Implicating E. coli PAL in the pathogenesis of Gram-Negative sepsis

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Implicating *E. coli* PAL in the pathogenesis of Gram-Negative sepsis

Bethany R. Novick

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry in the School of Chemistry and Materials Science, College of Science, Rochester Institute of Technology

May 2016
The M.S. Degree Thesis of Bethany R. Novick has been examined and approved by the dissertation committee as satisfactory for the thesis required for the M.S. degree in Chemistry.

Dr. Lea Michel, Thesis Advisor

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Dr. Thomas Kim

Dr. Ravinder Kaur

Date
Abstract

Just under half of all sepsis cases are caused by Gram-negative bacterial infections. Sepsis occurs when an infection results in a systemic inflammatory response, which can lead to organ dysfunction and death. Studies have shown that some outer membrane proteins, lipoproteins, and lipopolysaccharides are released from Gram-negative bacteria during sepsis; those proteins and LPS toxins are thought to induce the over-exuberant host inflammatory response, which is characteristic of sepsis. The ability to detect those proteins and determine the role they play in the development and severity of sepsis infection would be a powerful tool for immunotherapy. *Escherichia coli* (*E. coli*) are Gram-negative bacteria that release Lipopolysaccharide and several lipoproteins, including peptidoglycan-associated lipoprotein (Pal), which we propose is a bacterial mediator of Gram-negative sepsis. The goal of this study is to detect released Pal in human sera or urine of Gram-negative sepsis patients using purification and protein detection techniques in order to further implicate Pal in the pathogenesis of *E. coli* sepsis. Our findings suggest *E. coli* is capable of releasing Pal into both human sera and urine of patients with Gram-negative sepsis.
Acknowledgements

Firstly, I would like to acknowledge the School of Chemistry and Materials Science at RIT, for the constant direction and allowing me to pursue this project, especially my committee, Dr. Thomas Kim, Dr. Ravinder Kaur, and Dr. Suzanne O’Handley for providing me with the feedback and their vast knowledge of Biochemistry over the past two years. I also would like to thank Dr. Michael Pichichiero, Dr. Roberto Vargas and Dr. Robert Zagursky of Rochester General Hospital for collaborating on this project.

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None of this work would have happened without the love and support of my family. I would like to thank my siblings: Stephen, Lindsey and Lauren, my grandma and my godparents Aunt Kathy and Uncle Mike for supporting my mental sanity over the past two years and reminding me that “everything is going to be ok”. I would like to thank my parents for investing, not only in my education, but also in all my life’s ambitions and for the constant support to allow me to become whom I have today.

Finally, and most importantly, I must thank my research advisor, Dr. Lea Vacca-Michel. Lea has gone above and beyond the role of an advisor and I have had the pleasure of working with her for the past 6 years. Lea has supported all my ideas, even the not-so-great ones, throughout this project and allowed me to develop more as a scientist over the past two years than ever possible previously. I am grateful for my time and experience working in Lea’s Lab, as I know it will the fondest memory of my scientific career.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>GN</td>
<td>Gram-negative</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer Membrane Protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>GNS</td>
<td>Gram-negative sepsis</td>
</tr>
<tr>
<td>PAL</td>
<td>Peptidoglycan-associated Lipoprotein</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic Immune Response Syndrome</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>OmpA</td>
<td>Outer Membrane Protein A</td>
</tr>
<tr>
<td>MLP</td>
<td>Murein Lipoprotein</td>
</tr>
<tr>
<td>Lpp</td>
<td>Braun’s Lipoprotein</td>
</tr>
<tr>
<td>CLP</td>
<td>Caecal Ligation and Puncture</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDaltons</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodiumdodecylsulfate</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like Receptor 4</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like Receptor 2</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>KAN</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>NaPi</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline: Tween20</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish peroxidase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary Tract Infection</td>
</tr>
<tr>
<td>NTHi</td>
<td><em>Nontypeable Haemophilus Influenzae</em></td>
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1. Introduction

Sepsis in the United States

Sepsis is a disease caused by the effects of a systemic infection accompanied by host hyper-immune response. In the United States, there are an estimated 751,000 cases of sepsis annually, with a mortality rate of about 30% [1]. Approximately 51% of sepsis cases will receive intensive care treatment, and an additional 17% will receive ventilator support in an intermediate care unit or a coronary care unit. The burden of sepsis-related costs to healthcare comes to $17 billion each year [1]. The number of sepsis cases has increased over the last decade, making sepsis the tenth leading cause of death in the United States [2]. Older patients and those with chronic diseases or immune deficiencies are most susceptible to sepsis and are most likely to develop severe sepsis conditions [1-3].

Incidence of sepsis continues to increase due to the persistent use of antibiotics to treat infection and immunosuppressive drugs to treat cancer, HIV/AIDS and autoimmune/auto inflammatory disease, as well as the emergence of antibiotic resistant microbes [3]. Sepsis can also occur through burns, appendicitis, and pneumonia, but more than half of all sepsis infections are nosocomial, resulting from infiltrated intravascular catheters, non-sterile surgical equipment, and other hospital sources [4]. Historically, Gram-positive bacteria have caused the great majority of sepsis cases; however, in more recent years it has been shown that the number of sepsis cases caused by Gram-negative bacteria is increasing [5]. Of the Gram-negative bacteria, the two most common in causing sepsis infections are Escherichia coli (E. coli) and Pseudomonas aeruginosa [5].

Categorization and physiopathology of sepsis infection

Sepsis is categorized based on the level of severity of infection and the progression of host immune response. Systemic Immune Response Syndrome (SIRS) is the first indicator of an infection becoming septic and is diagnosed through white blood cell levels [6]. Severe sepsis will develop if the infection goes untreated, resulting in organ dysfunction [7]. Septic shock occurs when the infection develops further and is accompanied by hypotension, causing the patient’s blood vessels to collapse [8, 7]. At that point, the patient cannot maintain normal blood pressure,
thus requiring ventilator support and sometimes resulting in death [8, 7]. The renal, central nervous, respiratory, and cardiovascular systems are most commonly affected by sepsis [5].

Upon onset of infection, bacteria release toxins into the bloodstream of the host, which interact with the host cell pattern recognition receptors, including the toll-like-receptors. Those receptors then trigger the innate immune response [5]. In Gram-negative sepsis (GNS), the endotoxin lipopolysaccharide (LPS) is released into the plasma initiating the host immune response [9,10]. The innate immune response, usually localized to one area within the body, causes release of pro-inflammatory cytokines, including tumor necrosis factors and interleukins, in an attempt to control the level of infection [5]. When the pro-inflammatory mediators are introduced into the bloodstream, full body (systemic) and/or hyper-inflammation can occur, which can result in tissue damage, necrotic cell death, and multiple organ failures [11]. The immune system will react to the hyper-inflammation by releasing anti-inflammatory mediators, including serotonin and histamine, to control the overwhelming inflammatory response [8]. This period of immunosuppression following the state of hyper-inflammation results in hypotension and can lead the patient into septic shock.

The most critical determinants of recovery from sepsis are speed and appropriateness of therapy administered in the initial hours after diagnosis. Antibiotics must be administered within 24 hours of sepsis onset in order to combat the infection before the bacterial load becomes too great [12]. However, about one-third of patients being treated for sepsis receive inappropriate antibiotic therapy. Examples include those administered after 24 hours of sepsis onset or antibiotics to which the bacteria are resistant [4]. Over the last 30 years, many strains of bacteria have become resistant to the currently available antibiotics, due to their increased use in the United States. Thus, it can prove very difficult to successfully treat a patient with just one antibiotic. Immunotherapy targeted to LPS endotoxin was believed to be the most promising new GNS therapy for treatment [13]. However, to date, all antibody therapies targeting the endotoxin core have been unsuccessful [13], underlining the urgent need for new therapeutic targets to GNS.
LPS as a target for sepsis treatment

LPS, the highly conserved endotoxin that is released by Gram-negative bacteria during infection, has been extensively studied as a mediator of GNS [14]. LPS has been shown to activate host immune cells through Toll-like receptor 4 [9]. The structural core of LPS is highly conserved, while the O-polysaccharide side-chain is varied among different Gram-negative bacteria [14]. Because of its known ability to activate cytokines, LPS has been a leading candidate for anti-sepsis therapies using monoclonal antibodies.

In one pivotal 1982 clinical study, scientists showed that treatment with human antiserum raised against the heat-killed rough mutant strain of *E. coli* J5, which features the LPS core without the O-polysaccharide chains, reduces death by GNS [14]. Over the next several decades, multiple investigators tested the concept of treating GNS with antibody directed towards the endotoxin core by passively immunizing with antisera against the *E. coli* J5 rough mutant. In 1997, immunoblot analysis using the *E. coli* J5 antiserum showed that, surprisingly, the J5 antiserum did not bind LPS, as expected. [15]. However, Hellman and coworkers did observe antiserum binding to three major OMP’s present in both whole *E. coli* O18 and the purified OMPs from *E. coli* O18 [15], suggesting that antibodies to these OMP’s may have also contributed to the therapeutic nature of the J5 antisera. In addition, Hellman and coworkers hypothesized that these OMP’s may also contribute to the pathogenesis of GNS.

The J5 antiserum contained high titers of Immunoglobulin G (IgG), which had known interactions with OMP’s [15]. Specifically, the anti-J5 IgG antibodies bound to three OMP’s, which were later determined to be Outer membrane protein A (OmpA), Murein lipoprotein (MLP or Lpp), and Peptidoglycan Associated Lipoprotein (Pal). Hellman and coworkers showed that these proteins were released from *E. coli* in the sera of septic mice in complex with LPS, and thus were thought to be the targets for the observed antibody protection [13]. Much to the surprise of the scientists involved, neither monoclonal nor polyclonal antibodies against Lpp and Pal offered protection in a rat sepsis model. In light of the highly successful J5 antiserum treatment, scientists and medical professionals were puzzled by the lack of success in treating GNS patients with antibody against LPS, Lpp, and Pal individually.

A deeper look at this study reveals the major limitations that exist within the methods used. Prior to this study, the anti-Lpp and anti-Pal antibodies were not assessed for their ability to neutralize an inflammatory response caused by Lpp or Pal. The anti-Lpp and anti-Pal were also
administered prior to inducing sepsis in the mice; a more clinically appropriate method would consist of multiple antibody doses and a post-treatment regiment. Furthermore, the models employed in the study were acute and the majority of deaths were observed in the first 72 hours of the study. While the model is similar to that of human sepsis, it is not fully replicative. Patients usually survive the acute systemic inflammatory response and die later due to organ dysfunction. The combination of these limitations makes it impossible to determine if passive immunization against Lpp and Pal would prove therapeutic in humans with sepsis. Further studies are needed, using better sepsis models with post treatment strategies to deliver IgG. It is also crucial that the antibodies against Lpp and Pal be analyzed for their ability to neutralize the inflammatory response caused by these lipoproteins prior to introducing sepsis.

