *xg*, 2 minutes) to remove whole cells. All urine samples were combined with 2X Sample Buffer at 1:1 ratio and boiled for 10 minutes. All samples were separated by SDS PAGE, and Pal was detected via immunoblotting. Blood samples were plated on LB agar and incubated overnight at 37 °C. The plates were visually assessed for the presence of *E. coli* colonies.

Results and Discussion

The role of Pal in *E. coli* sepsis has been investigated by several scientists, including our collaborator, Dr. Judith Hellman. Hellman and coworkers demonstrated that Pal is released from *E. coli* in the presence of human sera (*in vitro*) and in several animal models of sepsis. Hellman and members of the Michel lab sought to detect Pal in the human sera of *E. coli* sepsis patients. This task, however, proved challenging, due in large part to the excess of proteins in sera.

We hypothesized that Pal, once released from *E. coli*, might be filtered by the kidneys. Kidneys filter excess salts and toxins from the blood, followed by excretion of those molecules in urine.[48] An added benefit to detecting Pal in urine is that there are far fewer proteins in urine compared to sera, allowing for a more efficient detection protocol and a better signal to noise ratio for Pal detection.

A previous graduate student in the Michel Lab was able to identify the limit of detection of Pal in urine by spiking healthy urine with different concentrations of purified Pal. In addition, that student detected Pal in the urine of several *E. coli* sepsis patients, thus corroborating our hypothesis that Pal is indeed filtered by the kidneys and excreted in urine.

We proposed to detect Pal in the urine of septic mice. To accomplish this goal, we employed a monomicrobial IP injection sepsis mouse model described by Okeke *et al*., with a few modifications.[49] As described above, mice were infected with *E. coli* by IP injection and then administered ampicillin or PBS. Urine was collected at different time points, post-infection.

First, we sought to determine the appropriate concentration of *E. coli* for infection. In our first experiment, 3 mice were injected a "high" concentration of *E. coli* (3×10^8 CFU/100 μL),

4 mice were injected with a "medium" concentration of *E. coli* (1.5 x 10^8 CFU/100 μL), and 3 mice were injected with a "low" concentration of *E. coli* (0.75 x 10^8 CFU/100 μL). Only one mouse injected with the lowest dose of *E. coli* survived the night. We did collect urine from two mice prior to infection (Figure 12, Healthy), as well as urine from the one surviving mouse the day after infection (Figure 12, "Low" concentration *E. coli*).

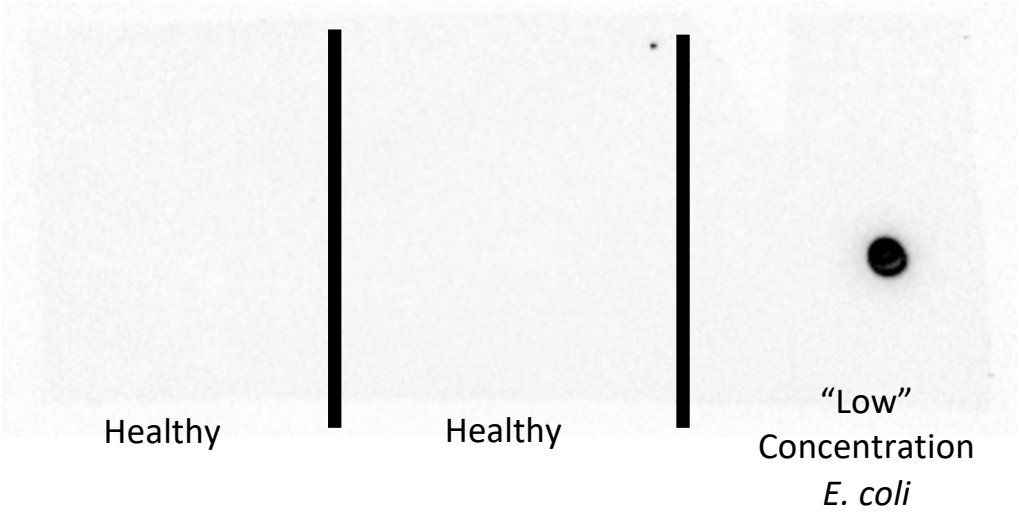

Figure 12. Urine from healthy mice (left and center panels) were collected prior to infection; one urine sample (right panel) was collected from a mouse ~24 hours post-*E. coli* infection. All urine samples were analyzed via immuno-dot blot with monoclonal anti-Pal.