**Outer Membrane Protein Release in GNS**

In 2000, scientists showed that the three OMP’s that interacted with J5 antisera were also released into human serum *in vitro* as an OMP-LPS complex [16]. This study also showed the individual release of at least one OMP protein with an estimated molecular mass of 18 kDa [16]. The three OMPs were estimated to have molecular masses of approximately 35, 18, and 5 to 9 kDa [16].

Through immunoblot analysis using protein deficient bacterial strains, the three OMP’s were identified as muerin lipoprotein (MLP), also known as Braun’s lipoprotein (LPP) (5 to 9kDa), outer membrane protein A (OmpA) (35-kDa), and peptidoglycan-associated lipoprotein (Pal) (18kDa) [16]. MLP, OmpA, and Pal proteins have been well studied in their native *E. coli*, and have been shown to be involved in the maintenance of cell-wall integrity via an OMP-peptidoglycan web-like interaction [14].

**Peptidoglycan-associated lipoprotein**

Pal is a highly conserved OMP among many different types of Gram-negative bacteria. *E. coli* Pal is a bacterial lipoprotein, with an N-terminal lipid moiety anchoring it to the outer membrane of the bacterium [10]. Like all Gram-negative bacteria, *E. coli* contains an inner and outer membrane, which is separated by an aqueous layer, the periplasm [17]. This aqueous layer contains peptidoglycan, which is a mesh-like layer of amino acids and sugars that provides the
cell with structural support [17]. E. coli Pal has been well studied for its role in the Tol-Pal complex, interacting with the peptidoglycan layer [18, 19], LPP [20] and OmpA in the outer membrane [21], TolB in the periplasm [22, 19], and TolA of the inner membrane [23, 24]. While known for years as the periplasmic protein that interacts with members of the Tol-Pal complex, Pal was more recently shown to exist in an alternate, surface exposed form, as well.

In 2013, the Michel group demonstrated that Pal’s homologue in nontypeable Haemophilus influenzae (NTHi), P6, is dual-oriented: in other words, one subpopulation of P6 was anchored into the outer membrane and faced out into the extracellular space (i.e., surface exposed), while another subpopulation of P6 was anchored into the inner leaflet of the outer membrane and faced into the periplasm, thus allowing it to interact with peptidoglycan [25]. Two years later, the Michel group showed that E. coli Pal was also dual oriented in a very similar manner to P6 [25]. Further, a biotinylation labeling experiment showed that the majority of Pal was oriented facing into the cell, and only ~5-25% of the Pal population was surface exposed (Figure 1).

Interestingly, LPP of E. coli has also been shown to exhibit dual orientation [26]. In light of the findings that both LPP and Pal are dual-oriented and released from E. coli, we proposed that Pal’s dual orientation might play an important role in allowing for its release from the surface of the bacterium. Further, we hypothesize that the two subpopulations of Pal (surface-exposed and internal) are released in two forms- one released as a complex with LPS, OmpA, and LPP and one released on its own.
**Figure 1:** Schematic of the dual orientation of Pal, in which there are two subpopulations of PAL in the outer membrane of *E. coli*: one in which Pal faces out toward the extracellular space (A) and one in which PAL faces in toward the periplasm and interacts with peptidoglycan (B).

**Pal release and the pathogenesis of Pal in GNS models**

In order to study the pathogenic nature of Pal, scientists employed the use of both *in vitro* and *in vivo* sepsis models. Examples of commonly used sepsis models include cecal ligation and puncture, macrophage-cultured supernatants, and live mouse models. Cecal ligation and puncture (CLP) in mice involves puncturing the intestines and allowing the naturally existing *E. coli* in the gastrointestinal tract to spill into the bloodstream. This model is often used to study sepsis, as it most closely resembles the progression and characteristics of human sepsis [27].

In 2002, a CLP mouse model study showed that Pal was released from *E. coli* into the bloodstream of the septic mouse. Plasma from two groups of mice (CLP mice and healthy mice) was collected 24 hours after puncture; Pal was detected via immunoblotting in the sera of CLP mice only. Pal was detected in 94% of CLP mice (~28 ng/mL), whereas no Pal was detected in the blood sera of healthy mice [27].

In a separate study, Hellman and coworkers showed that Pal was capable of stimulating an immune response in macrophages in cultured supernatant. Various doses of Pal were injected
into human macrophage-cultured supernatants and levels of interleukin-6 (IL-6) and tumor necrosis factor (TNF) (two cytokines released by macrophages in GNS) were measured. Data revealed that concentrations of Pal greater than 5ng/mL showed a dose dependent increase in production of both IL-6 and TNF, thus demonstrating that Pal stimulates an immune response in an *in-vitro* human model [27]. In a similar study, Pal was injected into mice to determine the cytokine response. One hour after Pal was injected, TNF-α, IL-6, and IL-1β levels were expressed at maximum levels, thus demonstrating that Pal is capable of inducing an inflammatory response in an *in-vivo* mouse model [27].

Another mouse model was implemented to show that Pal was toxic. In the study, mice were treated with D-galactosamine, a chemical that makes mice hyposensitive to the effects of LPS. By using D-galactosamine, scientists were able to study the immune response from Pal without an interfering response from LPS [27]. Mice injected with high concentrations of Pal showed a significantly lower survival rate compared to those injected with lower concentrations of Pal; however, Pal was shown to cause death at all concentrations [27].

The immune response stimulated in mouse sepsis models demonstrates the virulent nature of Pal in sepsis. A 2002 study sought to further implicate Pal in sepsis using Pal-nonsense and Pal-deficient mutants. The Pal-nonsense mutant contained a premature stop codon in the Pal gene, creating a truncated version of Pal, while the Pal-deficient mutant contained Pal protein at a reduced level [27]. LPS-responsive mice injected with the PAL mutants showed a significant increase in survival compared to the mice injected with wild-type (WT) *E. coli*. Infection by the Pal-nonsense mutant resulted in 100% survival of mice, compared to infection by the Pal-deficient mutant, which resulted in 33% survival and infection by wild-type *E. coli*, which resulted in 7% survival. The immune response against each of these mutants was also observed and compared to wild-type. Pal-nonsense *E. coli* stimulated no response from IL-6, while the Pal-deficient mutant stimulated an IL-6 response less than half of that observed with wild-type *E. coli*. When bacterial blood levels were measured, both mutants exhibited levels less than that of wild-type *E. coli* (by a factor of 10) [27]. Taken together, these studies point to Pal being toxic and involved in stimulating the immune system.

Scientists proposed that Pal would have synergy with LPS, due to the fact that they both activate Toll-like receptors. While the Toll-like receptors (TLRs) shared the same pathways, there were differences in the activation patterns and the signaling pathways between the TLRs.
Bone marrow-derived macrophages were collected from mice and incubated in cultured supernatants with three different supplements: one with Pal alone, one with LPS alone, and a third with Pal and LPS [10]. The cytokine responses of macrophages in each of these samples were analyzed. In the Pal only samples, IL-6 levels were about 2.5 ng/ml; in the LPS only samples, IL-6 levels were less than 1 ng/mL; in the LPS/PAL samples, IL-6 concentration was 7 ng/mL, confirming that synergy between Pal and LPS did exist and suggesting that multiple microbial components could be involved in the clinical onset of sepsis [10]

A similar study using human serum was performed and showed when LPS was incubated in vitro in 20% human serum, the cytokine response levels, specifically TNF, were measured. In healthy human serum, the levels of induced TNF were between 5000-10,000 pg/mL (compared to induced TNF levels in mice which were ~5000 pg/mL) [9]. In combination, Pal and LPS stimulated a similar response to that of LPS on its own in human serum. These differences suggest that mouse sepsis models may not be a close representation of human sepsis with regards to immune response.
Thesis Project Proposal

In summary, a significant amount of work has already implicated Pal in the pathogenesis of GNS in vitro in human model and in vivo in mice and rats. The main candidate for GNS immunotherapy has historically been LPS, which has known pathogenic properties; however, the J5 antisera studies demonstrated immunoglobulin in J5 binds Lpp and Pal, not LPS [14,15]. Although anti-Lpp and anti-Pal did not offer the same protection as seen with the J5 antisera, other studies showed that 1) Pal stimulates cytokines in vivo in mice and in vitro in human serum; 2) Pal is released in vivo in mice and in vitro in human serum; 3) Pal is toxic in mouse sepsis models; and 4) Pal and LPS act synergistically to a cytokine response in macrophages from mice.

The number of sepsis cases increases each year with the increase in number of medical procedures and use of immunosuppressant drugs; unfortunately, therapy for sepsis patients is limited and the death rate is close to 30%. Currently, sepsis costs the United States close to $17 billion each year, thus underlining the need to identify new immunotherapeutic targets in humans with sepsis.

Here, we describe the first study to look at bacterial Pal in human patients with sepsis. Using immunoprecipitation techniques, immunoblotting, and mass spectrometry, we aim to detect E. coli-released Pal protein in the sera and/or urine of patients with E. coli sepsis. While past studies have focused on Pal in sera, we have decided to include urine in our experiments. We hypothesize that toxic bacterial proteins circulating in the bloodstream may be filtered by the kidneys and into the urine during the sepsis condition. Since urine typically contains far fewer proteins compared to sera, we propose it will be easier to detect small amounts of Pal in urine. We also propose that the concentration of released Pal will be directly correlated to the patient’s severity of sepsis (i.e., increased bacterial load will result in a greater concentration of released Pal). The results of our studies will further implicate Pal in the pathogenesis of Gram-negative sepsis, with the long-term goal of developing therapeutics targeted to Pal.
2. Over-expression and Purification of Recombinant Pal protein

The overall goal of this project was to develop a technique to detect *E. coli*-released Pal in the serum and urine of GNS patients. In order to develop and optimize a method for detection of Pal in human serum, a recombinant Pal protein was needed as a control. The recombinant Pal allows us to supplement samples of healthy sera and urine test our methods for detection of Pal at higher concentrations to start before determining the lowest level of detection.

Methods

DNA Transformations of Topo151/PET28A

The DNA sequence that codes for the protein of interest, Pal in *E. coli*, was edited for purification purposes and ordered from GenScript in an ampicillin resistant DNA storage vector (TOPO151). A start codon and a 6-histidine tag were added to the N-terminal end of the Pal sequence. The DNA plasmid was then transformed into competent XL1-Blue (Agilent) and BL-21 (Invitrogen) *E. coli* cells, as described by the manufacturers. In summary, the lyophilized DNA received from GenScript was reconstituted with sterile water to a final concentration of 50 ng/µL; 1 µL DNA was added to the competent cells, incubated on ice, and heat shocked at 42°C (XL1-Blue: 45 seconds, BL-21: 30 seconds). Super Optimal broth with catabolite repression (S.O.C media) was added to the cells, followed by incubation for 1 hour at 37°C, shaking at 225 rpm. The transformed cells were plated onto Luria broth (LB) supplemented with ampicillin (50 µg/mL) and incubated at 37°C overnight. The following morning, a single colony was used to inoculate 5 mL of LB supplemented with ampicillin (50 µg/mL). The small culture was grown (37°C, shaking at 225 rpm) until the optical density at 600 nm is reached between 0.8-1.0. A freezer stock was prepared with 900 µL of cell culture and 100 µL of an 80% glycerol solution, flash frozen in liquid nitrogen, and stored at -80°C. A similar recombinant Pal gene was placed into a kanamycin-resistant plasmid (pET28a), and the DNA was prepared in a similar manner as described above.
**Pal Expression in BL-21 E. coli**

Recombinant Pal protein was over-expressed and purified as described for Pal’s homologue, P6, from nontypeable *Haemophilus influenzae* [25] with the following modifications. BL-21 cells containing the Pal plasmid (described above) were cultured on LB supplemented with Kanamycin (Kan) (50 µg/mL Kan: pET28a plasmid) or ampicillin (50 µg/mL Amp: TOPO151 plasmid). Liquid cultures were grown in 25 mL LB supplemented with Amp or Kan at 30°C, shaking at 180rpm for ~16 hours. Small growths were used to inoculate 1 L LB (supplemented with appropriate antibiotic), which was then incubated at 30°C, shaking at 180 rpm until the optical density at 600 nm was reached between 0.6-0.8; over-expression was induced with 1 mM IPTG, and the cultures were allowed to grow an additional 3 hours. The cells were harvested by centrifugation (5000xg, 15 minutes, 4°C), and the cell pellets were stored at -20 °C.

**Protein Purification**

Each frozen cell pellet (from 1 L of culture) was resuspended in 15 mL of 40 mM Tris pH 8.0 + 0.5% Triton X-100 buffer and sonicated on ice for a total of 15 minutes (15 seconds on and 45 seconds off) at a medium horn setting. The lysed cells were centrifuged (20,000xg, 25 minutes) and the supernatant was kept for further processing.

TALON resin (Clontech) was prepared as described by the manufacturer. Briefly, 2 mL beads equilibrated with 5-10 bead volumes of equilibration buffer (50 mM Sodium phosphate (NaPi), 300 mM NaCl, pH 7.0). The supernatant was incubated with the TALON beads, with end-over-end mixing, for 30-60 minutes at room temperature. The protein was eluted using Elution Buffer (50 mM NaPi, 300 mM NaCl, 150 mM imidazole, pH 7) and collected in 2-3 mL fractions. Optical absorbance (280nm) was used to determine which fractions contained protein, and a standard Bradford Assay was performed to determine the concentration of the purified protein. An SDS-PAGE gel was performed to confirm purity.
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE gels were prepared using a standard 10% recipe [resolving gel: 3.27 mL of 30% acrylamide/bis-acrylamide, 3.33 mL Tris/SDS (Tris/SDS solution: 182 g Tris base, 1.5 g SDS, pH 8.0), 1.38 mL nanopure water, 2.12 mL 50% glycerol, 100 µL 10% ammonium persulfate (APS), and 10 µL tetramethylethylenediamine (TEMED); stacking: 405 µL 30% acrylamide/bis-acrylamide, 775 µL Tris/SDS, 1.95 mL nanopure water, 20 µL 10% APS, and 5 µL TEMED]. A BioRad gel system was used to cast the gels, and the gel was run in standard buffers (10X Cathode buffer: 60.6 g Tris base, 89.6 g Tricine, 5 g SDS, 500 mL water; 10X Anode buffer: 121.1 g of Tris base, 500 mL water, pH adjusted to 8.9 using 12 M hydrochloric acid). The protein samples were prepared by mixing 1:1 with either 2X-sample buffer: 4 mL of 10% SDS, 2 mL Glycerol, 1.2 mL of 1 M Tris pH adjusted to 6.8, 2.8 mL of H2O, 0.001-0.002 g bromophenol blue; or 2x-reducing buffer: 900 µL 2X-sample buffer, 100 µL β-mercaptoethanol, and boiled for 10 minutes.

All protein samples were loaded onto the gel (14 µL) alongside a Kaleidoscope protein ladder (BioRad and ThermoScientific) (5 µL). Proteins were separated for between 30-45 minutes (120-155 V). Gels that were not transferred for Western blot analysis were incubated for 15 minutes in 15 mL of InstaBlue gel dye (Fisher).

Semi-Dry Transfer

Protein bands were transferred to a nitrocellulose membrane using a semi-dry transfer technique. 2 filter papers (8 cm x 10.5 cm), a nitrocellulose membrane (0.45 micron, 8 cm x 8 cm), and the SDS-PAGE gel were equilibrated in transfer buffer (2.91 g Tris base, 1.47 g glycine, 100 mL methanol, 400 mL of nanopure water) for at least 10 minutes. A “sandwich” was prepared on the bottom electrode plate of the BioRad Trans-Blot SD semi-dry transfer system: filter paper, nitrocellulose membrane, gel, filter-paper (layered bottom-up). Air bubbles were removed and the stainless steel cathode and lid were placed over the sandwich. The proteins were transferred at 15 V for 15 minutes.
**Western Blot Protocol**

Transferred nitrocellulose membranes were blocked in 7 mL 5% (m/v) evaporated milk in 1x Tris-buffered saline (TBS) (50 mL of 10X TBS: 80g NaCl, 2g KCl, 30g Tris Base dissolved in 950 mL H₂O pH adjusted to 7.4 with HCl, and sterile filtered; diluted to 500 mL with H₂O) for 30 minutes, followed by a one hour room temperature incubation with primary antibody [1 µL of Monoclonal anti-Pal or 1.5 µL of Polyclonal anti-Pal in 6 mL of 1% (m/v) evaporated milk in TBST (100 mL 1x TBS, 50 µL TWEEN-20, 1 g powdered milk)]. After two washes (1x TBST, 15 minutes each), the membranes were incubated in secondary antibody [0.5 µL of secondary antibody (2°): Goat anti-mouse IgG-H+L HRP conjugate (BETHYL laboratories) in 6 ml of 1% (m/v) evaporated milk in TBST] for 30 minutes. After three washes (1x TBST, 10 minutes each) and an additional two washes (1x TBS, 5 minutes), Pal was detected using a Lumiglo reserve HRP chemiluminescent substrate and a BioRad BioDoc system (Quantity One software).

**Mass Spectrometry Sample Preparation**

Protein bands (stained with InstaBlue dye) were excised from gels using a clean razorblade and placed in a microcentrifuge tube. The samples were kept cold, and analyzed via Liquid Chromatography-Mass spectrometry at the University of Rochester Mass Spectrometry (MS) Center. One sample of recombinant purified Pal was prepared: 5µM of protein dissolved in 0.1% MS grade formic acid, 20% MS grade acetonitrile and 80% water and analyzed via liquid chromatography mass spectrometry (LC-MS) at Rochester Institute of Technology.
Results and Discussion:

The TOPO151 vector was transformed into *E. coli* BL-21 cells and the promoter on the Pal gene was activated with IPTG to over-express Pal protein. A Western blot (Figure 2.1) shows the over-expression of Pal in the TOPO151 vector. The harvested cell pellet contains most of the expressed Pal protein, before and after sonication, while the supernatant/lysate shows much less Pal. Purified Pal shows up as two different molecular weight bands: ~25 kDa (non-monomer Pal) and ~17 kDa (monomer Pal).

![Figure 2.1. Pal over-expressed in TOPO151 plasmid.](image)

To further examine the two forms of Pal, we expressed Pal in *E. coli*, grown at 37 °C instead of 30°C. Also, an alternate buffer (40 mM Tris + 0.5% Triton X100, pH 8.0) was used to re-suspend the *E. coli* cells prior to sonication. Figure 2.2 shows detection of Pal in the cell pellet and cell lysate (after sonication). Two different molecular weight Pal bands are prominent in those samples. After purification via TALON beads, only a single higher molecular weight Pal (~25 kDa) was detected.
Figure 2.2. Pal over-expressed in TOPO151 plasmid, lysed in Tris/TritonX100 buffer. Two forms of Pal are detected in the cell pellet and lysate (after sonication), but only a single higher molecular form of Pal is detected after purification via TALON beads.

Several other attempts were made to obtain Pal at its correct molecular weight (~17 kDa). Briefly, *E. coli* Bl-21 (TOPO151 plasmid) were grown at 30°C and sonicated using the new Tris/Triton buffer. Samples were prepared in 2X-Sample buffer and incubated at 100°C, 37°C, or room temperature for 10 minutes. **Figure 2.3** shows that Pal was detected in both forms (17 kDa, 25 kDa) for all incubation temperatures; incubations at lower temperatures resulted in a larger percentage of the higher molecular Pal.
**Figure 2.3.** Pal over-expressed in TOPO151 plasmid, lysed in Tris/TritonX100 buffer; gel samples prepared at 100°C, 37°C, or room temperature. Two forms of Pal are detected in the cell pellet and lysate (after sonication) under all conditions, but a greater percentage of the higher molecular weight Pal was detected in samples prepared at lower temperatures.

In a second attempt to break up Pal-25 we added a reducing agent, β-mercaptoethanol, to the 2X-sample buffer. **Figure 2.4** shows that the lower molecular weight Pal-17 is only detected in the cell pellet after sonication. Only Pal-25 is detected in the cell lysate before and after purification.
Figure 2.4. Pal over-expressed in TOPO151 plasmid, lysed in Tris/TritonX-100 buffer; gel samples boiled in reducing sample buffer. Two forms of Pal are detected in the cell pellet after sonication; only Pal-37 is detected in the lysate before and after purification.