In the next study, four mice were infected with 1x10^7 CFU/100 μL *E. coli* via IP injections. One hour after infection, two mice were administered 5XMIC of ampicillin and two mice were left alone. Urine was collected at various time points and analyzed on immuno-dot blots with monoclonal anti-Pal (Figure 13).

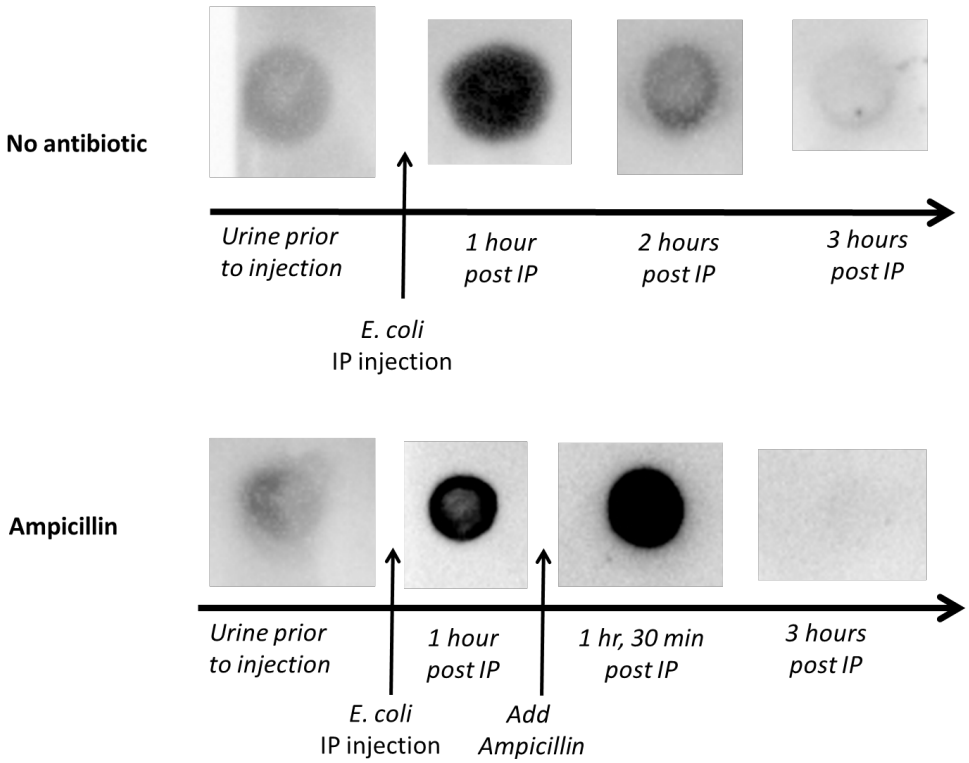

Figure 13. Immuno-dot blots of urine samples collected from *E. coli* infected mice treated with no antibiotic (top row) or 5XMIC of ampicillin (bottom row). The panels above were imaged on 3 western blots, which were developed together and detected using the same reagents.

These results revealed several interesting findings. First, healthy urine seemed to contain low levels of Pal. We continued to see this phenomenon (Pal in healthy urine) sporadically throughout our studies. We hypothesized that some of our mice were eating their own fecal matter, and thus processing/filtering *E. coli* Pal into their urine. Second, we observed what looked like a spike in Pal levels half an hour after the ampicillin injection, suggesting the ampicillin itself caused a spike in Pal release.

We repeated the same study (four mice were infected with $5x10^6$ CFU/100 μL *E. coli*; two mice administered ampicillin), but attempted to collect urine at more time points. These urine samples were filtered, run on SDS PAGE gels, and detected on immunoblots with anti-Pal (Figure 14). The Pal band volumes were quantified using ImageLab software (Figure 15).