The final optimal expression and purification conditions were determined, as follows: *E. coli* BL-21 (containing TOPO151 Plasmid with Pal gene) cells were grown at 30°C; a surfactant (Triton X-100) was added to the His-tag column equilibration buffer; fractions were collected off the TALON bead column, prepared in reducing buffer, and boiled for 10 minutes. Figure 2.5 shows Pal purified under these conditions. A single Pal-25 band is detected following purification. Further analysis determined that our methods yielded ~2 mg of purified Pal-25 per 1 L of culture.
Based on the amino acid sequence of the Pal gene, we estimated that the Pal monomer should be approximately 20.6 kDa (which includes the N-terminal His tag). After many failed attempts to break up the Pal-25 into its 21 kDa form (ex. using 2M, 4M, 6M and 8M Urea), we contacted GenScript. After analyzing the sequence of the Pal gene, we determined that there were two start codons in the plasmid, the first further up-stream than our designed start codon, thus creating a double His-tag at the N-terminal end of the protein and resulting in a 25 kDa protein (Figure 2.6).
A second gene was ordered from GenScript with a single His-tag, contained in the pET28a Kan-resistant vector. The same optimized over-expression and purification protocol developed for Pal-25 was used to purify the new pET28a version of Pal in *E. coli* BL-21 cells. Surprisingly, as shown in Figure 2.7, the new purified Pal traveled to ~25 kDa on an SDS-PAGE gel.

However, analysis of the protein via mass spectrometry suggested that the molecular mass of the new purified Pal was indeed 20.6 kDa, suggesting that the new Pal gene was correctly synthesized, but that the purified Pal protein traveled as a heavier protein during SDS-PAGE.
Figure 2.8. Western blot of purified “new” Pal (pET28a), detected with a monoclonal anti-Pal antibody. Monoclonal anti-Pal detects several versions of “new” Pal as it is eluted off the TALON bead column. Purified Pal runs between 20-25 kDa (red rectangle). A non-His-tagged lower molecular weight version of Pal is detected in the lysed cell pellet and cell lysate (supernatant), prior to purification (blue rectangle).

Pal was eluted off the TALON bead column in fractions, and samples were analyzed via immunoblotting. Monoclonal anti-Pal antibody (Figure 2.8) detects several different Pal populations, including what we propose to be dimerized Pal (~40 kDa). The majority of the purified Pal, though, still has molecular weight between 20-25 kDa, suggesting the Pal is represented as a heavier protein than expected. The same samples were detected using the polyclonal anti-Pal antibody (Figure 2.9), showing similar results. Surprisingly, both monoclonal and polyclonal anti-Pal appear to detect a lower molecular weight version of Pal (~17 kDa) in the lysed cell pellet and cell lysate (supernatant). During the purification process, this band disappears, suggesting this is a non-His-tagged version of Pal.
Figure 2.9. Western blot of purified “new” Pal (pET28a), detected with a polyclonal anti-Pal antibody. Polyclonal anti-Pal detects several versions of “new” Pal as it is eluted off the TALON bead column. Purified Pal runs between 20-25 kDa (red rectangle). A non-His-tagged lower molecular weight version of Pal is detected in the lysed cell pellet and cell lysate (supernatant), prior to purification (blue rectangle).

In summary, the optimal conditions for over-expression and purification of Pal were determined, as followed: a single His-tagged Pal gene in pET28a (Kan-resistant) was transformed into E. coli BL-21 cells; cells were grown at 30˚C and over-expression was induced with IPTG; thawed cell pellets were solubilized in equilibration buffer supplemented with Triton X-100, and sonicated for a total of 5 minutes; cell lysates were purified via TALON bead chromatography. All protein samples were analyzed via SDS-PAGE and/or Western blotting. To prepare the gel samples, protein was boiled in reducing 2X-sample buffer (with β-mercaptoethanol). Under these expression/purification conditions, we were able to purify ~1.5 mg/mL of Pal protein. Although purified Pal was ~20.6 kDa, verified by mass spectrometry, the “new” Pal typically ran between 20-25 kDa on an SDS-PAGE gel. We are unsure why our “new” Pal runs at a slightly higher molecular weight on the gel, but propose it may be due to a super-stable secondary structure that is not denatured during SDS/boiling. Interestingly, when “new” Pal is run on a Mini-
PROTEAN® TGX™ pre-cast gel (BioRad), the protein runs at its correct molecular weight (~21 kDa) (Figure 2.10). This finding suggests our recombinant Pal is indeed 21 kDa, but will run as a slightly higher molecular weight protein under certain conditions.

Figure 2.10. Pal runs to its correct molecular weight on a Mini-PROTEAN® TGX™ pre-cast gel
Chapter 2 Conclusions:

The optimal condition for over-expression of the Pal protein in *E. coli* BL-21 cells containing the PET28A plasmid grown at 30°C, solubilized in equilibration buffer supplemented with Triton X-100, sonicated 20 times and boiling the samples in 2X-sample buffer with β-mercaptoethanol prior to SDS-PAGE analysis. We found that the PET28A plasmid transformed in BL-21 *E. coli* cells using these conditions, yields approximately 1.5 mg of protein per 1 L of growth with binding affinity to both anti-Pal antibodies.

Adequate yields of purified soluble Pal have been obtained but the purified Pal travels differently based on the type of gel used. Mass spectrometry analysis confirmed the protein obtained has a molecular weight consistent with that expected, 20.6 kDa, based on the gene sequence and the additional mass from the His-Tag. At this time it is not clear as to why the protein has a marker weight of 25 kDa rather than 20 kDa. This could be due to a secondary structure or the protein not unfolding prior to gel analysis.

Another portion of this project is to look at the interaction between Pal and the peptidoglycan in *E. coli* by mutating the Pal-Peptidoglycan interaction sites. For this study a recombinant purified Pal was needed as a control to observe the changes of the mutants from that of WT. Because our purified Pal is detected at a marker range different than the expected 20 kDa, we decided to use *E. coli* pellet in all detection studies, to detect surface-exposed native Pal.
3. Detection of Pal in Human Serum/Plasma

Pal is one of two dual-oriented OM proteins in *E. coli*. While the biological relevance and function of lipoprotein dual orientation is not well understood, there is strong evidence linking Pal to the pathogenesis of *E. coli* sepsis. We propose that Pal’s dual orientation may play a role in its release into serum.

**Methods:**

*Pierce™ IgG Melon Bead Purification*

To purify IgG from human serum, we used IgG Melon beads (Thermo Scientific Melon Gel IgG Spin Purification Kit 45212), which were swirled to create a slurry. 500 µL bead slurry was added to each spin column (Pierce), and the storage buffer was discarded by centrifuging at 4000xg for 1 minute. The beads were washed twice with 300 µL Purification Buffer (Thermo Scientific Melon Gel IgG Spin Purification Kit 45212), centrifuged at 4000xg for 10 seconds. Serum was diluted 1:10 with the Purification Buffer (Thermo Scientific Melon Gel IgG Spin Purification Kit 45212) and a total of 500 µL sera/buffer was loaded onto each spin column and capped. The column was incubated for 5 minutes at room temperature with end-over-end mixing. The IgG sample was eluted by centrifugation (4000xg, 1 minute), and the column was re-capped. The column was incubated with 500 µL of 2X Sample Buffer for 5 minutes at room temperature with end-over-end mixing, sealed and boiled for 10 minutes. The “bound” samples (non-IgG proteins) were eluted from the column by centrifuging at 10,000xg for 1 minute.

*Millipore PureProteome™ NHS FlexiBind Magnetic Bead Kit*

Monoclonal anti-Pal was conjugated to magnetic beads (Millipore PureProteome NHS FlexiBind Magnetic Bead Kit LSKMAGN01-2) following the manufacturer’s protocol. Briefly, 100uL of slurried magnetic beads were added to a microcentrifuge tube. A magnet was used to pellet the beads so the storage buffer could be removed. 500 µL of Equilibration Buffer (1 mM Tris HCl) was vortexed with the beads for 20 seconds, and the magnet was used to pellet the
beads and remove the buffer. 30 µL of monoclonal anti-Pal antibody was incubated with the beads for 1 hour (or overnight) at room temperature with end-over-end mixing. The antibody was then removed from the tube (and kept for future use in immunoblotting), and the beads were washed five times with 500 µL Quench buffer (100 mM Tris HCl, 150 mM NaCl, pH 8.0). After the final wash, 500 µL Quench buffer was incubated with the beads for at least 1 hour at room temperature with end-over-end mixing. The buffer was removed using the magnet, and the beads were resuspended in 100 µL wash/coupling buffer (PBS) and stored at 2-8ºC for future use (used within a few months of preparation).

To purify the serum samples, 20 µL anti-Pal conjugated beads were washed with 500 µL Wash/Coupling buffer (PBS) and vortexed for 20 seconds. The buffer was removed and 100µL of the sera sample was added to the beads and incubated at room temperature for 2 hours with end-over-end mixing. The “unbound” sample was removed and kept for further analysis (Elution 1). The beads were washed three times with 500 µL Wash/Coupling buffer (PBS), vortexed for 20 seconds, and the buffer was removed from the tube using the magnet to pellet the beads. 50 µL either 2X-sample buffer or reducing buffer was added to the tube and boiled for 10 minutes to elute the sample (Elution 2).

*Pierce*® *Albumin/IgG Removal Kit*

To deplete human urine of albumin and IgG, 170 µL of the Immobilized Cibacron Blue/Protein A Gel (ThermoScientific™Pierce™ Albumin/IgG Removal Kit 89875) was added to a spin column (Pierce). The column was placed in a microcentrifuge tube and centrifuged at 10,000xg for 1 minute to remove the storage buffer. The column plug was placed on the spin column, and the diluted sample (10 µL Urine + 65 µL Binding/wash buffer (ThermoScientific™Pierce™ Binding/Wash buffer)) was added to the gel bed. The sample was incubated with end-over-end mixing for 10 minutes at room temperature. The sample was removed, and the sample was eluted via centrifugation at 10,000xg for 1 minute. The column plug was placed on the spin column and the filtrate was re-added to the gel bed and incubated with end-over-end mixing for 10 minutes at room temperature. The final sample was eluted (10,000xg for 1 minute) into a clean tube. 75 µL Binding/wash buffer was added to the gel bed, and the column was centrifuged at 10,000xg for 1 minute; the filtrate was collected in the
microcentrifuge tube that contained the previous elution. The final sample (~150 µL) contained the urine, which should be depleted of albumin and IgG.

The column with the gel was washed with 100 µL Binding/wash buffer centrifuged at 10,000xg for 1 minute and the filtrate was discarded. The column was plugged and 100 µL 2X-SDS sample buffer was added to the column. The column was capped and incubated with end-over-end mixing for 10 minutes at room temperature. The final sample containing Albumin, IgG and Pal proteins was collected in a microcentrifuge tube by centrifuging the column at 10,000xg for 1 minute.