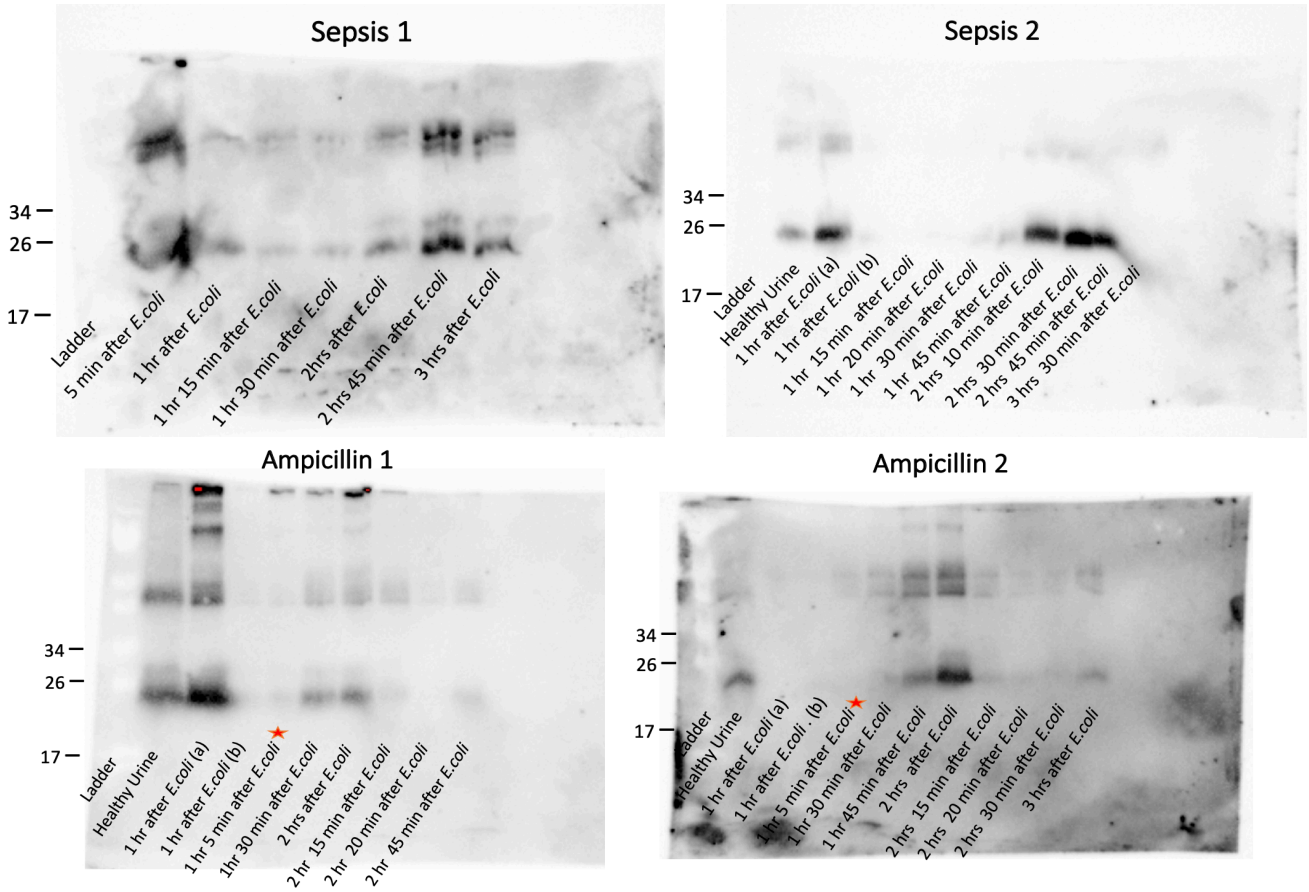

Figure 14. Pal immunoblots of urine samples collected from *E. coli* infected mice treated with no antibiotic (Sepsis 1 and Sepsis 2) or 5XMIC of ampicillin (Amp 1 and Amp2). The star indicates the timepoint at which ampicillin was administered. As is typical in these studies, Pal was detected as a monomer (~18kDa) and dimer (~36kDa).

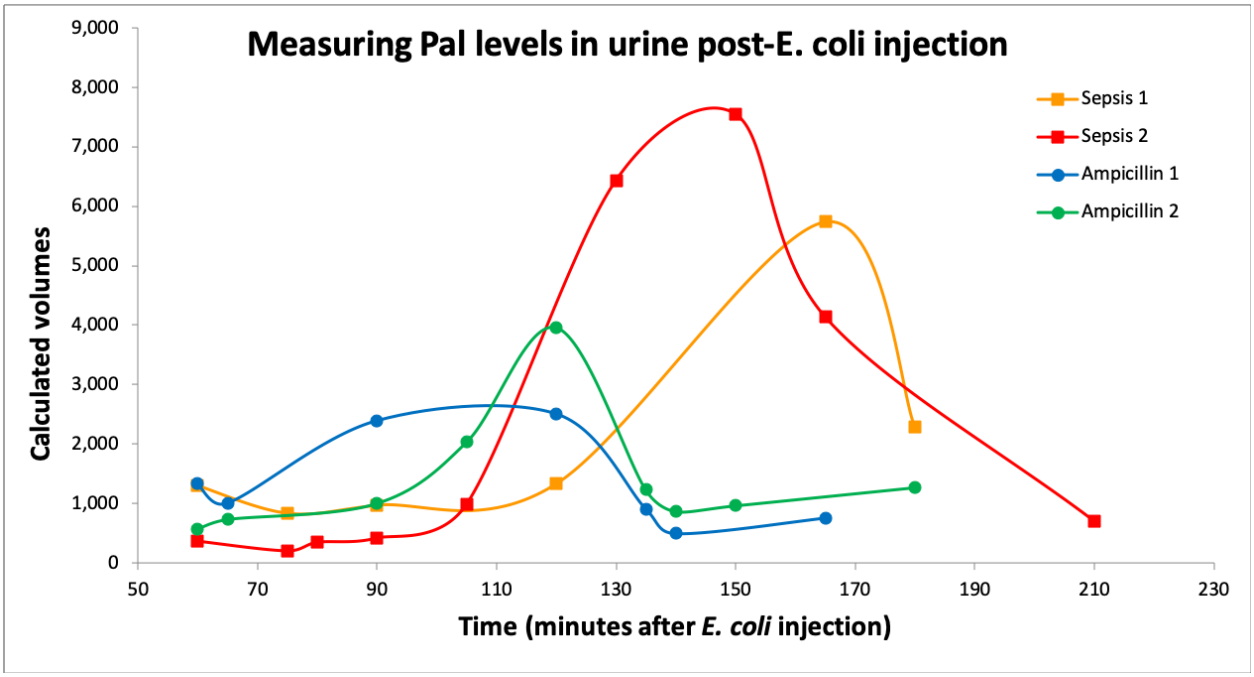

Figure 15. Pal band volumes were calculated from Pal immunoblots of urine samples collected from *E. coli* infected mice treated with no antibiotic (Sepsis 1 and Sepsis 2) or 5X MIC of ampicillin (Amp 1 and Amp2).

Results from these studies suggest that mice treated with ampicillin had a spike of Pal in their urine at earlier time points compared to untreated mice. Overall Pal levels (as reflected by Pal band volumes) appeared to be lower in ampicillin mice compared to untreated mice. However, we did not normalize the Pal levels to overall protein levels or creatinine levels in urine; therefore, we could not make clear conclusions about overall Pal levels. If Pal levels are indeed lower in ampicillin mice, we propose that ampicillin is killing the *E. coli* and/or contributing to its faster clearance in mice.

In our final experiment, we harvested the kidneys and liver of all four mice to see if we could detect Pal in the homogenized samples (Figure 16). We were able to detect Pal in the supernatant of homogenized kidneys, but did not detect Pal in homogenized livers, suggesting that *E. coli* and/or released Pal are indeed processed by the kidneys