**Pierce Protein G Antibody Purification**

The Protein G Antibody purification buffers and columns (*Prod. 89949*) were brought to room temperature. The columns were uncapped, placed in 2mL collection tubes, and centrifuged for 1 minute at 5000xg to remove storage buffer. The column was washed with 400 µL Binding Buffer (5000xg), and then incubated with 500µL of sera sample at room temperature with end-over-end mixing for 10 minutes. The sample was eluted into a clean collection tube (5000xg, 1 minute). The final sample, containing sera depleted of IgG proteins, was concentrated using a VivaSpin Turbo 10,000MWCO filter to 50 µL, and then purified using the anti-Pal conjugated magnetic beads.

**E. coli Pal release study in Human Serum**

*E. coli* cells were grown on 10 mL LB media at 37 ºC, shaking at 225 rpm to an OD$_{600nm}$ of ~0.85. The culture was then divided into 7-1 mL aliquots labeled A-F. The cells were pelleted by centrifugation at 5000xg for 5 minutes and resuspended in 1 mL LB (Sample A), 1 mL LB supplemented with 200 µg AMP (Sample B), 1mL LB supplemented with 20 µg AMP (Sample C), 1 mL serum (Sample D), 1 mL serum supplemented with 200 µg AMP (Sample E), 1 mL serum supplemented with 20 µg Amp (Sample F), or 1 mL serum supplemented with 2 µL monoclonal anti-Pal antibody (Sample G). The samples were incubated at 37 ºC, shaking at 225 rpm for an additional 2 hours. The cells were then pelleted via centrifugation at 5000xg for 5 minutes. The following samples were kept for analysis: the pellets from samples A and D resuspended in PBS and mixed 1:1 with 2X-sample buffer; all supernatants were mixed 1:1 with
2X sample buffer. All samples were boiled for 10 minutes prior to being loaded on the gel, and analyzed via a Western blot.
Results and Discussion:

In 2000, scientists showed that Pal, along with 2 other outer membrane proteins, was released into human serum \textit{in vitro} as an OMP-LPS complex [16]. Pal has been well known for its interactions with peptidoglycan and other OMPs, including LPP and OMPA, in the Tol-Pal complex [18,19, 20]. Scientists have also shown, \textit{in vitro} and \textit{in vivo} mouse models, that Pal and Lpp most likely play a role in the pathogenesis of \textit{E. coli} GNS. In a 2002 study, Pal was shown to be released from \textit{E. coli} into the sera of 94% of CLP mice (~28ng/mL), while no Pal was detected in the sera of healthy LPS hypo-responsive (C3H/HeJ) mice [27].

Pal is also capable of stimulating an immunological response in macrophage-cultured supernatants. When various concentrations of Pal were injected into supernatants cultured with human macrophages, there was dose dependent production of IL-6 and TNF [27]. Pal was also injected into mice; 1 hour after Pal injection, the TNF and IL-6 levels were expressed at a maximum level [27]. Mice injected with wild-type \textit{E. coli} (expressing normal Pal) died, while Pal deficient and Pal-nonsense \textit{E. coli} mutants resulted in improved survival rates in mice.

Taken together, there is strong evidence demonstrating that Pal is released in serum during \textit{E. coli} infections, has the ability to stimulate an immune response, and can be toxic to C3H/HeJ mice at any concentration. Based on this evidence, we proposed that we should be able to detect Pal in the serum of humans with \textit{E. coli} infections/sepsis. We sought to collect sera samples from confirmed \textit{E. coli} sepsis and other GNS patients at Rochester General Hospital (RGH). We anticipated challenges such as the abundance of other human serum proteins, the predicted low concentration of released Pal (<10^1 ng/mL), and the ability of Pal to form stable complexes with other OMPs (and potentially with human IgG).

In human serum, albumin and globulin can be present at very high concentrated levels, typically between 6.0 to 8.3 g/mL; in contrast, we predict released Pal levels to be on the order of 10^1 ng/mL. In other words, Pal is likely present at concentrations 10^7 fold lower than the human proteins in sera. To increase our signal to noise ratio, we have used several purification methods to amplify the Pal signal and/or reduce the background protein noise. For example, we can remove ~80% of the total human proteins in sera using different purification and immunoprecipitation methods.
Magnetic bead Purification

Following the protocol established by our collaborator, Dr. Judith Hellman (University of California, San Francisco), we used the Millipore PureProteome NHS FlexiBind Magnetic Bead Kit to isolate Pal from other proteins in serum. The magnetic beads were conjugated to our monoclonal anti-Pal antibody, which had a free N-terminus to allow for conjugation. To determine the sensitivity of Pal detection in sera, post purification using the magnetic beads, we spiked different concentrations of our recombinant, purified Pal into negative (healthy) sera. As seen in Figure 3.1, we were able to detect Pal at 13,000 ng/mL and 5,000 ng/mL. We also detected IgG in our purified samples, suggesting that Pal may have been pulling IgG out of the sera. As seen in Figure 3.1, the reducing SDS buffer (used in this method of purification) dissociated the IgG into its light chain (25 kDa) and heavy chain (50 kDa) fragments. Unfortunately, the recombinant purified Pal ran close to the light chain IgG band. We were, however, able to detect the spiked Pal in sera at these high concentrations, just above the light chain IgG band.

Figure 3.1: Recombinant Pal spiked into negative sera and purified using Anti-Pal conjugated magnetic beads. Recombinant Pal bands were detected just above the light chain IgG bands.
Before we switched to other purification techniques, we analyzed a single *E. coli* sepsis patient sample, obtained from Rochester General Hospital (RGH). The sample was prepared using the Anti-Pal conjugated magnetic beads and eluted in SDS reducing buffer. As seen in Figure 3.2, we detected similar protein bands in the negative (healthy) sera and the *E. coli* sepsis patient sera. The two bands detected in the blot are likely heavy and light chain IgG.

![Western blot](image)

**Figure 3.2: Healthy and *E. coli* sepsis patient sera, purified using Anti-Pal conjugated Magnetic Beads.** Similar bands are detected in healthy and GNS patient sera.

We observed streaking in our Western blots when we ran the unbound samples (due to remnants of the magnetic beads in the samples), so we decided not to run the unbound samples on subsequent westerns. The same purified sera samples from Figure 3.2 were re-prepared and analyzed via Western blot, with 1000 ng of recombinant purified Pal loaded as a control (Figure
3.3). We again observed no significant differences between the negative sera and *E. coli* sepsis patient sera. If Pal was present in the *E. coli* sepsis patient sera, we were unable to detect it.

**Figure 3.3: Healthy and *E. coli* sepsis patient sera, purified using Anti-Pal conjugated Magnetic Beads (unbound samples removed).** Similar bands are detected in healthy and GNS patient sera.

Based on the data collected (Figures 3.2 and 3.3), we decided that the light chain IgG ran too close to where we might detect Pal, so we stopped using reducing sample buffer to elute the samples. *E. coli* sepsis patient 2 and a new negative (healthy) sera sample were prepared using the anti-Pal conjugated magnetic beads and eluted in non-reducing SDS buffer. The samples were analyzed on a Western blot with 500 ng recombinant purified Pal loaded as a control. As seen in **Figure 3.4**, we detected a prominent band around 150 kDa in both the negative sera and patient sample, which is most likely whole IgG protein. We did detect different bands in the negative control and GNS patient sample, but none of the bands appeared to be at the molecular mass we expected for Pal (18 kDa). The majority of the bands were detected in negative controls.
and patient sera, most likely due to non-specific binding of our anti-Pal antibody to non-Pal proteins.

Figure 3.4: Healthy and *E. coli* sepsis patient sera, purified using Anti-Pal conjugated Magnetic Beads and eluted in non-reducing buffer. We did not detect proteins in patient sera at the molecular weight predicted for Pal (18 kDa).

We also used Amicon protein concentrators (10,000 MWCO) to concentrate the sera samples with the goal of increasing the ratio of Pal to other sera proteins. As seen in Figure 3.5, the bands in the purified sera samples were more prominent, but we were not able to detect a protein of similar molecular weight to Pal.
Figure 3.5: Healthy and *E. coli* sepsis patient sera, purified using Anti-Pal conjugated Magnetic Beads and eluted in non-reducing buffer and concentrated in an Amicon concentrator. We did not detect proteins in patient sera at the molecular weight predicted for Pal (18 kDa).

A second *E. coli* GNS patient sample was obtained from RGH, prepared with the anti-Pal conjugated magnetic beads, and eluted with non-reducing SDS buffer. Three positive controls were used for analysis in this study: recombinant purified Pal (50 ng), WT *E. coli* pellet, and a negative sera sample with 500 ng recombinant purified Pal added. As seen in **Figure 3.6**, we did not detect Pal in the patient sample, but we did observe a dark band around 150kDa (likely IgG). In the sample of healthy sera with Pal added, we detected two prominent bands, including a band ~ 50 kDa. We propose that in the presence of sera, Pal forms stable complexes with itself or other proteins in the sera.
Figure 3.6: Healthy and *E. coli* sepsis patient sera, purified using Anti-Pal conjugated Magnetic Beads and eluted in non-reducing buffer. We did not detect proteins in patient sera at the molecular weight predicted for Pal (18 kDa). We propose that recombinant Pal formed at least one stable complex (50 kDa) when spiked into healthy sera.

Since the magnetic bead purification protocol required large quantities of the monoclonal anti-Pal antibody (which was in limited supply), we decided to pursue other purification techniques to amplify signal:noise ratio.
**Melon bead Purification**

The Pierce™ Melon bead kit was used to remove IgG, one of the major proteins that yielded non-specific bands in our blots. 500 µL beads were loaded onto a column and incubated with 100 µL serum. The beads bound to all non-IgG, resulting in two eluted fractions: unbound sample, containing IgG, and bound sample, containing non-IgG proteins including Pal. We eluted the bound sample by incubating with reducing SDS sample buffer (β-mercaptoethanol) and boiling for 10 minutes. We used recombinant purified Pal spiked into sera to determine the lowest level of Pal detected using this method. As seen in Figure 3.7, we can detect 750 ng to 5000 ng of Pal spiked into 100 µL of healthy sera (final Pal concentrations: 7,500 ng/mL to 50,000 ng/mL). As expected, Pal was eluted in the bound fractions. We also were able to see a faint Pal band when as low as 650 ng (6,500 ng/mL), as shown in Figure 3.8. As seen in both figures, a prominent band just below the Pal band (likely light chain IgG) obscured our ability to visualize Pal.

We purchased new Melon Beads and we were able to detect recombinant Pal spiked into sera at even lower levels. As seen in Figure 3.9, we detected 125 ng recombinant Pal spiked into healthy sera, purified using the new Melon beads (1,250 ng/mL). We also used a non-reducing SDS buffer to elute the sample so as to eliminate the interference from the light chain IgG band.
Figure 3.7: Detection of recombinant Pal in sera, purified using Melon beads. We were able to detect as low as 7,500 ng/mL of Pal in sera using this purification method.

Figure 3.8: Detection of recombinant Pal in sera, purified using Melon beads. We were able to detect as low as 6,500 ng/mL of Pal in sera using this purification method.
Figure 3.9: Detection of recombinant Pal in sera purified using new Melon beads and non-reducing sample buffer. We were able to detect as low as 1,250 ng/mL of Pal in sera using this purification method.

To further examine the effect of elution buffer composition on Pal detection level, we performed similar Pal spiking experiments into healthy sera, eluted in non-reducing buffer (Figure 3.10A) and reducing buffer (Figure 3.10B). Pal was detected to lower levels in reducing buffer, but the band was close to overlapping the light chain IgG band, making it difficult to differentiate between the two bands. Although the non-reducing buffer eliminates the light chain IgG band, the Pal bands are not as prominent, resulting in worse detection.
Based on our experiments, we decided to elute our samples in non-reducing sample buffer. We used Melon beads to purify a serum sample from an *E. coli* GNS patient (#2). As seen in Figure 3.11, we did not detect any bands near the molecular weight of Pal but did detect two prominent bands at the top of the blot (molecular weight ~150kDa). These bands may be the result of non-specific binding to our monoclonal anti-Pal antibody, or the bands may contain Pal complexed with larger proteins. For example, we have seen that Pal released from *E. coli* into sera (in an *in vitro* experiment) will complex with anti-Pal antibodies, and will maintain those complexes on an SDS PAGE gel. We hypothesize that Pal formed stable complexes with human anti-Pal antibodies in patient sera, and therefore was undetectable at its expected molecular weight.
Figure 3.11: Melon Bead Purification of *E. coli* sepsis Patient #2, eluted in non-reducing SDS buffer. No lower molecular weight bands were detected, but several high molecular weight bands were detected in patient sera that were not detected in healthy sera.

The same protocol was repeated with the same patient and negative sample, however this time the samples were eluted and boiled in reducing sample buffer prior to running the Western blot analysis. The goal of this experiment was to break apart the immunoglobulin at the top of the blot. As seen in Figure 3.12, we detected many streaked bands around 26-35 kDa in the patient sample that were not present in the negative sera sample. However, we were unable to definitively identify Pal in these bands without access to mass spectrometry facilities.
Another GNS sample, *E. coli* patient 4, was obtained from RGH and analyzed alongside *E. coli* Patient 2 and negative control. The samples were purified using Melon beads, eluted in reducing sample buffer, and detected using our mouse Polyclonal anti-Pal antibody (prepared at RGH using purified recombinant Pal). As seen in Figure 3.13, we did not detect any major differences between the patient samples and the healthy sera. We predict that the spiked Pal was too low in concentration to be detected in negative sera.
Figure 3.13: Sera from *E. coli* sepsis patients 2 and 4, purified using Melon beads, eluted in reducing buffer, and detected using our mouse polyclonal anti-Pal antibody. No major differences between sepsis patient sera and healthy sera were detected.

**Alternative methods**

With the goal of breaking up the Pal-IgG complex, without fragmenting IgG into its light and heavy chain parts, we employed several different protocols. We were advised by colleagues to use urea sample buffers and to incubate the sample at different temperatures. None of the urea buffers or temperature modifications changed the IgG band at the top of our blots. In an attempt to remove non-specific binding caused by our HRP labeled secondary antibody, we added an incubation step with a non-conjugated Goat-Anti-Mouse antibody, prior to incubating the membrane in the monoclonal anti-Pal primary antibody. We were successful at removing some of the background protein signal, but the amount of antibody required to make a significant difference was not practical for our purposes.

Because the darkest band detected after the magnetic bead or melon bead purifications was human IgG (150 kDa), we attempted to decrease the noise from this band by incubating the Western blot with anti-Human IgG prior to the anti-Pal primary incubation. We thought that the anti-Human IgG would bind to the human IgG in the sera and therefore decrease the non-specific
binding to our primary/secondary antibodies. Unfortunately, even using high concentrations of anti-human IgG did not decrease the intensity of the IgG band in our samples.

We also used an Albumin/IgG purification kit with the goal of removing 80% of the human background proteins in sera. Cibacron beads from the kit were designed to bind Albumin and IgG to the beads and allow all other proteins to flow through. Purification of sera using this method resulted in greater background than the other immunoprecipitation methods, because recombinant Pal eluted in the bound sample, most likely due to its interaction with IgG (data not shown).

We also used Protein G bead purification in tandem with the magnetic beads purification. Protein G beads are designed to bind and remove IgG from sera. A negative sera sample spiked with 750 ng of purified recombinant Pal was incubated on the Protein G column. The eluted sample was concentrated to 50 μL and incubated with the anti-Pal conjugated magnetic beads. After the magnetic bead purification, the sample (eluted in non-reducing buffer) was detected using monoclonal anti-Pal antibody. Figure 3.14 shows the data collected from this experiment; we were successful at detecting a prominent band where we expected to see recombinant Pal.

Figure 14: Recombinant purified Pal spiked into Negative plasma and purified using the Protein G purification kit and anti-Pal conjugated magnetic beads. We were able to detect a purified Pal band, but also numerous of other non-Pal bands from our healthy sera sample.
We employed the same Protein G/magnetic bead approach to purify two GNS patient sera samples. The patients were infected with \textit{E. coli} and \textit{Klebsiella pneumoniae}. As seen in Figure 3.15, we were unable to detect differences between either of the GNS patient samples and the negative sample. For reasons unknown, this procedure resulted in higher background than we saw with either the magnetic bead or melon bead preparations. Due to high background and the extensive time involved in this sample preparation method, we decided that this method was not optimal for detecting Pal in patient samples.

![Figure 3.15: Sera from \textit{E. coli} Patient 4 and \textit{K. pneumoniae} Patient 3, purified using the Protein G purification kit and anti-Pal conjugated magnetic beads. We observed no significant differences between patient and healthy sera samples.](image)

\textit{Analyzing non-\textit{E. coli} sera samples}

We obtained our monoclonal anti-Pal antibody from our collaborator, Dr. Judith Hellman, University of California San Francisco. The monoclonal anti-Pal antibody interacted with Pal from \textit{E. coli} as well as Pal homologues from any member of the \textit{Enterobacteriaceae} family. For example, a BLAST sequence search for Pal homologs in \textit{K. pneumoniae}, another GN enterobacterium naturally found in the intestines, shows that Pal from \textit{K. pneumoniae} has >65\% homology to Pal from \textit{E. coli}. 
Two *K. pneumoniae* patient sera samples were analyzed alongside a sera sample from an *E. coli* GNS patient (1) and a Gram-positive sepsis patient (Group A Strep). After Melon beads purification of the sera samples, we were able to detect proteins using our monoclonal anti-Pal antibody (Figure 3.16). We detected a 26-34 kDa protein band, outlined in pink, in every patient sample, including the Gram-positive sample. We cannot conclude the identity of this band, although its presence in the Group A Strep patient suggests it was not Pal.

![Figure 3.16: Sera from E. coli sepsis patient 1, K. pneumonia patients 2 and 3, and Group A Strep patient 6 was purified using Melon Beads and eluted in reducing buffer. We detected a prominent protein band just above the light chain IgG band in all the patient samples, but we were unable to identify the protein.](image)

Based on the data collected from the serum detection studies, it was not clear at what molecular weight Pal would be detected. For this reason we performed an *E. coli* release study, similar to the *in vitro* studies described in the literature, to determine where Pal would travel on an SDS PAGE gel. For the release experiment, multiple positive and negative controls were prepared. Three samples were prepared in LB media or LB supplemented with different 200 µg/mL
ampicillin (AMP), sera from healthy donors or sera supplemented with 200 µg/mL AMP, or urine from healthy donors or urine supplemented with 200 µg/mL AMP (Figure 3.17A). *E. coli* does not release native Pal in LB or urine and only minimally in sera from a healthy donor (as seen in previous studies). AMP was added to the *E. coli* to kill the cells by breaking up the peptidoglycan. Pal was released at all three growth conditions with AMP, suggesting that either breaking open the cells, disrupting the peptidoglycan, or both lead to release of Pal. In another experiment, *E. coli* cells were incubated in negative sera supplemented with 2 µL of the monoclonal anti-Pal antibody. From the blot (Figure 3.17B), we concluded that Pal formed a stable complex with the anti-Pal antibody, which was not dissociated by boiling or separation via SDS PAGE. These results suggest that if there is enough antibody against Pal in sepsis patient serum, Pal could form a stable complex with antibody that could not be dissociated and therefore detected as a complex in the molecular weight range >150 kDa.

**Figure 3.17: E. coli Pal release study.** A: We detected Pal bands in cell pellets and the supernatants of growths supplemented with ampicillin and sera alone. B: When *E. coli* was grown in sera and anti-Pal, we detected a band ~150kDa, suggesting Pal formed a stable complex with anti-Pal antibody.
Chapter 3 Conclusions:

While the monoclonal primary antibody used in these studies has high fidelity to Pal, the human proteins in sera are in such high abundance, **both the primary and secondary antibodies demonstrate non-specific binding to non-Pal proteins**. The stable Pal-immunoglobulin complexes formed in human sera have also made the isolation and detection of Pal extremely difficult. We have shown that Pal is capable of forming stable complexes with anti-Pal, which may result in released Pal in patient sera “hiding” among the 150 kDa IgG bands. In summary, we have tried multiple purification, separation, and immunoprecipitation techniques to “clean up” sera to detect Pal in sepsis patient samples. While our blots have improved in cleanliness, background noise, and Pal detection limits, we have still not been able to detect Pal in GNS patient sera.

Another issue that complicates our study is that we don’t know where Pal will appear on a gel. Based on its monomer sequence, Pal is predicted to be between 17-20 kDa, but if it is in a stable complex with itself, another OMP, or immunoglobulin, it would be detected at a higher molecular weight. The release study performed with the spiked anti-Pal in sera suggests that if a patient has a high enough concentration of anti-Pal in their sera, then Pal will form a stable complex with anti-Pal that is not disrupted during sample preparation or gel electrophoresis. We propose that Pal could bind to antibody in patient sera and therefore be detected with a high molecular mass at the top of the gel instead of as a monomer ~18 kDa.