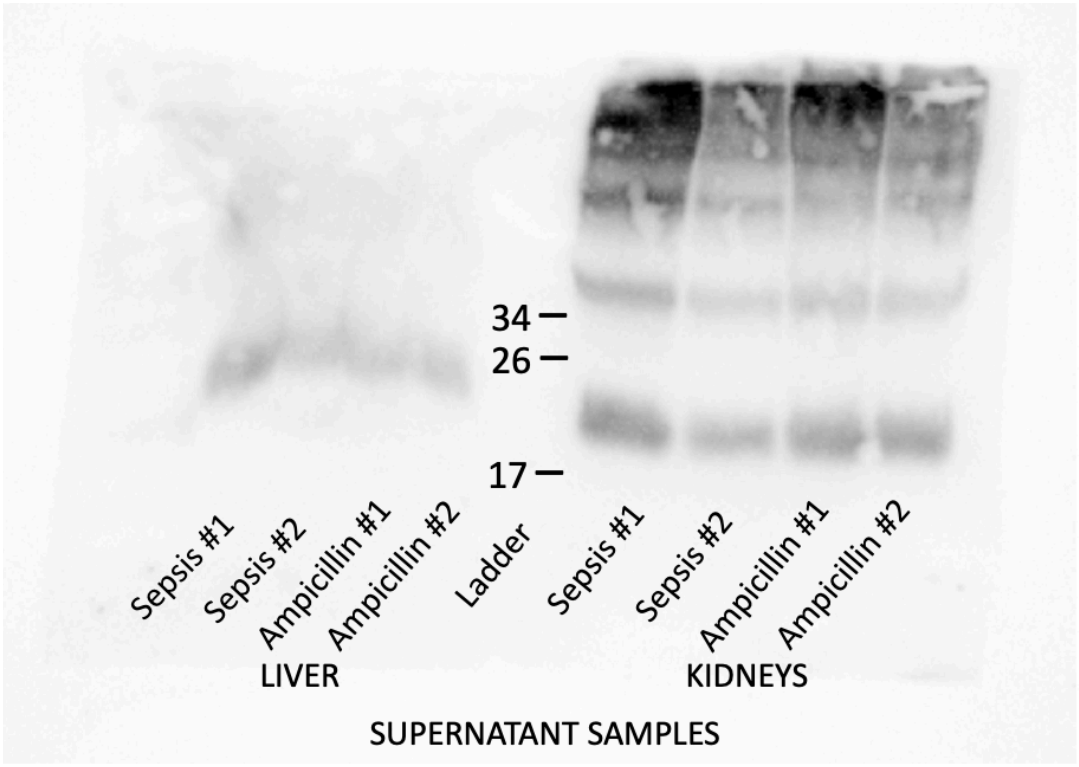

Figure 16. Pal immunoblots of the supernatants of homogenized kidney and liver samples from *E. coli* infected mice treated with no antibiotic (Sepsis 1 and Sepsis 2) or 5x MIC of ampicillin (Amp 1 and Amp2).

Section Summary and Conclusions

In summary, we were able to detect Pal in the urine of mice with *E. coli* sepsis. Preliminary results suggest that Pal levels spike earlier when mice are treated with ampicillin compared to untreated mice. These results corroborate our *in vitro* studies, which showed that ampicillin enhances the release of Pal from *E. coli*.

In the *in vivo* studies, there were some caveats: we discovered that detecting *E. coli* Pal in mouse urine is far more challenging than expected, due to the inherent difficulties in collecting mouse urine AND the realities of fecal contamination. We would like to improve our mice sepsis model, as currently our sepsis model mimics an acute infection/sepsis; many human patients experience a much slower progression of infection and sepsis. We would also like to perform experiments with a larger group of mice in order to obtain statistically significant results. Also, we cannot rule out the possibility that our blots were detecting light and/or heavy chain mouse IgG instead of Pal; since we used goat anti-mouse HRP as our secondary antibody, we may have inadvertently directly detected mouse IgG fragments, which would appear at ~25 and 50 kDa. Our detected "Pal" bands ran close to 25 kDa, and therefore may actually be light chain IgG instead of Pal. We will run further experiments using different antibody combinations to verify our findings. Finally, we aim to use protein controls, like creatinine, to normalize our Pal levels in urine, and to perform similar experiments with different combinations of antibiotics.

Final Conclusions and Future Work

In both our *in vitro* and *in vivo* studies, we showed that ampicillin, a beta-lactam antibiotic, enhances Pal's release from *E. coli*. We propose that ampicillin inhibits the synthesis of peptidoglycan, thus allowing for more efficient Pal release, since it is no longer tethered to the cell wall. We were able to show that Pal is ***likely*** released inside OMVs, although Pal may also be released among the cellular debris, especially when the cells are treated with ampicillin.

Many questions about Pal and its role in sepsis remain unanswered. First, we do not know the exact mechanism of Pal release. We do not know how or if Pal becomes untethered to peptidoglycan prior to its release from *E. coli*, or if part of the peptidoglycan comes along with Pal during release. We also do not know what specifically triggers Pal's release from *E. coli*, or how Pal might be sequestered inside an OMV. Further studies are required to better understand the mechanism of Pal release and its relationship with antibiotics, other than the three antibiotics tested in this work.

Furthermore, our mouse model of sepsis requires much optimization before more definitive conclusions can be made. For example, we aim to implement a monomicrobial mouse model of sepsis that more closely follows the typical, slower progression of sepsis in humans. We propose using a clot model in which *E. coli* is embedded in a blood clot, which is placed into the mouse body cavity via IP injection. We also propose using different antibiotics, lower doses of antibiotics (closer to the MIC values) and administering antibiotics in several doses.

The long-term goal of this work is to A) confirm the role of Pal in sepsis; B) elucidate the detailed mechanism of Pal's release from *E. coli*; and C) to assess the viability of Pal as a urine biomarker for the detection of *E. coli* sepsis in human patients.

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