Although we have not successfully detected Pal in the serum of a GNS patient, we have developed two purification techniques to minimize human protein background binding. We have also shown that Pal forms stable complexes with IgG. More patient samples are needed to optimize our purification and detection. Additionally, we are looking into using more sensitive techniques to detect Pal in sera, such as targeted quantification of low level (ng/mL) proteins using Mass Spectrometry, a technique created by Dr. Wei-Jun Qian (Pacific Northwest National Laboratory). From our own studies and those of our collaborator, Dr. Judith Hellman, we are confident that Pal is released from *E. coli* in the presence of human sera (especially with the
addition of peptidoglycan-targeted antibiotics such as ampicillin). We propose that we have been unsuccessful at detecting Pal in patient sera, because:

A) Pal may be at low ng/mL levels and therefore undetectable using standard immunoblotting (even with our purification techniques);

B) Pal may be forming stable complexes with itself; other OMP’s or anti-Pal antibodies naturally produced by the patients during infection, and therefore is not detectable at its normal monomeric molecular weight;

C) Pal may be filtered out from the sera into the spleen or liver and excreted into the feces or urine of the patient.
4. Detection of Pal in Human Urine

The function of the kidney in the body is to filter the blood of excess salts, urea and toxins, that can damage blood cells, then excrete these toxins in the form of urine. Typically the nephrons do not filter cells and protein into the urine, unless the protein is of a small enough molecular weight. We postulated that we might be able to detect Pal in the urine if the kidneys filter the Pal from the blood. Since urine contains far fewer proteins, we anticipated lower background from nonspecific binding and a better signal to noise ratio for Pal detection.

Methods:

Low-level detection sample preparation
Different concentrations of purified Pal (0-2000 ng) were spiked into 20 µL of urine from a healthy young adult donor. The sample was prepared in SDS sample buffer and boiled for 10 minutes. Samples were analyzed on an SDS-PAGE gel and Pal was detected via Western blot.

E. coli Sepsis patient urine sample preparation
Urine samples from E. coli sepsis patients were provided by Dr. Roberto Vargas (Department of Infectious Disease at Rochester General Hospital) and assigned ID numbers. The samples were assigned a number in the chronological order in which they were received from RGH. Patient urine samples were prepared in SDS sample buffer (1:1) and boiled for 10 minutes. 14 µL of each sample was loaded onto a SDS-PAGE gel for analysis via Western blot and Coomassie staining.
Albumin/IgG depletion of Urine samples

A Pierce® Albumin/IgG Removal Kit was employed to purify Albumin and IgG from human urine. Immobilized Cibacron/ Protein A gel was swirled to create a slurry. 170 µL bead slurry was added to each spin column (Pierce); storage buffer was eluted from the beads by centrifuging at 10,000xg for 1 minute. The bottom of the column was capped, and urine (10 µL) was diluted with 65 µL Binding/Wash Buffer (25 mM Tris, 25 mM NaCl, 0.01% sodium azide; pH 7.5) and loaded onto the capped spin column/beads. The column was incubated for 10 minutes at room temperature with end-over-end mixing. The bottom cap was removed and centrifuging the column at 10,000xg for 1 minute eluted the unbound sample. The bottom of the column was then re-capped and the sample was re-applied to the spin column. The column was incubated an additional 10 minutes at room temperature with end-over-end mixing. The bottom cap was removed and centrifuging the column at 10,000xg for 1 minute again eluted the unbound sample. An additional 75 µL Binding/Wash buffer was added to each column and centrifuging the column at 10,000xg for 1 minute eluted the final sample. Both the first and second elutions (~75 µL each) were combined for analysis.
Results and Discussion

We first determined our detection limit of Pal in urine. Purified Pal protein was spiked into urine from a healthy young adult (age 18-45), which was negative for *E. coli*. Concentrations of purified Pal protein varied from 250 ng to 2000 ng in the first study (Figure 4.1). The purified Pal protein ran to the 26 kDa protein marker and was detectable at all levels in 50 uL of urine, using the monoclonal Anti-Pal antibody and standard Western blot analysis. We prepared lower level Pal samples (10 ng to 250 ng) to determine our lowest level of detection (Figure 4.2). We were able to detect Pal at levels between 10 ng and 25 ng per 50 µL of urine (0.2 ng/µL). According to the Mayo Clinic, normal protein levels in healthy urine are typically ~20 mg/mL, about 10^5 greater than our lowest limit of detection for Pal in urine. These data suggested that if Pal were present in “normal” concentrations in patient urine, we should be able to detect it.

*Figure 4.1: Detection of Purified Pal (2000 ng to 250 ng) in 50 µL of healthy urine.* Purified Pal was detected at the expected molecular weight at levels as low as 250 ng.
Figure 4.2: Detection of Purified Pal (250 ng to 10 ng) in 50µL of healthy urine. Purified Pal was detected at the expected molecular weight at levels as low as 10-25 ng.

Our first *E. coli* Patient sample (Sample #3) was diluted in 2X sample buffer and analyzed via SDS PAGE and Western blotting. Negative urine from a healthy young adult (negative control) and diluted recombinant purified pal (positive control) were also analyzed. As seen in Figure 4.3, anti-Pal immunoblotting detected a protein in Patient Sample #3 that traveled to between the 17-26 kDa markers; no protein bands were detected in the negative urine sample. The protein band appears at the molecular weight of what we would expect for native Pal (monomer) from *E. coli*. As a reminder, our purified Pal, although ~20 kDa in weight, consistently travels at a higher molecular weight than expected.
Figure 4.3: Western Blot Analysis of *E. coli* GNS Patient #3 urine developed using monoclonal Anti-Pal antibody. We detected a single prominent band at ~18-20 kDa, the molecular weight of monomer Pal from *E. coli*.

We tested the possibility that whole cell *E. coli* (containing attached Pal) was a contaminant in the sample. To remove any intact *E. coli* cells, we gently pelleted the samples (5000xg) or filtered the samples using a 0.2 µm syringe filter. As seen in Figure 4.4, after pelleting or filtering the sample, we were still able to detect a prominent band between 17-26 kDa.
Figure 4.4: Western Blot Analysis of *E. coli* GNS Patient #3 urine developed using monoclonal Anti-Pal antibody. To remove any potential intact cells from the patient urine, we filtered the urine (prior to boiling in 2X SDS buffer) or spun down the sample at 5000xg. We detected a prominent band at ~18-20 kDa, the molecular weight of monomer Pal from *E. coli*, in both prepared urine samples and no proteins in negative/healthy urine.

The anti-Pal reactive protein band (around 20 kDa) was excised from an SDS-PAGE gel and sent off-site for mass spectrometry analysis (Promega Mass spec services). No bacterial proteins were detected in the sample, suggesting that any bacterial proteins in the sample were below the lowest level of detection. Typically, immunoblotting is far more sensitive in detecting low level proteins compared to mass spectrometry.

To test the non-specific reactivity of our samples with secondary antibody (Goat-Anti-Mouse HRP), we prepared an immunoblot with secondary antibody only (no primary antibody). As seen in Figure 4.5, the blot shows there was no significant background binding of secondary antibody to proteins in the patient urine at molecular weights lower than 140 kDa.
Figure 4.5: Western Blot of *E. coli* GNS patient urine (spun down or sterile filtered) using Goat-Anti-Mouse HRP antibody only (no primary). Secondary antibody non-specifically bound to a few proteins in patient urine with higher molecular weights than Pal.

We also used the Albumin/IgG depletion kit to remove more background proteins from the *E. coli* sepsis patient urine sample. The beads in the kit bind to non-albumin and non-IgG proteins, so we expected to see Pal in the “bound” sample. As seen in Figure 4.6, two bands were detected in the column-bound sample. A high molecular weight band was detected, as well as the ~19 kDa band we predict to be Pal. The higher molecular weight band may be remnant IgG in the sample and/or a larger Pal complex that remained intact. The same protocol was performed on healthy urine; no proteins were detected in either healthy urine sample. Since 10 µL of the *E. coli* sepsis patient (#3) was purified using the Albumin/IgG depletion kit, the band is not as prominent as seen with the non-purified urine samples.
Figure 4.6: *E. coli* sepsis patient urine sample depleted of Albumin and IgG proteins and compared to healthy urine. A higher molecular weight protein and a 19 kDa protein were detected with anti-Pal in the *E. coli* sepsis patient urine sample (bound to beads). No proteins were detected in the healthy urine.

Two additional *E. coli* sepsis patient urine samples (patients #1 and #2) were obtained from Rochester General Hospital and analyzed in the same manner as Patient 3 sample, which was also re-analyzed for comparison. As seen in Figure 4.7, an anti-Pal immunoblot detected a prominent band in patients #1 and #3 samples. These bands line up with our recombinant Pal protein, but not with our native *E. coli* Pal band. We did not detect proteins in the healthy urine or in the urine of patient #2. We did note that patient #2 had clear and colorless urine, perhaps indicative of a failing renal system, in which case no toxins/proteins would be filtered by the kidneys.
**Figure 4.7:** Urine from *E. coli* sepsis patients #1-3 were analyzed via immunoblotting and an anti-Pal antibody. A band at 26 kDa was detected in the urine of patients #1 and #3. The original 19 kDa band in patient #3 was not detected in this blot, which was run X months after the original experiment.

A discrepancy in patient 3 sample is seen in Figure 4.7, when compared to an earlier blot (Figure 4.4) of the same sample. In Figure 4.4, a clean dark band around 19 kDa reactive for anti-Pal antibody was detected; however, in Figure 4.7, this band disappears and a new band is detected around 26 kDa. About 3 months passed between the processing of these blots, during which the Patient 3 sample was stored in the refrigerator. We postulate that during this time, the Pal structure/stability may have changed and resulted in Pal forming a complex with itself or another protein. These changes would explain why the new band is shifted to a higher molecular weight. Without access to a mass spectrometry facility we cannot determine unequivocally whether or not these bands are Pal, Pal bound to another molecule, or another protein that interacts nonspecifically with our monoclonal anti-Pal antibody.
Kidney function declines with age, and therefore the majority of elderly people (60 or older) will have more protein in their urine due to decline in kidney function. Here, we used both the monoclonal and polyclonal anti-Pal (Figures 4.8A and 4.8B) to detect Pal in urine from elderly donors. We detected a few proteins at higher molecular weight in donor #2 and #4 samples, but no other significant proteins near the molecular weight of Pal.

Figure 4.8: (A) Western Blot of elderly donor urine samples using Polyclonal Anti-Pal Primary (B) Western Blot of elderly donor urine samples using monoclonal Anti-Pal primary. Only a few higher molecular weight proteins were detected in the elderly donor urine samples using anti-Pal.

We also considered the possibility that the patient had a urinary tract infection (UTI). In that case, *E. coli* cells could be releasing Pal directly into the patient’s urine. A urine sample from a patient with a UTI was analyzed before and after being filtered through a 0.2 µm filter. As seen in Figure 4.9, we were able to detect a band of similar molecular weight to Pal in the unfiltered UTI sample; however, the corresponding band was not detected in the syringe filtered (0.2 µm) UTI sample. Since the 20kDa band was moderately faint, we concentrated the urine sample using a 10 kDa molecular weight cut-off concentrator (Amicon). We re-ran the samples alongside lysed *E. coli* pellets as controls.
As seen in Figure 4.10, a prominent band ~20 kDa can be seen in the unfiltered UTI sample, but is not detected in the filtered UTI sample. Surprisingly, the UTI sample band (unfiltered) is shifted slightly higher than the Pal band from the *E. coli* pellet. These results suggest the following: 1) Pal (or a homologue to Pal) was present in the bacteria found in the urine sample (our monoclonal anti-Pal antibody interacts with Pal from any enterobacteria, so we cannot determine the source of the bacteria in the sample); 2) Pal (or a homologue to Pal) was not released from the UTI bacteria into urine, as it is not present in the filtered sample; 3) the presumed Pal detected from the UTI sample travels to a slightly higher molecular weight compared to our *E. coli* Pal control, suggesting that the sequence of the UTI Pal is not identical to *E. coli* Pal. We are doing further studies to determine whether or not Pal is released from *E. coli* in the presence of urine.

Figure 4.9: Western blot analysis of urine from a non-*E. coli*-infected sick patient and a UTI patient. Pal was detected in the unfiltered urine of the UTI patient, but not in the filtered urine sample. No detectable proteins were found in the non-*E. coli*-infected sick patient (negative control).
Patients with sepsis often have a plethora of immunological proteins in their system (as a response to their infection). Therefore, comparing a healthy person’s urine to a GNS patient’s urine is not the best comparison. For a better negative control, we collected urine from a donor who was hospitalized for a non-sepsis inflammatory infection. As seen in Figure 4.9, the sick non-sepsis patient’s urine contains no proteins that were detectable using our anti-Pal antibody. In the future, we will collect urine from SIRS (systemic inflammatory response syndrome) patients to be used as negative controls. As long as those SIRS patients do not have *E. coli* (or other enteric bacterial) infections, we do not anticipate proteins in their urine will be detected using our anti-Pal assay.

**Figure 4.10: Western blot analysis of concentrated urine samples from UTI patient.** Both the unfiltered and filtered urine samples were concentrated. A prominent band was detected by anti-Pal (at a molecular mass slightly higher than our wild-type *E. coli* Pal) in the unfiltered sample; no proteins were detected in the filtered sample.
A Pal release study (data not shown) was performed in our lab using human urine. The study showed that a minimal amount of Pal was released from *E. coli* into urine in the presence of antibiotics (ex. ampicillin). The UTI patient sample used in our study was collected before the patient began an antibiotic regimen, which likely explains why Pal was only detected in the urine and removed via filtration. It is very likely that the GNS patients at RGH were all receiving antibiotics at the time our samples were collected. Therefore, we cannot rule out the possibility that the Pal we detected in the urine samples was released from *E. coli* directly into the urine (as opposed to being released from *E. coli* in the blood and filtered by the kidneys into the urine).
Chapter 4 Conclusions:

Our recombinant purified Pal does not run to its true molecular weight of 20.6 kDa, but instead to a molecular weight ~26 kDa. We are unsure of why our recombinant Pal runs to a higher molecular weight, but we propose that the protein exhibits secondary/tertiary structure that is not unfolded via SDS and boiling. Using our recombinant Pal spiked into healthy urine, we determined that we could detect Pal at concentrations as low as $10^1$ ng per 10 µL urine.

Using this method, we were able to detect a protein of the same molecular weight as Pal, using anti-Pal antibody, in the urine of a single *E. coli* sepsis patient. A similar protein was not detected in any of our negative control urine samples (from healthy young adults, elderly donors, or patients with inflammation, but no *E. coli* infection). We also detected a higher molecular weight protein in a second patient that aligned with our recombinant Pal. Due to the tendency of Pal to bind other OMPs, it is possible that this higher molecular weight protein is indeed a Pal complex. At this time, without the proper mass spectrometry facilities, we were unable to determine if this band was Pal complexed with itself, Pal complexed with another protein, or a non-Pal protein that nonspecifically bound to anti-Pal. A similar band of the same molecular weight was detected for Patient 3; three months prior, a 19 kDa band was detected in Patient #3’s urine. It is possible that after a long period of storage at 4°C, Pal forms stable complexes and is therefore detectable at higher molecular weights.

We were able to detect a protein of similar molecular weight to Pal in the urine of a UTI patient who did not receive antibiotics. The band disappeared after filtration of the urine using a 0.2 µm filter, suggesting the Pal was detectable in *E. coli* whole cells only (and not as released Pal).

Taken together, our data suggest that urine may be an effective method to detect released Pal from *E. coli*, but further analytical studies must be performed. We believe that Pal may be forming complexes in urine. Protein matrix characteristics including ionic strength, composition, turbidity, and presence of ligands can affect a protein’s conformation (29), and thus could be causing Pal in samples to “change” over time and run “heavier” than we would expect on a gel. However, without access to more sensitive Mass Spectrometry facilities or high-performance
We used liquid chromatography and size exclusion chromatography, but have been unable to definitively identify Pal in GNS patient urine.
5. Final Conclusions and Future Ideas

The recombinant purified Pal does not run to its true molecular weight of 20.6kDa, as indicated by Mass Spectrometry, but instead to a molecular weight ~26 kDa. We are unsure of why our recombinant Pal runs to a higher molecular weight, but we propose that the protein exhibits secondary/tertiary structure that is not unfolded via SDS and boiling. This recombinant protein is needed to study the Pal: Peptidoglycan interaction but is not essential for the detection of Pal in sera/urine studies. Instead of using recombinant Pal as a control in our detection studies, we have been and will continue to use diluted *E. coli* pellet (which contains native Pal).

For detection of released Pal in sera, the monoclonal anti-Pal antibody we used demonstrated non-specific binding due to the high abundance of human proteins. The Pal-immunoglobulin complexes formed in human sera have made the isolation and detection of Pal extremely difficult. It has also proven difficult to break the interaction between Pal and immunoglobulin; specific immunoprecipitation techniques, Pal purification techniques, and varying buffers were unable to break the Pal-IgG complex. The immunoprecipitation methods we used lowered the levels of human proteins and allowed us to detect purified Pal more easily. In fact, compared to our collaborator’s initial studies to detect Pal in human patient sera, we have been able to optimize the purification protocol and produce cleaner, more sensitive immunoblots. However, even with these improvements, we have not been able to detect Pal in the sera of GNS patients.

Using our recombinant Pal spiked into healthy urine, we determined that we could detect Pal at concentrations as low as $10^1$ ng per 10µL urine. Using the same method, we detected a protein with the same molecular weight as Pal in a single *E.coli* sepsis patient. In all the negative control urine samples (healthy young adult, elderly donor, or patient with inflammation not caused by an *E.coli* infection), no protein with that molecular weight was detected with the anti-Pal antibody.

We believe that Pal is likely forming complexes in both urine and sera. During long-term storage, protein compositions can be altered and stable complexes can form, thus causing the Pal to appear “heavier” than expected when analyzed via gel electrophoresis/immunoblotting. We
also must take into account the likelihood that antibodies in the sera will bind to the released Pal protein. Because Pal is a known virulence factor of sepsis, it is likely that immunoproteins will bind to released Pal to neutralize and remove it from the body, thus forming stable complexes that would make Pal appear at higher molecular weights than expected.

Our data suggest that our current methods for detection of Pal in sera are not sensitive or specific enough to detect released Pal, but urine has proven a more sensitive and effective method to detect released Pal from *E. coli*. The Michel group will continue to analyze urine and sera samples from *E. coli* sepsis patient samples. We have detected a protein of the same molecular as Pal (using the anti-Pal antibody) in the urine of two *E. coli* sepsis patients, but we have been unable to detect a similar protein in 4 additional *E. coli* sepsis patients. Thus, data from more patients are necessary.

Dr. Wei-Jun Qian, Pacific Northwest National Laboratory, has developed a method for targeted quantification of low-level protein concentrations (ng/mL) in human serum using Mass Spectrometry. Dr. Qian has agreed to develop an assay targeted for low level quantifications of Pal in human serum using our recombinant Pal protein. Once the assay is developed, we will use the Mass Spectrometry facilities at Pacific Northwest National Laboratory perform the analysis on our patient samples using this assay.

Our collaborators have demonstrated the release of Pal from *E. coli in vivo* in mouse/rat models and *in vitro* in human sera. We have duplicated the *in vitro* release studies in human sera in our lab, and are currently working on an assay to determine the effect(s) of different antibiotics on Pal’s release. Most sepsis patients will have been given antibiotics while in the hospital, so it is important that we understand how these antibiotics affect the release of Pal from *E. coli*. We have initiated a similar release study in human urine to determine if *E. coli* can release Pal into urine in the bladder.

We are also pursuing another study in the lab, which will analyze at the interaction between Pal and peptidoglycan and its effect on Pal release. Site-directed mutants of recombinant Pal will be analyzed for their effect on the Pal:Peptidoglycan interaction. Interesting Pal mutants will be
further studied for their effect on Pal release in sera/urine. Our hypothesis is that the Pal:Peptidoglycan noncovalent interaction prevents Pal from release from \textit{E. coli}, unless peptidoglycan-targeted antibiotics are present.

We postulate that P6, the homolog to Pal in \textit{NTHI}, may also exhibit characteristics similar to Pal in a GNS model. The Pal release studies will be adapted for P6/\textit{NTHI} to determine whether or not P6 is released from \textit{NTHI} in sera. While GNS caused by \textit{NTHI} is rare in developed countries, many cases of NTHi sepsis are reported each year in developing countries.

A long-term goal related to this project is to determine the biological role of dual oriented proteins, including Lpp and Pal in \textit{E.coli}, in the virulence and development of GNS. Lpp, like Pal, has been shown to be released from \textit{E.coli} in sera (as a complex with LPS, Pal, and OmpA) and is a known virulence factor in GNS. The Michel group will continue studies to determine the role of Lpp and Pal on cytokine production and inflammation, with the goal of using the proteins or their corresponding antibodies to treat and/or diagnose GNS.
References:


