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The Characterization of a Putative Protease Expressed by Sneathia amnii

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CHARACTERIZATION OF A PUTATIVE PROTEASE EXPRESSED BY *SNEATHIA AMNII*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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Dedication

       I would like to dedicate this work to my parents for their unyielding love, support,

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My successes are due to the endless love and support my loved ones have given me.

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List of Abbreviations

°C .... degrees centigrade

μg ....microgram

μl .... microliter

AA.. amino acids

Amp ....ampicillin

Amp^r... ampicillin resistance

BHI...brain heart infusion

B-ME ....beta-mercaptoethanol

bp .... base pair

BSA...bovine serum albumin

BV .... bacterial vaginosis

C..cytosine

C- section .... Cesarean section

$CaCl_2$... calcium chloride

CFU... colony forming unit

Cm... chloramphenicol

Cm^r ....chloramphenicol resistance

CRH-R1 .... corticotropin releasing hormone receptor

DMEM .... Dulbecco's modified eagle media

DNA...deoxyribonucleic acid

dNTP...deoxynucleotide triphosphate

ECM...extracellular matrix

E. coli...*Escherihia coli*

FIRS ....fetal inflammatory response syndrome

g .... gram

G..Glycine

IgA .... Immunoglobulin A

IL-1a...interleukin-1a

IL-1b interleukin-1b

IL-6 ....interleukin-6

IL-8 ....interleukin-8

IL-10 ....interleukin-10

IPTG.. isopropyl β-D-1-thiogalactopyranoside

IUD .... intrauterine device

K.. Lysine

KCl..potassium chloride

kDa...kilodalton

L.. Leucine

L...liter

L.crispatus..*Lactobacillus crispatus*

LB .... Luria-Bertani

M ....molar

MIAC ....microbial invasion of the amniotic cavity

Mbp... megabase pair

ml .... milliliter

mM .... millimolar

MMPs...matrix metalloproteases

MW .... molecular weight

MWCO...molecular weight cut off

NaCl .... sodium chloride

NaP... sodium phosphate

nm .... nano meter

OD...optical density

PAMPs .... pathogen associated microbial patterns

PBS .... phosphate buffered saline

PBST...PBS supplemented with 0.05% Tween 20

PCR... polymerase chain reaction

Peg1 ....Sn35's putative zinc endopeptidase

pH...power of Hydrogen

pmol .... picomole

PPROM ....preterm premature rupture of the membranes

PVDF ....polyvinylidene fluoride

Q... Glutamine

RNA ....ribonucleic acid

rRNA..ribosomal ribonucleic acid

rpm ....revolutions per minute

S.amnii .... *Sneathia amnii*

S. aureus..*Staphylococcus aureus*

SBHI ....supplemented brain heart infusion

SDS ....sodium dodecyl sulphate

Sn35 ....*S.amnii* isolate

SOC...super optimal broth

TAE.. tris-acetate EDTA buffer

TBE .... tris-borate EDTA buffer

TEMED..tetramethylenediamine

UV...ultraviolet

VCU ....Virginia Commonwealth University

V..volt

VK2..human vaginal mucosal epithelial immortalized cells

WSW...women who have sex with women

ZnCl$_2$ .... zinc chloride

Abstract

CHARACTERIZATION OF A PUTATIVE PROTEASE EXPRESSED BY *SNEATHIA AMNII*

By: Rana Mehr, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Preterm birth, birth prior to 37 weeks gestation, is the leading cause of neonatal mortality and morbidity worldwide. While the uterine cavity and amniotic fluid largely remain sterile throughout gestation, bacterial infections can occur and are associated with preterm birth and/or preterm premature rupture of the fetal membranes (PPROM). *Sneathia amnii* can be detected as a component of the vaginal flora in healthy women; however, it's also associated with bacterial vaginosis and preterm birth. Sn35, an isolate of *S.amnii*, was identified and sequenced through the Vaginal Human Microbiome Project at VCU. Our objective was to classify potential

virulence determinants in Sn35 and we successfully identified a putative zinc endopeptidase. The zinc endopeptidase appeared to cleave itself in a site-specific manner under calcium-depleted conditions, resulting in a truncated protein. The truncated protein did have collagenase activity and bacteriolytic activity as well.

Chapter 1
Introduction

Clinical Significance of Preterm Birth

Preterm birth, defined as birth prior to 37 weeks gestation, is the leading cause of

neonatal morbidity and mortality worldwide (Jefferson, 2012; Wiktin, 2014). The clinical

definition of preterm labor is regular contractions that are accompanied with cervical change in

less than 37 weeks gestation and is now believed to be a syndrome that is initiated by multiple

routes (Goldenberg et al., 2008). Preterm birth can occur where labor is induced or infant is

delivered through C-section, spontaneous preterm labor while fetal membranes are intact, or

preterm premature rupture of the membranes, PPROM (Goldenberg et al., 2008). It is associated

with 70% of neonatal death, 75% of neonatal morbidity and 50% of long-term neurological

sequelae (Lacovidou et al, 2010; Whidbey et al., 2013). Classifications of preterm birth are as

follows; extremely preterm is defined as birth after 20 weeks gestation but before 28 weeks of

completed gestation, very preterm is defined as birth after 28 weeks but before 32 weeks of

completed gestation, and moderate preterm is defined as birth after 32 weeks but prior to 37

weeks (Lawn et al., 2010). There are multiple physical risk factors associated with preterm birth

including: tobacco and drug use, carrying multiples, uterine abnormalities, extremes in maternal

age, systemic and/or intrauterine infections, bacterial vaginosis, and cervical length (Ganu et al.,

2012; Romero et al., 2006; Stout et al., 2013;Wen et al., 2013). The greatest risk factor for preterm birth is having had a previous preterm birth (Jefferson, 2012). Non-physical factors that are associated with preterm birth are low socioeconomic status and race (Fettweis et al., 2014; Goldenberg et al., 2008)

The annual cost of health care associated with premature birth in the United States was at minimum $26 billion in 2005, and in 2004 12.5% of births in the U.S. were preterm (Behrman et al., 2007; Hyman et al 2013). While the U.S. is a developed country with modern medicinal technology, it currently holds a higher rate of preterm birth than the average rate, 7.5%, for developed countries and has an equal rate of preterm births with countries that are least developed. Additionally, within the U.S., African American women have an increased risk of delivering preterm; 18.4% of African American women delivered preterm and 11.7% of non-Hispanic white women delivered preterm.

Intrauterine and Amniotic Cavity Invasion by Vaginal Bacteria

While preterm birth is caused by a multitude of factors, between 25-40% of preterm births are caused by intrauterine infection and inflammation (Wen et al., 2013; Rubens et al., 2010; Gravett et al., 2010). There is strong evidence supporting that infection induces preterm birth; experimental infection or LPS injection in mice has been shown to induce preterm birth, intrauterine infections among women correlates with preterm birth, systemic infections among women is associated with preterm birth and chorioamnionitic tissue isolated from women who had spontaneous labor at less than 30 weeks were infected with bacteria (Hirsch et al., 1995; Schantz-Dunn et al., 2009; Goldenberg et al., 2000; Hillier et al., 1988). Pregnant women with systemic malaria infections were shown to have inflammatory responses that correlate with

preterm birth; however, it has been shown that the majority of preterm birth is due to bacterial intrauterine infections (Jefferson, 2012; Romero et al., 2001). The accepted theory is that vaginal bacteria ascend through the cervix to cause infection and inflammation (Jefferson, 2012). Chorioamnionic tissue sampled from women undergoing C-section for spontaneous labor indicated that 75% of tissue was culture positive (Goldenberg et al., 2008; Jefferson, 2012). Bacteria isolated from preterm tissue and amniotic fluid has been found in the vagina as well (Romero et al., 1989). While oral flora can be found in amniotic fluid, the general notion is that the oral species first enters the vagina through oro-genital contact and then begins ascension in the uterine cavity to invade the amniotic cavity rather than hematogenous spread (Figure 1.1) (Jefferson, 2012; Kim et al., 2009). The association of bacterial vaginosis, otherwise known as BV, and preterm birth therefore is not likely due to vaginal pathology, but ascension of BV-associated bacteria from the vagina into the uterine cavity (Jefferson, 2012).

During gestation, the cervical mucus plug becomes more viscous due to the presence of progesterone to form a mechanical barrier. Additionally, alpha and beta defensins, antimicrobial compounds like lactoferrin, secretory IgA, and host immune system factors like complement are present within the cervical mucus plug (Jefferson, 2012). Once bacteria have crossed the cervical mucus plug, they can remain in the choriodecidual space or invade the chorion, cause chorioamnionitis, traverse the fetal membranes or enter the amniotic cavity and are capable of eliciting a chronic infection that results in an inflammatory host response which can lead to preterm birth (Goldenberg et al., 2000; Jefferson, 2012). Triggering the inflammatory response mediates the release of tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-1a, IL-1b and granulocyte colony-stimulate factor (Estrada- Guiterrez et al 2010; Goldenberg et al., 2000; Jefferson, 2012). The release of the cytokines stimulates secretion of other cytokines,
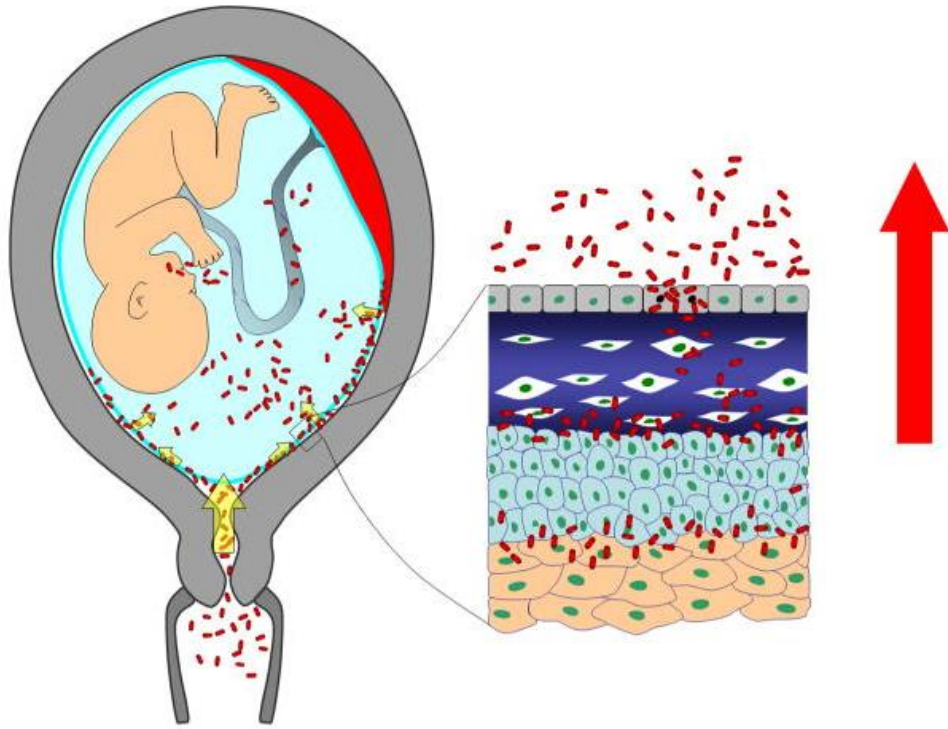
Figure1.1: Schematic representation of bacterial ascension into the amniotic cavity.

Bacterial ascension occurs at the vagina where select bacteria are able to traverse the fetal

membranes and enter into the amniotic cavity to cause infection. Adapted from (Kim et al.,

2009) and reproduced with permission of publishing journal.

prostaglandins, matrix metalloproteinases, which weaken the fetal membranes and eventually lead to PPROM.

Therefore it is hypothesized that bacteria that contain certain virulence factors can contribute to preterm birth and/or PPROM. The most frequently isolated BV-associated bacteria accompanying infection and preterm birth are *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Gardnerella vaginalis*, *Sneathia amnii*, and *Bacteriodes* (Goldenberg et al., 2000; Goldenberg et al., 2008; Jefferson, 2012). Since numerous bacteria associated with microbial invasion of the amniotic cavity (MIAC) are difficult to culture, the utilization of molecular techniques, like PCR, has aided research of bacterial species that are present in amniotic fluid and fetal membranes (Goldenberg et al., 2008).

Bacterial Vaginosis

Bacterial vaginosis, otherwise known as BV, is a complex polymicrobial disorder that is caused by a chemical and biological imbalance in the normal flora of the vagina. The virulence factors associated with BV are poorly understood and it is frequently misdiagnosed as trichomoniasis or a yeast infection. BV is associated with an overgrowth of commensal anaerobic bacteria where none is the clear pathogen. It is the most common vaginal infection worldwide and affects 29% of women in the United States (Allsworth et al., 2007; Fluery, 1981). It is associated with preterm birth, low birth weight, increased risk of acquiring HIV-1 and other sexually transmitted disease, pelvic inflammatory disease, and post-operative infections (Allsworth et al., 2011; Lambert et al., 2013; Lamont et al., 2011; Madden et al., 2012; Schellenberg et al., 2012). While the etiology is unknown, risk factors for BV include; race, douching, IUDs, and sexual activity (Madden et al., 2012).

BV is characterized by a decrease in *Lactobacillus* species, particularly those that are able to produce hydrogen peroxide; the depletion of *Lactobacillus* allows for an increase in anaerobic bacteria (Muzny et al., 2013). *Lactobacillus spp* are gram positive rod shaped bacterium that are facultative anaerobes and are the primary colonizer of the vagina. In addition to producing hydrogen peroxide, which inhibits the growth of other bacterial species, *Lactobacillus* produce lactic acid by fermentation lowering the overall pH (< 4.5) of the vagina to favor the vaginal microbiota composition towards acidophiles (Fettweis et al., 2012). A shift in *Lactobacillus* composition allows for anaerobic bacteria like *Gardnerella vaginalis, Prevotella bivia*, *Mycoplasma hominis, Mobiluncus spp., Atopobium vaginae, Fusobacterium spp.*, and *Sneathia amnii* to flourish (Harwich et al., 2012; Munzy et al., 20113).

Research has indicated that race plays an important role for BV and consequently preterm birth. While 10-20% of Caucasian women experience BV, African American women have a 30-50% rate. This racial disparity is also seen in preterm birth; African American women have a twofold increased risk of experiencing preterm birth and a threefold risk of extremely preterm birth (Sobel, 2005). While both races are colonized by *Lactobacillus*, there is a difference in colonization that attributes to BV and preterm birth. There are six species of *Lactobacillus* that colonize the vagina; *Lactobacillus crispatus*, *Lactobacillus iners, Lactobacillus jensenii, Lactobacillus gasseri, Lactobacillus vaginalis* and *Lactobacillus johnsonii* (Fettweis et al., 2014; Jefferson, 2012). These species of *Lactobacillus* vary in their stability and their ability to protect the vagina from BV associated bacterial colonization (Tamrakar et al., 2007). More stable species are not easily displaced from changes in pH, hormonal changes, nutrient sources and semen deposition. Women of European decent are predominantly colonized with *L. crispatus*, which is more stable and protective against BV. Women colonized with *L.crispatus* have a

fivefold decreased risk of developing BV. African American women however, tend to be colonized with *L.iners* which is less stable and less protective than *L.crisptaus* and therefore women colonized with *L.iners* have a tenfold increased risk of developing BV (Fettweis et al., 2014; Verstraelen et al., 2009). Furthermore, African American women have a higher diversity of microflora that is associated with microbial invasion of the amniotic cavity and preterm birth (Fettweis et al., 2014).

Another associated risk factor of BV is douching, since it mechanically causes an imbalance in normal flora through physical and chemical irritants; douching solutions often contain surfactants that lyse the normal flora and allow for other bacterial growth. Additionally, the use of intrauterine devices is a risk factor for BV. It is thought that the presence of the IUD string in the vagina allows for colonization of anaerobic bacteria associated with BV (Madden et al., 2012). Finally, for reasons not completely understood, having multiple sex partners increases the risk for BV especially women who have sex with women, WSW, have a higher BV prevalence than women who do not (Koumans et al., 2009; Munzy et al., 2013).

Clinical diagnosis of BV is done either by the Amsel criteria or by the Nugent scoring system. For the Amsel criteria, three of the four criteria characterizes a positive diagnosis of BV: a thin, homogenous grey-white discharge; pH greater than 4.5; presence of clue cells on wet mount; positive whiff amine test where a fishy smell is present with 10% KOH is added to sample of discharge (Amsel et al., 1983; Jefferson, 2012; Srinivasan et al., 2012). The Nugent system utilizes the gram stain, which is the gold standard of BV diagnosis (Srinivasan et al., 2012). The scoring system is based on the presence of lactobacilli, curved rods and small gram negative rods (Nugent et al., 1991). In the majority of cases, women who test positive for BV are asymptomatic, so it is unclear why or what factors promote symptoms (Srinivasan et al., 2012).

Treatment for BV includes either oral or topical administration of clindamycin or metronidazole; however, relapse among women is very high (Hay, 1989; Srinivasan et al., 2012). This complex polymicrobial syndrome includes many species of bacteria, like *Sneathia amnii*, that play a role in both vaginal and gestational health.

Sneathia amnii strain Sn35

Sneathia amnii, formerly classified under *Leptrotrichia*, is an anaerobic gram negative, non-motile bacterium that is part of the normal urogenital flora of women (Harwich et al., 2012). This species, however, is associated with serious medical complications such as; preeclampsia, spontaneous abortion, bacterial vaginosis, preterm birth, preterm premature rupture of the fetal membranes and post-partum bacteremia in both mother and neonate (Bachy et al., 2011; Harwich et al., 2012). The uterine cavity and amniotic fluid are thought to remain sterile throughout gestation; however bacterial infections can occur and *S.amnii* is frequently isolated from the amniotic cavity. (Harwich et al., 2012) *S.amnii* infiltration of the fetal membranes and amniotic cavity can lead to chorioamnionitis and/or amnionitis (Han et al., 2009). Other studies have shown that *Sneathia* colonization of the male urogenital tract can occur, suggesting that this organism can be sexually transmitted (Nelson et al., 2010). Additionally, through the Vaginal Microbiome Project at VCU, mid-vaginal isolates of 736 women were sequenced and *Sneathia* was found to be present in 43.4% of women with an abundance threshold of 0.1% of total reads (Harwich et al., 2012). *S.amnii* is a fastidious bacterium that requires blood or human serum for growth. Growth can occur on chocolate agar and BHI agar with supplemented blood; however it cannot survive on Brucella Sheep's blood agar, making this organism human specific (Harwich et al., 2012).

Strain Sn35, a mid-vaginal isolate, was from an African American woman in her early 20's enrolled in the Vaginal Microbiome Project at VCU who had extremely preterm birth at 26 weeks (Harwich et al., 2012; Fettweis et al., 2011). Sequencing and identification was done using the V1-V3 regions of the 16S rRNA gene and was shown that *S.amnii* has high homology to another vaginal bacterium, *Sneathia sanguinegens*; which often co-occurs in the vagina (Harwich et al., 2012). *Sneathia amnii* has the smallest genome of the Fusobacteriaceae family with a genome size of approximately 1.34 Mbp and has 28% GC content. *S.amnii* has 1,282 protein encoding genes, making up 92% of the genome and has at least 110 overlapping genes (Harwich et al., 2012).

With scanning electron microscopy, *S.amnii* was found to have a variety of morphologies; it has been seen as a long rod, short rod, and both amorphous rods and cocci. The shorter morphologies have been identified in other bacterial species such as, *Streptococcus monilformis* and are referred to as "L forms" where the cell wall is deficient (Dienes, 1947). "L forms" of *S.amnii* were typically found in older cultures and active cultures were dominated by bacillus (Harwich et al., 2012). Occasionally, the rod shaped bacterium exhibits bulbous protrusions at the ends of the rods (Harwich et al., 2012).

Preliminary research on *Sneathia amnii* identified that it has weak alpha hemolysin activity, and has the ability to ferment glycogen, maltose and glucose while is unable to ferment starch, mucin, galactose, sucrose or fructose (Harwich et al., 2012). Similar to other pathogenic bacteria, *S.amnii* does not have enzymes necessary for synthesis of most amino acids; however, it does have enzymes required to convert L-amino acids to D-amino acids and the enzymes necessary for the conversion of L-aspartate to fumarate, L-asparagine and oxaloacetate. Additionally, genes required for de novo amino acid biosynthesis have not been identified in the

species; however, salvage pathways for purine and pyrimidine synthesis are present (Harwich et al., 2012). A putative DNA restriction modification system was identified; however, it lacks a complete system of genetic competence (Harwich et al., 2012). Furthermore, the sequence of *S.amnii* identified a putative Type II secretion system, and has genes related to ion transport and multi-drug/lipid/protein pumps (Harwich et al., 2012).

While most gram negative bacteria are inherently vancomycin resistant, *Sneathia amnii* is vancomycin sensitive. It is also sensitive to metronidazole, a common drug used to treat bacterial vaginosis; however it is resistant to nafcillin, ciprofloxacin, and tetracycline. Women who are treated for BV with metronidazole during gestation still have a twofold increased risk of preterm birth. During BV, biofilm formation on the vaginal epithelium can occur which can lead to antibiotic resistance; *S.amnii* can become resistant or tolerant of antibiotic measures if associated with a biofilm (Harwich et al., 2012; Swidsinski et al., 2008). While this species is part of the normal urogenital microflora of women little is known about this organism; however, *Sneathia amnii* seems to be a significant opportunistic pathogen that plays a role in vaginal health, MIAC, and preterm birth.

Research Objectives

Sneathia amnii is part of the vaginal normal flora; however, it has serious clinical implications such as bacterial vaginosis and preterm birth. Since bacterial vaginosis is a risk factor for preterm birth and the greatest risk factor for preterm birth is having previously had preterm birth, it is essential to understand the ability of species like *S. amnii* to play a part in both clinical pathologies. Additionally, most extreme preterm births arise from intrauterine infections and inflammation and pose a more serious threat to the neonate. While *Sneathia amnii* can be

found in 25% of amniotic fluid samples of patients presenting with preterm birth, the ability of *S.amnii* to traverse the cervical mucus plug and enter into the amniotic cavity remains unknown. Recent studies in our lab indicating that *S.amnii* is cytotoxic to ME-180 cervical epithelial cells show a small glance of the virulence potential of *S.amnii*. The goal of this project is to utilize a *Sneathia amnii* mid-vaginal strain isolated from a woman who gave birth at 26 weeks to better elucidate details of *S.amnii*'s virulence determinants and its ability to traverse the fetal membranes. First, to characterize protease interactions with fetal membrane substrates and secondly, to explore bacterial ascension into the uterine cavity. These objectives have resulted in the characterization of a novel protease expressed by *S.amnii*, a potential virulence factor.

CHAPTER 2

Materials and Methods

Bacterial Strains and Growth Conditions

The *S. amnii* and *E.coli* strains used throughout this study are listed in Table 1. *Sneathia amnii* was maintained under anaerobic conditions at 37ºC on SBHI blood plates. SBHI blood plates were composed of brain heart infusion (BHI) agar (Oxoid, Cheshire, England) + 10% gelatin + 0.5% starch + 0.5% dextrose + 5% yeast and 10% female pooled human blood. Liquid media was composed of SBHI broth and pooled human serum (MP Biomedicals, Santa Ana, CA). SBHI broth was composed of brain heart infusion broth + 10% gelatin + 0.5 % starch + .5% dextrose + 0.5% yeast. After autoclaving, 10% human serum was added to the SBHI broth and then filter sterilized prior to *S.amnii* inoculation. Anaerobic conditions were met through the Shell Lab BacBasic Anaerobic Chamber with anaerobic gas mixture; 5% carbon dioxide, 5% hydrogen and 5% nitrogen. *Escherichia coli* strains were grown aerobically at 37ºC on Luria-Bertani broth (LB) agar plates containing the appropriate antibiotic. *E.coli* liquid cultures were grown in LB, incubated aerobically at 37ºC, 200 rpm. Appropriate antibiotics were incorporated at the following concentrations 100µg ampicillin (Amp)/ml for *E. coli*, 35µg chloramphenicol (Cm)/ml for *E. coli*.

Table 1. Bacterial Strains

Strain	Description	Source
CH3 Blue	Chemically competent cells derived from *E.coli* K12 cloning strain (Amp^r when transformed with plasmid	Bioline (Tauton, MA)
BL21-CodonPlus (DE3)-RIL	Chemically competent cells derived from *E.coli* Bl21(Amp^r when transformed with plasmid, Cm^r)	Bioline (Tauton, MA)
BL21+pET32xT+peg1	BL21-CodonPlus(DE3)RIL cells containing peg1 from Sn35 *in trans* using pET32xT (Amp^r , Cm^r)	This study
Sn35	Mid-vaginal isolate from woman in her early 20's presenting with extreme preterm birth at 26 weeks	Vaginal Microbiome Consortium, VCU
Lactobacillus crispatus	Mid-vaginal Isolate	Vaginal Microbiome Consortium, VCU

Polymerase chain reaction (PCR) and product purification

For all PCR amplifications of DNA, general conditions were used with some modifications that were dependent upon the DNA template in use, the melting temperature (T_m) of the primer, and the size of the product being amplified. Reactions were composed of a 50µL total volume, 1 µL 10mM dNTPS, 1 µL template,1µL of both forward and reverse primer (10pmol dilutions in sterile water), 1µL Phire II polymerase (Thermo Scientific, Waltham, MA), 10 µL 5X Phire buffer and 25 µL of sterile water. Primers utilized in this study are listed in Table 2 and primer design was based off *S.amnii* isolate Sn35. Thermocylcer reaction conditions were programmed as the following: heated lid at 105 ºC, initial denaturation 99ºC for 2 minutes, followed by 31 cycles of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 seconds, and extension at 68ºC for 2 minutes (+2 minutes, depending upon size of amplified region), and an additional cycle of final extension at 68ºC for 10 minutes. PCR products were then held at 4ºC in thermocylcer or placed in -20ºC freezer until ran on DNA gel electrophoresis and subsequently purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA) using manufacturer's instructions.

DNA Agarose Gel

1% DNA agarose gels were made with 1X Tris-acetate- EDTA (TAE) buffer (Life Technologies, Waltham, MA), agarose, and 0.15µg/mL of ethidium bromide. 5X Loading DNA Buffer (Bioline, Taunton, MA) was added to DNA samples for a final concentration of 1X. Agarose gels were electrophoresed at 124 volts in 1X TAE buffer and examined under UV light. The size of the product was determined against Bioline Hyperladder 1kb weight markers (Bioline, Taunton, MA).

Table 2. Primer List

Primer Name	Sequence
PCR4TOPO T3	5´-ATTAACCCTCACTAAAGGGA-3´
PCT4TOPO T7	5´-TAATACGACTCACTATAGGG-3´
Sn35Coll2FWD	5'-GGCCGGGGATCCTTTTCAGATGCTCGTATTGATAAGAATAAAAGA-3'
Sn35Coll2REV	5'GGCCGGCTCGAGTTATAGATACTCATAGGATTTACAATG-3'

<u>Plasmid DNA purification and modification</u>

Plasmid purifications were done using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) using the manufacturer's instructions. Single colony transformants of *E. coli* were grown overnight in 3 ml LB with 100µg ampicillin/ml. Plasmid modification/ DNA manipulation restriction enzymes, buffers, and BSA were purchased from New England Biolabs (Ipswich, MA) and were used according to the manufacturer's protocols. Plasmid restriction digests were done using BamHI and XhoI, and the 5' phosphoryl groups were removed using Antarctic Phosphatase (New England Biolabs, Ipswich, MA) to prevent re-ligation of plasmid.

<u>Plasmid Construction</u>

DNA fragments were subcloned into PCR4-TOPO (Life Technologies, Waltham, MA) using manufacturer's recommended procedure and were transformed into CH3 Blue chemically competent *E.coli* (Bioline, Taunton, MA). After incubation overnight on LB agar with ampicillin, single colonies were selected and grown overnight in LB broth with ampicillin. Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and digested with New England Biolabs restriction enzymes and buffers according to the manufacturer's recommendations. Digests were electrophoresed on DNA agarose gel. Bands that were the appropriate insertion size, based off Hyperladder 1kb (Bioline, Taunton, MA) were gel extracted and purified. Ligation of purified DNA fragment insert into digested pET vectors was done using Ready-To-Go T4 DNA Ligase and Ligase Buffer (Amersham Biosciences, Piscataway, NJ). Ratio used for ligation was a 3µL insert to 1µL plasmid, at 16ºC for 1 hour.

<u>Transformation of chemically competent *E. coli*, CH3 Blues</u>

A vial of chemically competent *E.coli* cells was thawed on ice, 3µL of pET32xT plasmid ligation reaction was added to the cells and reaction was incubated on ice for 30 minutes. The tube was placed in a 42ºC water bath for 30 seconds, immediately afterward 200µL of warmed SOC media (85.5mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 2% tryptone, 0.5% yeast extract, and 20mM glucose) was added to the reaction. The cells recovered on a shaker at 37ºC for 1 hour. Cells were plated on LB plates containing 100µg/mL of ampicillin.

<u>Transformation of chemically competent *E. coli*, BL21 DE3 RIL</u>

A vial of electrocompetent *E.coli* cells was thawed on ice; 0.7µL of β-mercaptoethanol was added to the cells. The reaction was incubated on ice for 10 minutes and gently swirled every 2 minutes before adding 3µL of ligated plasmid and incubating on ice for 10 minutes. SOC media (85.5mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 2% tryptone, 0.5% yeast extract, and 20mM glucose) was pre-warmed in a 42ºC water bath. The reaction was heat pulse in a 42ºC water bath for 45 seconds and 250µL of warmed SOC media was immediately added to the reaction. The reaction was incubated for 1 hour at 37ºC, shaking at 200 rpm.

<u>Induction of BL21 DE3 RIL- Coll2 bacterial cells</u>

Once plasmid was sequenced to confirm proper insertion of template into pET32xT, plasmid containing bacterial cells were inoculated in 6 mL overnight cultures containing LB, 100µg/ml ampicillin (Amp) and 35µg/ml chloramphenicol (Cm) at 37ºC on shaker. The overnight cultures were diluted 1:200 in 1L of fresh LB containing 100µg/ml ampicillin (Amp) and incubated at 37ºC for 4 hours. The culture was induced with 1mM IPTG and incubated for

an additional 2 hours at 37ºC. Prior to induction, 500μL of the culture was collected, the bacteria were obtained through centrifugation. After 2 hour induction, 500μL of culture was collected, the bacteria were obtained through centrifugation. Uninduced and induced controls were electrophoresed on a NuPAGE SDS-PAGE Gel system (Invitrogen, Grand Island, NY) at 200V for 30 minutes in 1X NuPAGE Running Buffer. Lanes were stained with Imperial Protein Stain (Thermo Scientific, Waltham, MA) for 1 hour and then destained with water until bands were clearly visible. Bacterial cells were collected from cultures by centrifugation at 8,000 rpm at 4ºC for 15 minutes. Pellets were suspended in 25 mL of 1X sterile PBS (Invitrogen, Grand Island, NY) and were subsequently harvested by centrifugation at 4,000 rpm at 4ºC for 15 minutes. The pellet was frozen at -80ºC.

Polyacrylamide gel electrophoresis

All protein gels used the NuPAGE SDS-PAGE Gel system (Invitrogen, Grand Island, NY). Samples were mixed using a 30:10:4 ratio, 30 μL of sample, 10μL of Novex NuPAGE LDS Sample Buffer 4X (Life Technologies, Waltham, MA) and 4μL of NuPAGE Sample Reducing Buffer (Invitrogen, Grand Island, NY). Samples were boiled for 10 minutes and loaded into the wells of a NuPAGE 4-12%Bis-Tris 1.0mm Gel (Invitrogen, Grand Island, NY). Gel electrophoresis was for 30 minutes at 200Vs in 1X NuPAGE Running Buffer. Gels were then stained with Imperial Protein Stain for one hour and destained in water until bands were visible. Peptide size was determined using Precision Plus Protein Kaleidoscope Standard (BioRad, Hercules, CA).

Protein Purification

The bacterial pellet from 1 L induction was thawed on ice and was resuspended in a solution of 25 mL 50mM NaP, 300mM NaCl buffer and one Complete, EDTA Free Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN). The resuspended pellet was then lysed by passing through a French Pressure Cell Press (Thermo Scientific, Waltham, MA) 4 times. The solution was then lysed by sonication on ice 6 times with 30 second sonication periods and 1 minute rests between each pulse, bubbles were not introduced. The lysed solution was then centrifuged at 12,000 rpm at 4ºC for 20 minutes. The lysate was filtered with 0.45µm filter and immediately loaded onto a 3 mL HisPur Cobalt Resin (Thermo Scientific, Waltham, MA) column. After flow through collection, the column was subjected to 6 washes consisting of 10 mL of 50mM NaP + 300mM NaCl and 10mM imidazole. Washes were collected and incubated on ice, wash 7 consisted of 10 mL 50mM NaP + 300mM NaCl and 20mM imidazole. Final elution was consisted of 3 mL of 50mM NaP + 300mM NaCl and 250mM imidazole. The Final elution was performed in 3, 1 mL washes where 1 mL of elution buffer was rocked gently in resin for 5 minutes before eluate was collected. A280 reading was collected on elute. The Eluate was dialyzed overnight at 4ºC against 3L of 20mM Tris-HCl pH 8.0 with 150mM NaCl using a 3,500 MWCO Slide-A-Lyzer Dialysis Cassette G2(Thermo Scientific, Waltham, MA).

Thrombin Cleavage

The protein was removed from overnight dialysis cassette, A280 reading was recorded. Thrombin CleanCleave Kit (Sigma Aldrich, St. Louis, MO) was used for removal of the purified protein's Thio-HIS tag. Thrombin cleavage was performed according to the manufacturer's instructions except the following deviations; 150 mM of $CaCl_2$ was added to purified protein,

thrombin beads, and cleavage buffer. Cleavage reaction was rocked at 25ºC for 2 hours. The reaction was centrifuged at 4,000 rpm for 2 minutes; supernatant was collected and ran through HisPur Cobalt Resin (Thermo Scientific, Waltham, MA) column. Flow thru was collected and centrifuged at 4,000 rpm for 2 minutes, elute was collected. The 3 mL final collection of purified protein was then diluted with 12 mL sterile water and was concentrated using Amicon Ultra-4 Centrifugal Filters, 30k MWCO (Millipore, Billerica, MA) at 4,000 rpm until 500μL of elute remained. Thrombin cleaved purified elute was either stored at -80ºC or immediately used.

Casein/Mucin/ Gelatin Zone of Activity Plates

General conditions were used for all zone of activity plates, with the exception of the substrate used; casein, gelatin from cold water fish skin (Sigma, St. Louis, MO), and mucin (Sigma, St. Louis, MO). 1% agarose, 1% substrate plates were made by dissolving agarose in 1X Tris-acetate-EDTA (TAE) buffer (Life Technologies, Waltham, MA). The agarose was heated until boiled; substrate was added and poured in 35x10mm plates (Fisher Scientific, Pittsburg, PA). After plates were solidified, 10 μL of sample were pipetted 1 inch apart. Samples included; 1X sterile PBS (Life Technologies, Waltham, MA), sterile water, uncleaved purified protein, cleaved purified protein, proteinase K (1:10 dilution, in sterile water) (Qiagen, Valencia, CA). Plates were placed in 37ºC incubator overnight. The plates were observed to identify zones of activity.

Biotinylation of Collagen Type I and Collagen Type IV

Both collagen type IV (Sigma Aldrich, St. Louis, MO) and collagen type I (Sigma Aldrich, St. Louis, MO) were from human placental tissues. Collagen types were dissolved in 0.5

M acetic acid to achieve 2mg/ml. Solutions were gently vortexed until completely dissolved, 242.5 µL of 5M NaOH was added to neutralize the solutions. Appropriate calculations were done to identify the amount of biotin necessary for each collagen type. EZ-link Sulfo-NHS Biotin kit (Thermo Scientific, Waltham, MA) was used to biotinylate collagen type I and IV. 10mM Biotin was prepared with ultrapure water; 71.9 µL of biotin solution was added to each collagen solution. Reaction was then incubated on ice for 2 hours. Reaction clean-up was done with Zeba Spin Desalting Columns (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Biotinylated collagen type I and IV were aliquoted in 100 µL in Siliconized Polypropylene Extended Capacity Tubes (Sigma Aldrich, St. Louis, MO) and were stored at -80ºC. Dot blot was performed on biotinylated collagen type I and IV to verify Biotinylation. Collagen type I and IV were spotted onto Pure Nitrocellulose Blotting Membrane (Pall, Pensacola, FL) in 2 µL aliquots in various dilutions. Membrane was left to dry and blocked in 5% non-fat milk in PBS for 1 hour. Afterward 1.1 µL of Streptavidin- HRP (Thermo Scientific, Waltham, MA) was added to the membrane and shook for 1 hour at 25ºC. Membrane was washed 3 times in 0.005% PBST and subsequently incubated with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and developed.

Collagenase Assay

Samples used in collagenase assay included; cleaved purified protein, 1X sterile PBS (Life Technologies, Waltham, MA), 1:10 dilution Proteinase K solution (Qiagen, Valencia, CA) and overnight cultures of *Sneathia amnii* isolate, Sn35. Biotinylated collagen type I and IV were diluted to a 1:25 working concentration in 1X sterile PBS (Life Technologies, Waltham, MA), 10 µL of sample was incubated with collagen in Siliconized Polypropylene Extended Capacity

Tubes (Sigma Aldrich, St. Louis, MO) overnight at 37ºC. Samples were boiled for 10 minutes

and were loaded NuPAGE 4-12% Bis-Tris 1.0mm Gel (Invitrogen, Grand Island, NY). Gel

electrophoresis ran for 30 minutes at 200Vs in 1X NuPAGE Running Buffer. After

electrophoresis, gel was rinsed with deionized water. A 0.45µm PVDF membrane (Invitrogen,

Grand Island, NY) was soaked in 100% methanol while sponges and Criterion Blotter Filter

Paper (BioRad, Hercules, CA) were presoaked in 1X NuPAGE Transfer Buffer (Invitrogen,

Grand Island, NY) with 10% methanol. Sponges, filter paper, gel and membrane were stacked

upon one another in the Invitrogen transfer system so the arrangement was; 4-5 sponges, 1filter

paper, gel, PVDF membrane, 1 filter paper, and finally 4-5 sponges, more sponges allowed for

tight contact between the PVDF membrane and the gel. The transfer was run at 45V for 90

minutes on ice in 1X NuPAGE transfer buffer with 10% methanol. Membrane was blocked

overnight at 4ºC in 5% nonfat milk in 1X PBS and subsequently probed with 1:15,000-diluted

Streptavidin-HRP (Thermo Scientific, Waltham, MA) and 1X PBST (PBS + 0.05% Tween 20)

for 1 hour, rocking at 25ºC. The membrane was washed 3 times for 4 minutes with 1X PBST and

developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare,

Buckinghamshire, UK), where membrane was immersed in solution for 1 minute, covered in

plastic wrap and exposed to Blue Devil Interleaved Autoradiography Film (Genesee Scientific)

and developed.

Fibronectin assay

       Samples were incubated with fibronectin from Human Plasma (Sigma Aldrich, St. Louis,

MO) at 37ºC overnight. Fibronectin was diluted 1:10 with sterile 1X PBS (Invitrogen, Grand

Island, NY) and each incubated reaction had 10µL of fibronectin. Fibronectin was incubated in a

variety of salt solutions; 10mM $CaCl_2$ + 15mM $ZnCl_2$, 15mM $ZnCl_2$ +10 µL of cleaved purified

protein, 2mM $CaCl_2$ + 5mM $ZnCl_2$, 2mM $CaCl_2$ + 15mM $ZnCl_2$ + 10 µL of cleaved purified

protein, 5mM $ZnCl_2$ +10µL of cleaved purified protein diluted 1:5, 5mM $ZnCl_2$ +10 µL of

cleaved purified protein diluted 1:25, 5mM $ZnCl_2$ +10 µL of cleaved purified protein diluted

1:125. Samples were mixed using a 30:10:4 ratio, 30 µL of sample, 10 µL of Novex NuPAGE

LDS Sample Buffer 4X (Life Technologies, Waltham, MA) and 4 µL of NuPAGE Sample

Reducing Buffer (Invitrogen, Grand Island, NY). Reactions were boiled for ten minutes and then

loaded onto a NuPAGE 4-12%Bis-Tris 1.0mm Gel (Invitrogen, Grand Island, NY). Gel

electrophoresis ran for 30 minutes at 200Vs in 1X NuPAGE Running Buffer where band size

was determined using Precision Plus Protein Kaleidoscope Standard (BioRad, Hercules, CA).

Gels were then stained with Imperial Protein Stain (Thermo Scientific, Waltham, MA) for one

hour and destained in water until bands were visible. Degradation of fibronectin was evaluated

based on complete disappearance of fibronectin peptide band, 220kDa.

Laminin Assay

Samples were incubated with laminin from Human Placental Liquid (Sigma Aldrich, St.

Louis, MO) at 37ºC overnight. Laminin was diluted 1:10 with sterile 1X PBS (Invitrogen, Grand

Island, NY) and each incubated reaction had 20µL of laminin. Laminin was incubated in a

variety of salt solutions; 10mM $CaCl_2$ + 15mM $ZnCl_2$, 15mM $ZnCl_2$ +10 µL of cleaved purified

protein, 2mM $CaCl_2$ + 5mM $ZnCl_2$, 2mM $CaCl_2$ + 15mM $ZnCl_2$ + 10 µL of cleaved purified

protein, 5mM $ZnCl_2$ +10 µL of cleaved purified protein diluted 1:5, 5mM $ZnCl_2$ +10 µL of

cleaved purified protein diluted 1:25, 5mM $ZnCl_2$ +10 µL of cleaved purified protein diluted

1:125. The samples were mixed using a 30:10:4 ratio, 30 µL of sample, 10 µL of Novex

NuPAGE LDS Sample Buffer 4X (Life Technologies, Waltham, MA) and 4µL of NuPAGE Sample Reducing Buffer (Invitrogen, Grand Island, NY). The reactions were boiled for ten minutes and then loaded onto a NuPAGE 4-12%Bis-Tris 1.0mm Gel (Invitrogen, Grand Island, NY). Gel electrophoresis ran for 30 minutes at 200Vs in 1X NuPAGE Running Buffer where band size was determined using Precision Plus Protein Kaleidoscope Standard (BioRad, Hercules, CA). Gels were then stained with Imperial Protein Stain (Thermo Scientific, Waltham, MA) for one hour and destained in water until bands were visible. The degradation of laminin was evaluated based off complete disappearance of laminin peptide bands at 50 kDa and 130-160kDa.

Coagulase Assay

BBL Coagulase Plasma Rabbit (BD, Franklin Lakes, NJ) vials were rehydrated in sterile purified water; one vial was aliquoted into six separate tests. Reactions with coagulase test included; 1X Sterile PBS (Invitrogen, Grand Island, NY), live overnight culture of *Sneathia amnii* isolate SN35, cleaved purified protease + 5mM $ZnCl_2$, cleaved purified protease, 5mM $ZnCl_2$, 2mM $CaCl_2$. The reaction vials were gently mixed and incubated in 37ºC water bath. The tubes were examined by tipping the tube to identify clotting after 4 hours and then again at 24 hours to identify any weak enzyme production.

Turbidity Assay

A 2mL overnight culture of SN35 was grown and subcultured into 10mL of fresh media. Culture grew for 6 hours to reach exponential stage and was removed from the anaerobic chamber. Culture was divided into two 5mL cultures. One 5 mL culture was centrifuged at

4,000rpm for 4 minutes. Supernatant was removed and pellet was washed with sterile water and centrifuged. The pellet was resuspended in 4 mL of sterile water and aliquoted into two 2 mL tubes. Non-centrifuged culture was separated and aliquoted into two 2 mL tubes. The initial sample optical density readings, OD_{600nm}, were determined using a spectrophotometer; sterile SBHIs and sterile water were used as controls. Afterward, 100μL of cleaved purified protease was added to 1 tube of Sn35 in SBHI media and 1 tube of Sn35 in sterile water; 100μL of dialysis buffer was added to other Sn35 tubes. The optical density was determined immediately, then after 30 minutes, 1 hour, 2 hours, and 24 hours.

Collagen Type IV Biofilm Assay

Cell Adhesion Human Collagen Type IV Strips (Millipore, Temecula, CA) were rehydrated in 200 μL of 1X sterile PBS (Invitrogen, Grand Island, NY) for 15 minutes at room temperature; PBS was removed from the strips. Overnight culture of Sn35 was grown and diluted until OD_{600nm} was 1. The strip wells were inoculated as such; SBHI alone, 100 μL of 1:10 dilution Sn35 OD=1, 100 μL of 1:100 dilution Sn35 OD=1, 100 μL of 1:10 dilution Sn35 OD=1 boiled for 5 minutes, 100 μL of cleaved purified protease, 100 μL of heat killed Sn35 + cleaved purified protease, SBHI alone, and last lane was left as BSA. The collagen strips were kept under anaerobic conditions at 37ºC overnight and suspensions were discarded and well strips were washed gently 2-3 times with 200 μL of PBS. The wells were stained with 100 μL of safranin; adherence was determined by visualization of the degree of safranin staining.

Edman Degradation

The protein was purified and cleaved using methods mentioned above. Thrombin treated protein was run on an 4-12% Bis-Tris 0.1mm NuPAGE gel for 30 minutes at 200V. Gel was electroblotted onto PVDF membrane using methods mentioned previously. PVDF membrane was then stained using Imperial Protein Stain (Thermo Scientific, Waltham, MA) for 1 hour. The membrane was destained for 15 minutes using 15% methanol/10% acetic acid and was rinsed under deionized water for 4 hours. The band of interest was excised and placed in microcentrifuge tube with deionized water. Edman degradation was performed by Iowa State University Protein Facility; membrane was washed 6 times in deionized water and sample was analyzed by 494 Procise Protein Sequencer/140C Analyzer from Applied Biosystems, Inc.

Bacteriolysin Assay

A 10% SDS PAGE separating gel containing 0.003g of dry *Micrococcus luteus* was poured, allowed to solidify and a 4% SDS PAGE stacking gel was prepared and poured on top of the separating gel. After the gels polymerized, cleaved purified protein was mixed with 5X Loading buffer and was electrophoresed at room temperature at 175V for 40 minutes in 1X SDS PAGE running buffer. Gels were rinsed for 1 hour under a slow stream of sterile water for one hour and then immersed in 100mM phosphate buffer pH 7.0 + 10mM $MgCl_2$. Gels remained in buffer, rocked overnight at 37ºC. Gels were rinsed with sterile water and subsequently stained with Imperial Protein Stain (Life Technologies, Waltham, MA) for one hour. Gels were destained in sterile water until banding patterns were visible.

Antiserum Production

Cleaved purified protein (> 1.5mg/ml) was produced using methods mentioned previously and shipped to New England Peptides. Prior to immunization, 5 mL of pre-immune sera was collected from one rabbit (New Zealand White- SPF), the rabbit was immunized with protein 3 times. Boosts occurred at day 14 and 28, bleeds occurred on days 35 and 40. Upon arrival, sera were aliquoted into cryovials and stored at -80ºC.

Antisera Detection and Subsequent Westerns with antisera

All western analysis performed with antisera had a positive control sample consisting of frozen cleaved purified protein. Samples were electrophoresed with SDS NuPAGE gel as previously mentioned in methods. After electrophoresis, gels were rinsed with deionized water. 0.45µm PVDF membranes (Invitrogen, Grand Island, NY) were soaked in 100% methanol while sponges and Criterion Blotter Filter Paper (BioRad, Hercules, CA) were presoaked in 1X NuPAGE Transfer Buffer (Invitrogen, Grand Island, NY) with 10% methanol. Sponges, filter paper, gel and membrane were stacked upon one another in the Invitrogen transfer system so the arrangement was; 4-5 sponges, 1filter paper, gel, PVDF membrane, 1 filter paper, and finally 4-5 sponges, more sponges allowed for tight contact between the PVDF membrane and the gel. Transfers were run at 45V for 90 minutes on ice in 1X NuPAGE transfer buffer with 10% methanol. Membranes were blocked overnight at 4ºC in 5% nonfat milk in 1X PBS and subsequently probed with anti-sera, 1:2,500 in 1X PBST(PBS + 0.05% Tween 20) for one hour; rocking at room temperature. Membranes were washed 4 times for 15 minutes with 1X PBST and then probed with 1:15,000 diluted Goat-Anti-Rabbit IgG (H+L) Horseradish Peroxidase Conjugate (Life Technologies, Waltham, MA) for 45 minutes; rocking at room temperature.

Membranes were washed 4 times for 15 minutes in 1X PBST. The membrane blots were

incubated in ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire,

UK) for 1 minute, covered in plastic wrap and developed with autoradiography film.

Sn35 Growth Curve Assay

A 250 mL culture of SBHI + 10% Human Serum was inoculated with a 5 mL overnight

culture of Sn35 under anaerobic conditions at 37ºC. At 4 hours, 100 mL of growing culture was

collected and centrifuged at 4,000 rpm for 20 minutes. The supernatant was discarded and pellet

was stored at -80 ºC. Additional time points of 6, 8, 12, 16, 24, and 48 hours were collected,

centrifuged, and stored at -80º C as well. For each time point the culture collected was halved so

that at 48 hours 1.56 mL was collected. Pellets were resuspended in NuPAGE SDS Loading

Buffer and boiled for 10 minutes, afterward A280 reading was performed to confirm protein

concentration. Samples were loaded into wells so that each contained equivalent amounts of

protein. SDS page gels were electrophoresed, transferred onto PVDF membrane and

subsequently probed with antisera.

Adhesion Binding Assay

A 200 mL culture of SBHI 5% human serum was inoculated with SN35 and grown

overnight. Bacterial cells were harvested by centrifugation at 3,000g for 20 minutes at 4ºC.

Supernatants were discarded and pellets were gently resuspended in 7 mL of TSE buffer

(200mM Tris-HCL pH 8.0, 500mM sucrose, 1mM EDTA, protease inhibitor cocktail). Cells

were incubated on ice for 30 minutes and then centrifuged at 16,000g for 30 minutes at 4ºC.

Supernatants were transferred to an ultracentrifuge tube and centrifuged at 60,000g for 1 hour at

4ºC. The final spin pellet contained outermembrane proteins while the supernatant contained periplasmic proteins. Pellet was resuspended in 200 µL of TSE buffer, if not immediately used pellet was stored at -80ºC. Eight CellStar T75 Flasks (Greiner Bio-One, Monroe, NC) of VK2/E6E7 (ATCC, Manassas, VA), human epithelial vaginal mucosal cells, in 1X Keratinocyte Serum Free Media (Life Technologies, Waltham, MA) accompanied with Supplements for Keratinocyte Serum Free Media (Life Technologies, Waltham, MA) were prepared. Once cells were grown to 80-90% confluency, outermembrane cell surface proteins were biotinylated and extracted using Pierce Cell Surface Protein Isolation Kit (Thermo Scientific, Waltham, MA). Manufacturer's instructions were followed for Biotinylation, Cell Lysis, and Isolation of Labeled Proteins. After the streptavidin beads were washed in final wash buffer, they were washed with 1X sterile PBS (Life Technologies, Waltham, MA) and centrifuged at 1000g, this was repeated 3 more times. Outer membrane surface proteins isolated from Sn35 were added to streptavidin beads and rocked for 30 minutes at room temperature with end-over-end rotator. Flow thru was collected via centrifugation at 1000g for 1 minute. The streptavidin beads were subject to a series of stringent washes where each wash was collected by centrifugation at 1000g for 1 minute. All washes were done twice and consisted of 500µL. Washes went in the following order; 10mM Tris-HCl pH 8.0 + 10mM NaCl, 10mM Tris-HCl pH 8.0 + 25mM NaCl + 10mM KCl, 10mM Tris-HCl pH8.0 + 100mM NaCl + 25mM KCl, 10mM Tris-HCl pH 8.0 + 150mM NaCl + 50mM KCl, 10mM Tris-HCl pH 8.0 + 150mM NaCl + 200mM KCl. The final wash was Novex NuPAGE LDS Sample Buffer 4X (Life Technologies, Waltham, MA). Samples, including beads, were boiled and run on a NuPAGE SDS gel. The gel was rinsed with sterile water, stained with Imperial Protein Stain for one hour and destained until band patterns were visible.

<u>Placental Invasion Assay</u>

Full term healthy caesarean section, c-sectioned, placentas were provided by consenting women who gave birth at VCU Medical Center through IRB approval #HM10906_CR1 through Dr. Scott Walsh. Placental tissues were immediately placed in a sterile hood using sterile gloves and 4cm x 4cm sections of placental tissues were cut and submerged in a solution of 1X DMEM (Life Technologies, Waltham, MA) and 1mg/mL Penicillin Streptomycin (Life Technologies, Waltham, MA) for 2 hours in Series II Water Jacket CO_2 incubator (Thermo Scientific) at 37ºC. Sectioned tissues were washed 5 times with 1X sterile PBS (Life Technologies, Waltham, MA). Washed tissue sections were then placed on the outside of a 0.4µm Sterile cell Strainer (Fisher Scientific, Pittsburg, PA) that did not have a bottom strainer. The tissue was kept in placed with a sterile rubber band. Cell strainer + tissue were them placed in a 6 Well Tissue Culture Plate (Grenier, Nurtingen, DE). Each well had 7 mL of antibiotic free DMEM (Life Technologies, Waltham, MA) and 10% pooled human serum. The 6-well plate was incubated overnight in BacBasic Anaerobic Chamber overnight. Live cultures of SN35 and *Lactobacillus crispatus* were inoculated into separate strainers and were left under anaerobic condition overnight. Placental tissues were removed from the cell strainer and were gently washed in 1X sterile PBS 4 times. Tissues were then submerged in 4% paraformaldehyde + 2% glutaraldehyde in 0.1M Cacodylate Buffer. Tissues were embedded in paraffin, sectioned and mounted on microscope slides, and observed under light microscopy. Media from the top portion of cell strainer and the media at the bottom of the well were inoculated onto SBHI Blood agar plates and grown under anaerobic conditions, CFUs were noted.

<u>ME180 Cytotoxicity Assay</u>

A 6-well plate (CellStar) of human epithelial cervical carcinoma ME-180 cells (ATCC, Manassas, VA) were grown to 99% confluency in McCoy's 5A Media Modified (Cassion Labs, Logan, UT). Cells were incubated in CO_2 incubator at 37º C during growth. Wells were washed 3 times with 1X sterile PBS (Life Technologies, Waltham, MA) and subsequently incubated in 2 mL of 1X sterile PBS. Wells were then either inoculated with cleaved purified protease, cleaved purified protease + 50mM $ZnCl_2$, 50mM $ZnCl_2$, or 2mM $CaCl_2$. The plates were incubated overnight in CO_2 incubator at 37º C. Monolayers were examined via light microscopy.

CHAPTER 3
Analyses of Pathogenicity Potential of *Sneathia amnii* isolate Sn35

<u>**Introduction**</u>

Once bacteria have invaded the amniotic cavity; the bacterial infection is able to elicit an inflammatory response. Pathogen associated molecular patterns, PAMPs, are recognized by toll-like receptors and activate the innate immune response and the production of cytokines like IL-1α, IL-1β- IL-6, and TNF-α are stimulated (Chiesa et al., 2015; Goldenberg et al., 2008). An increase in IL-6 is a hallmark of preterm birth and the fetal inflammatory response syndrome; while IL-6 levels are modest during term labor, they are excessively higher in preterm births (Chiesa et al., 2015; Romero et al., 2007). IL-6 is produced by a variety of cells and functions to regulate the immune response, inflammation and the acute phase response (Chiesa et al., 2015). The concentration of IL-6 serves as an indicator of the severity of inflammation and if highly elevated levels of IL-6 exist, the fetal inflammatory response syndrome (FIRS) is activated (Chiesa et al., 2015; Jefferson, 2012). Furthermore, IL-6 recruits neutrophils and other leukocytes to the fetal membranes to secrete pro-inflammatory cytokines.

The activation of pro-inflammatory cytokines is a normal process during parturition and leads to the increased activity of matrix metalloproteinases and production of prostaglandins (Goldenberg et al., 2008). Throughout gestation, IL-10, an anti-inflammatory cytokine, is expressed to prevent premature responses; however the production of IL-10 decreases towards the end of gestation and allows for increased activity of other cytokines to induce labor (Hanna et

al., 2000).   In order for labor to occur, the cervix must soften, shorten and dilate, and the fetal

membranes have to become weakened in order to rupture for birth. During labor estrogen,

prostaglandins, and oxytocin hormones work together to cause the uterus to contract forcefully;

birthing the fetus (Jefferson, 2012). The decrease in IL-10 allows for corticotropin releasing

hormone receptor (CRH-R1) to be induced and thereby increases prostaglandin and placental

estrogen release (Jefferson, 2012). In addition to cytokines, women with MIAC will have

elevated amniotic fluid levels of matrix metalloproteinase-8 (Combs et al., 2014). Matrix

metalloproteinases, MMPs, like MMP-8 and MMP-9 can degrade collagen which allows for the

fetal membranes to weaken and aides in the onset of labor (Estrada-Gutierrez et al., 2010).

The fetal membranes are composed of collagens type I, III, IV and V and allow for high

tensile strength. Collagen is the most abundant protein found in mammalian bodies and is typed

based upon the structure it forms. Collagen type IV forms the basal lamina found in the basement

membranes, collagen type V is found in the placenta, collagen type III is typically found with

collagen type I which is found in organs and ligaments. The collagen in fetal membranes allows

for resilience; however, at the end of term labor MMPs degrade collagen to induce labor.

PPROM and preterm birth have been correlated with elevated activity of collagenases (MMP-8)

as well as other MMPs (Maymon et al., 2001). The increase in MMPs can be due to

inflammation, but also bacteria. MMP-9 has elevated expression in the fetal membranes in the

presence of bacteria. Additionally, select bacterial species are capable of producing their own

matrix metalloproteinases in order to degrade collagen (Jefferson, 2012). The work done on this

study was to analyze the *Sneathia amnii* genome to identify MMPs that could be responsible for

fetal membrane destruction and PPROM.

Results

Invasion of human fetal membranes by *Sneathia amnii*

It has been determined that MIAC is a cause of preterm birth. Invasion of the amniotic cavity depends upon the bacteria's ability to produce virulence factors to promote entry into the fetal membranes and cause infection. Previous data showed that *Sneathia amnii* is detectable in the amniotic cavity and can cause chorioamnionitis and amnionitis leading to preterm birth (Harwich et al., 2012). Therefore it is thought that *Sneathia amnii* is ascending from the vagina and ultimately flourishing in the amniotic cavity. Placental tissues from full term healthy c-sectioned patients were incubated with isolate Sn35 in anaerobic conditions at 37ºC to mimic the *in vivo* environment. We found that placental tissues incubated with Sn35 had extensive damage to the chorion layer causing the cells to round up (Figure 3.1) .Additionally, tissues incubated anaerobically with no bacteria saw no damage to the chorion layer; however, tissue incubated with Sn35 no longer had tight cell to cell contact. This observation supported previous hypothesis of bacterial ascension and additionally these results determined that Sn35 can invade the placental tissues over a short amount of time. However, media plated from the bottom portion of the well did not have any microbial growth from either *Lactobacillus crispatus* or Sn35, suggesting that the bacteria did not traverse the fetal membranes within 24 hours. While media plated from the top of the cell strainer did have colony growth suggesting that the bacteria survived the experimental conditions. Previous results suggest that *L.crispatus* does not traverse the fetal membranes. The media plated from the top was to ensure that bacterial survival could occur in DMEM 10% Human serum. The lack of Sn35 in the bottom portion of the well indicated that while Sn35 has the ability to traverse the first layer of the fetal membranes, it may need additional time to fully traverse the fetal membranes.
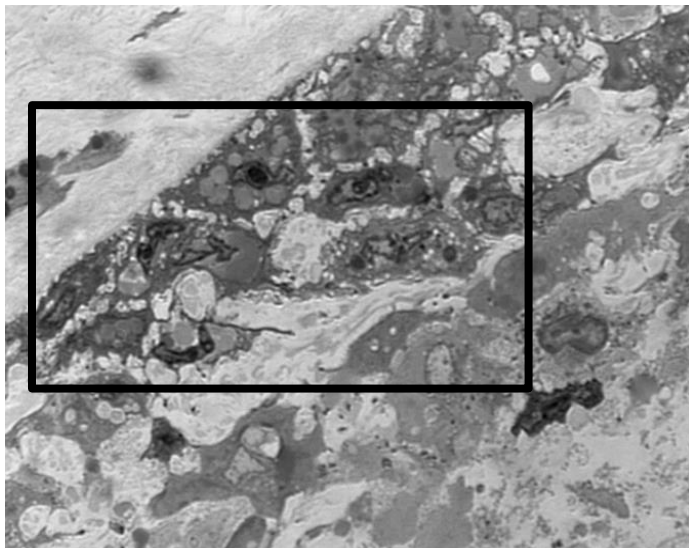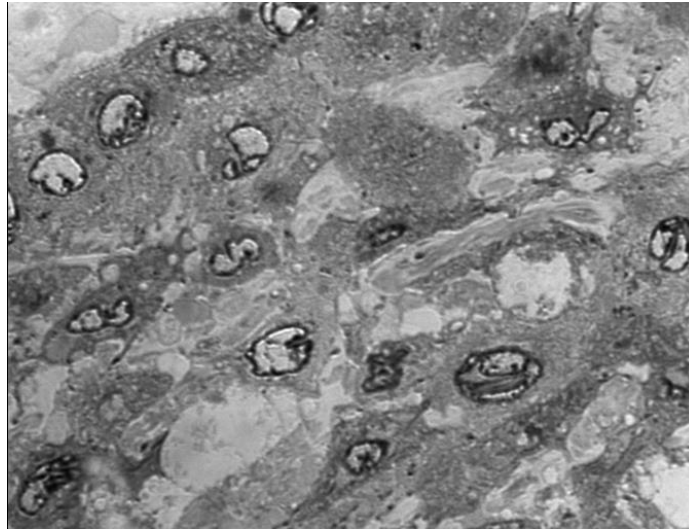
Figure 3.1: Fetal membranes incubated with or without Sn35. Placental tissue not incubated with bacteria (top image) remained intact and did not have cell or tissue damage. Placental tissue incubated with Sn35 (bottom image) had extensive tissue and cell damage. Highlighted in the box, the chorion layer no longer possessed tight cell:cell contact and cells appeared to be rounding; indicating that Sn35 was able to traverse the chorion layer.

Sn35 adhesion capability with VK2 cell line

Bacterial adhesion to a eukaryotic cell always precedes invasion. In order to identify

ligand: ligand receptor interaction, outermembrane proteins from both Sn35 and VK2 cells were

isolated. VK2 outermembrane proteins were biotinylated and bound to streptavidin beads while

outermembrane proteins from SN35 were added to the VK2 beads. A series of stringent salt

solutions was used to wash the beads in hopes of identifying a bacterial peptide with high affinity

for a eukaryotic protein. However, we did not detect a peptide that remained on the streptavidin

beads following the initial washes. Therefore we did not isolate a Sn35 adhesin.

Selection of virulence determinant expressed by Sn35

Bioinformatic analysis revealed a gene of interest in Sn35 labeled as a putative zinc

endopeptidase with homology to the M23 peptidase family comprised of glycine glycine

endopeptidases and matrix metalloproteinases.   Analysis using SignalP4.1 suggested that the

putative zinc endopeptidase had a signal peptide for secretion via the sec pathway. The

isoelectric point of the putative zinc endopeptidase is 10.63 pH(I). With the signal peptide, the

gene is 1311 bp and 436 amino acids and is 48.95 kDa .Additionally, it also has an active site

characteristic of the M23 peptidase family, HXXXD, where HXXXD is HAILD, and is found

AA site 165-169 (Figure 3.2) This putative gene was of interest to our research since it could be

a potential virulence determinant that allows Sn35 to traverse the fetal membranes .The zinc

endopeptidase gene, without its signal sequence, was then amplified by PCR with specific

primers, and was named protein encoding gene 1 (Peg1). Serial cloner indicated that the gene is

1233 bp (Figure 3.3).

```
ATG AAA ATA ACT GGG AAG TGT AGT GTA TTA CTT CTT TCA TTA GCA CTC TCT ATA
 M   K   I   T   G   K   C   S   V   L   L   L   S   L   A   L   S   I
                                                                          18


CCA TAT ATG ACA TTT TCA GAT GCT TTT TCA GAT GCT CGT ATT GAT AAG AAT AAA
 P   Y   M   T   F   S   D   A   F   S   D   A   R   I   D   K   N   K
                                                                          36


AGA AGA ATA GCA CAA ATA AAT AGA CAA GTT ACA CAA AAC AGA AAT CTT ATT ATA
 R   R   I   A   Q   I   N   R   Q   V   T   Q   N   R   N   L   I   I
                                                                          54


AAA AAC AAT AGA CAA ATT GTT GTT ACT AAA AGA ACA CAA GCA AGT TTA CAA TCA
 K   N   N   R   Q   I   V   V   T   K   R   T   Q   A   S   L   Q   S
                                                                          72


GAA ATC AAT GAC TTG AAT TTT CAA ATT AAC AAG CTA CAA AAA GAA TAT AAT GTT
 E   I   N   D   L   N   F   Q   I   N   K   L   Q   K   E   Y   N   V
                                                                          90


CTA GAA CAA AGA TAT ATA GAA CTA TTA AAA GCA ATT GGA GCT AAT GAA AAA GAT
 L   E   Q   R   Y   I   E   L   L   K   A   I   G   A   N   E   K   D
                                                                          108


ATT AAA GCA TCA ATT TCT AAA ATT CAA AGT AGT AAT GCT GAA ATA AAA GTT AAT
 I   K   A   S   I   S   K   I   Q   S   S   N   A   E   I   K   V   N
                                                                          126


AAA GAT GAA TAT AAC ACA AAG ATA AGA AAT TAT GAT TTA ATT AGA AGA GCA AGA
 K   D   E   Y   N   T   K   I   R   N   Y   D   L   I   R   R   A   R
                                                                          144


AAT ATT AAT CAA AAT AGT GGT TTA GTT GGT GCA AGA CGT GAT AAA TTA AGA CAC
 N   I   N   Q   N   S   G   L   V   G   A   R   R   D   K   L   R   H
                                                                          162


GAT GCA CAT GCT ATA CTA GAC CTA CAA ATC AAT AAA ATA AAG GGC ATA GAG ACA
 D   A   H   A   I   L   D   L   Q   I   N   K   I   K   G   I   E   T
                                                                          180
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TAT AAA AAA GGT GTA GAA CAA GAT AAG ATT AAG GTT GAA GGA ATA AAA CAA AAG
 Y   K   K   G   V   E   Q   D   K   I   K   V   E   G   I   K   Q   K
                                                                        198


AAT CAA TCT GAA GCT ACT AAA GTT ATG AGT GCT AGA AAT GAT TTA GAA AAT AAG
 N   Q   S   E   A   T   K   V   M   S   A   R   N   D   L   E   N   K
                                                                        216


AAG AAA GAA CTT AAT ACT GCT GTT GCA AGA AAG AAT AAA GCA GTA GCT GAA TTA
 K   K   E   L   N   T   A   V   A   R   K   N   K   A   V   A   E   L
                                                                        234


AGA GAC TTA CAA AAT AAA TTA AGA AAA GAA AAT ATT ACT ATT GAA AAA AAT AAT
 R   D   L   Q   N   K   L   R   K   E   N   I   T   I   E   K   N   N
                                                                        252


AGT AAA TTA GCT TCT GAA AGA AAT AGA TTA GAA GCA CAA ATT AAT GCA ATT ATA
 S   K   L   A   S   E   R   N   R   L   E   A   Q   I   N   A   I   I
                                                                        270


CGA CAA AAT GCA ATA AGA CAA CAA CAA CTT GAA AGA CAA AGA CAA TTA GCA TTA
 R   Q   N   A   I   R   Q   Q   Q   L   E   R   Q   R   Q   L   A   L
                                                                        288


CGT CGT AAA CAA CAA CAA GGT TCT GCT AAA CAT GAA AAT ATA AAT GTT TCA CAA
 R   R   K   Q   Q   Q   G   S   A   K   H   E   N   I   N   V   S   Q
                                                                        306


GAA CCA ATA AAT GTT GTA AAA GGA ACT GGA TCA TTA ATT TAT CCT ATT AGA GGT
 E   P   I   N   V   V   K   G   T   G   S   L   I   Y   P   I   R   G
                                                                        324


AGG GTA GTT GTA GGA TTT GGT ACT GAA AAA GTT CAA GGA TTA AAG AGT AAA GGT
 R   V   V   V   G   F   G   T   E   K   V   Q   G   L   K   S   K   G
                                                                        342


ATA GAA ATA TTA GGT ACA TTA GGT CAA TCT GTA TCT GCT GCT GAT ACT GGA ACT
 I   E   I   L   G   T   L   G   Q   S   V   S   A   A   D   T   G   T
                                                                        360


GTA ATA TAT TCA GGA AGT CTT GGA GGA TTA GGA AGA GTT GTA ATA ATA AGC CAC
 V   I   Y   S   G   S   L   G   G   L   G   R   V   V   I   I   S   H
                                                                        378
```

38

```
GGT TCA CTA ATT ACA GTT TAT GGT AAT TTA GCA TCT ATA AGG GTA TCA AGA AAT
 G   S   L   I   T   V   Y   G   N   L   A   S   I   R   V   S   R   N
                                                                          396


GAA GCT GTT CGT AAA GGA CAA AGT ATA GGA ACT TTA GGA AGA GAT TCT GAA ACT
 E   A   V   R   K   G   Q   S   I   G   T   L   G   R   D   S   E   T
                                                                          414


AAG AAA CCA ACA CTT TAC TTT GAA GTT AGA CAT GGT GTT AAC ATT GTA AAT CCT
 K   K   P   T   L   Y   F   E   V   R   H   G   V   N   I   V   N   P
                                                                          432


ATG AGT TAT CTA TAA
 M   S   Y   L   *
                    436
```

Figure 3.2 Gene Map of Putative Zinc Metalloprotease. The nucleotide sequence is shown

first and the amino acid sequence is directly below it. The number, seen at the end of every line,

correlates to the position of the last amino acid on the line. The signal peptide is highlighted in

green, the active site HAILD is highlighted in blue, and the M23 peptidase is highlighted in

orange.
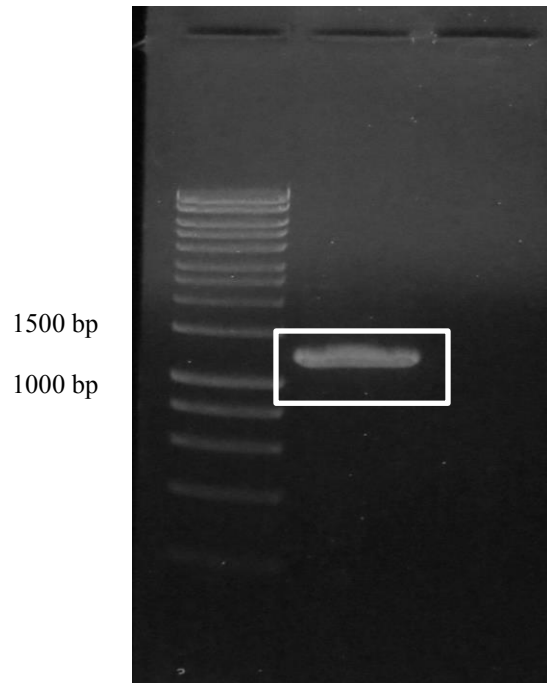
Figure 3.3: DNA gel of Peg1 PCR amplification. PCR amplification of Peg1 was done using Peg1 specific primers. Lane 1 (left most lane) contains Hyperladder 1kb, Lane 2 contains PCR product Peg1, 1123kb which is highlighted in the box. Molecular weight markers indicated. DNA sequence of Peg1 is noted below DNA gel.

Expression and purification of recombinant Peg1

Peg1 was cloned into pET32xT (generously donated by Dr. David Williams, VCU, Richmond, VA) in BL21 DE3 RIL *E.coli* cells, clones were restriction digested (Figure 3.4) and sequenced to ensure successful cloning. The vector, pET32xT, is an IPTG inducible vector which attached a C-terminal Thio-HIS tag to the protein. Expression of the Thio-His-tagged Peg1 product was confirmed through SDS PAGE gel with the presence of the protein at approximately 64.1kDa upon induction with 1M IPTG (Figure 3.5). Peg1 is 46.1kDA while the His-tag is 18kDA. The expressed recombinant protein was isolated through column purification using cobalt resin (Figure 3.6) and the Thio-His tag was removed by thrombin cleavage and the addition of calcium (Figure 3.7). If 150mM of $CaCl_2$ was not added to the thrombin cleavage reaction, Peg1 rapidly degraded in a site specific fashion leaving two peptide bands at 35kDa and 12kDA (Figure 3.8). The degradation appeared to be autocatalytic as no contaminating proteases were detected. While Peg1 does not have an obvious (HEXXH) zinc binding motif, which is common in M23 peptidases, our results determined that $CaCl_2$ does hinder Peg1's autocatalytic degradation. Additionally, these results determined that the protein degrades itself in a site specific manner to produce a C-terminal peptide and an N-terminal peptide.

Edman Degradation

Site specific cleavage is also seen in a zinc endopeptidase expressed by *Bacteriodes fragilis* (Freer et al., 1999). The protein is cleaved into a proprotein and a mature protein between an Arginine (211) and Alanine (212). We hypothesized that since *Sneathia amnii* is also a zinc metalloprotease, Peg1could cleave itself at the same motif. Sn35 has an Arginine Alanine site where if cleaved at this site it would produce a protein around 35 kDa. However, when the
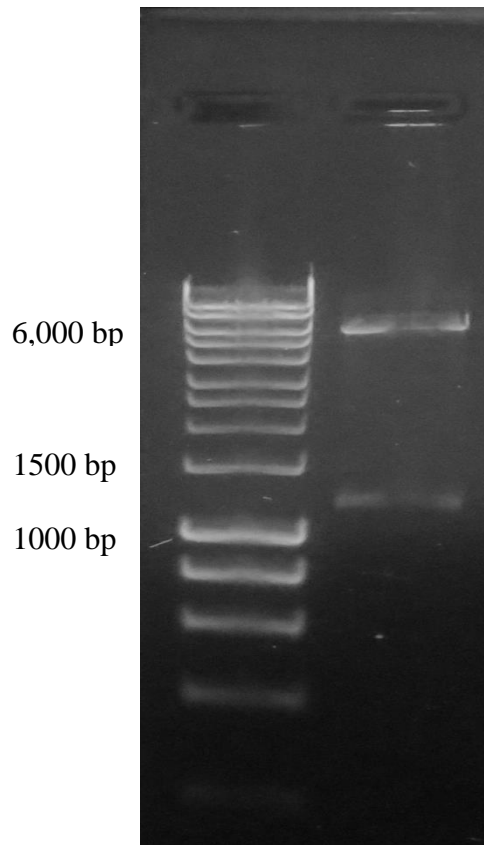
Figure 3.4F: DNA agarose gel analysis of restriction digest of BL21 DE3 RIL *E.coli* cells containing pET32xT+Peg1. Lane 1 contains HyperLadder 1kb, lane 2 contains BL21 DE3 RIL colony miniprep that has been digested with BamHI and XhoI. The fragments 5900 bp (pET32xT) and 1123 bp (Peg1) indicate successfully cloning by restriction digest confirmation.
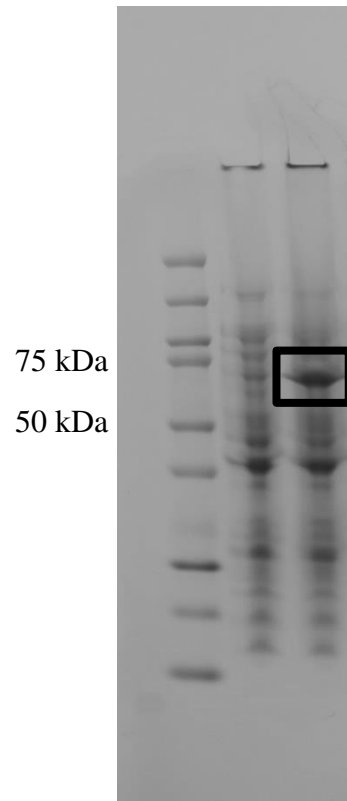
Figure 3.5: Confirmation of induced Peg1 with 1mM IPTG. Imperial stained SDS-PAGE of uninduced (Lane 2) and induced (Lane 3) cell lysates of E.coli BL21 DE3 RIL cells containing pET32xT+ Peg1. Molecular weight markers are shown above. Peg1 with Thio-HIS tag is 64.1 kDa, indicated in box, is induced under IPTG.
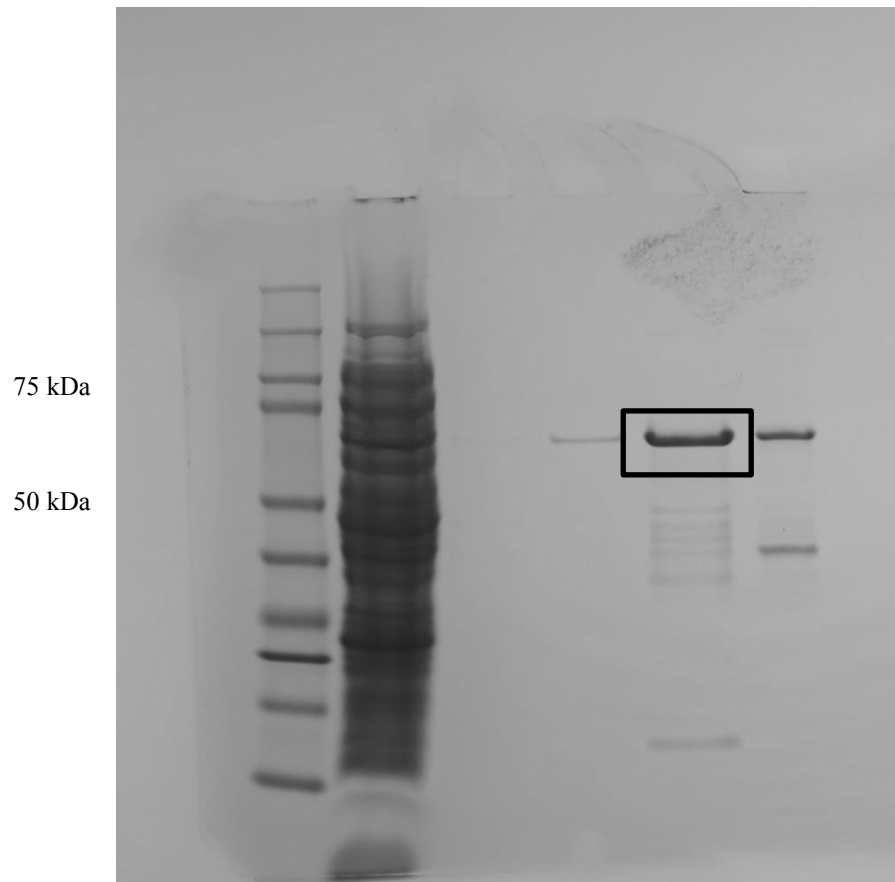
Figure 3.6: Purified protein by cobalt resin column confirmed with SDS PAGE. Lane 5 indicates purified protein was collected after 250mM imidazole wash, peptide band boxed in is Peg1 which is 64.1 kDa. Molecular weights are indicated. Lane 2 is flowthrough, Lane 3and 4 are 10mM imidazole washes.
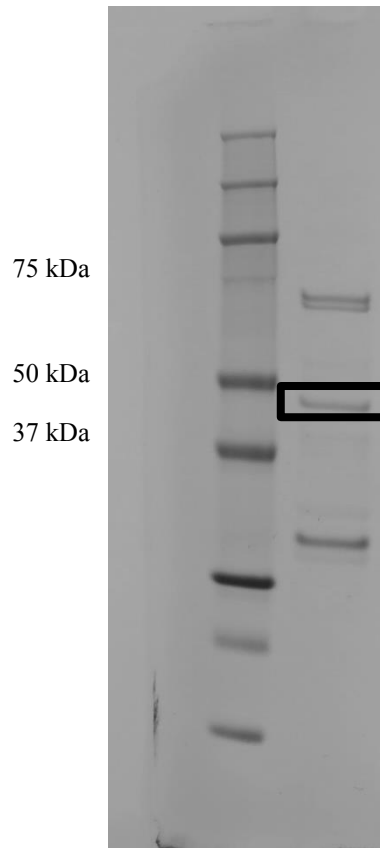
Figure 3.7: Thrombin cleavage of Thio-HIS tag with CaCl₂. SDS-PAGE gel shows

supplementation of CaCl₂ in the thrombin reaction allowed for recovery of 46.1 kDa peptide,

boxed in Lane 2. Molecular weight markers are noted.

75 kDa

50 kDa

37 kDa

15 kDa
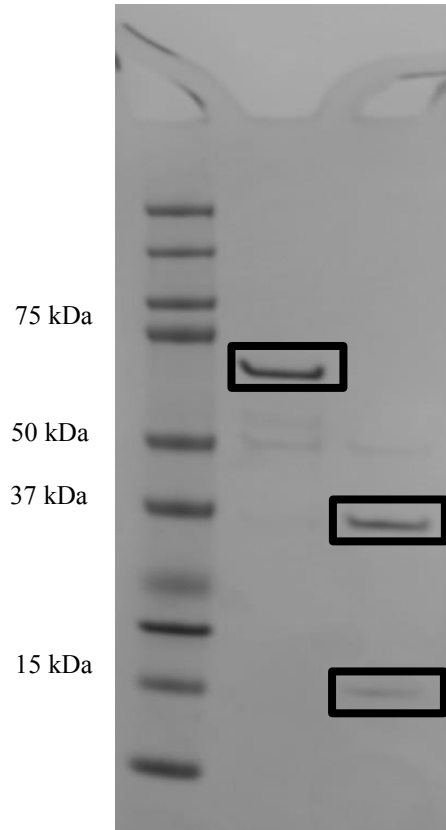
Figure 3.8: Thrombin cleavage without CaCl₂ allows for site specific degradation. Imperial

stained SDS PAGE gel depicts autodegradation of Peg1 into 2 peptides sized 12 kDa and 37 kDa

in Lane 3. Lane 2 is thrombin treated Peg1. Molecular weight markers are indicated.

11.98 kDa peptide was sent for N-terminal sequencing, it was found that Peg1 cleaves itself

between a Glutamine $_{(335)}$ (Q) and Glycine $_{(336)}$ (G) before a Leucine Lysine (LK)

motif leaving the N-terminal peptide at 312 AA and the C-terminal peptide at 92 AA (Figure

3.9). The N-terminal peptide is 34.12 kDa while the C-terminal peptide is 11.98 kDa.

Extracellular Matrix Component Degrading Activity

We hypothesized that Peg1 would be able to degrade a substrate that was present in the

fetal membranes. First, the protease was tested against a universal protease assay where casein

acted as the substrate. Zone of activity plate assays indicated that proteinase K, a broad spectrum

serine endopeptidase, was able to degrade casein; however, Peg1 was unable to degrade casein.

This same method was tested with gelatin and mucin and the results were the same, proteinase K

was able to degrade the three substrates while Peg1 was unable to. Mucin was tested as a control

since it was already shown that Sn35 was unable to degrade mucin (Harwich et al., 2012).

Since Blastx indicated homology to matrix metalloproteinases, we next hypothesized that

the substrate of Peg1 would be a component in the extracellular matrix. The ECM consists of

extracellular molecules secreted by cells that allow for structural support for the surrounding

cells, tissues repair, and intercellular communication. Matrix metalloproteases, serine proteases

and threonine proteases aide in the dynamic changes of the ECM by degrading and remodeling

the ECM. The ECM components include collagen, fibronectin and laminin. Fibronectin is a

glycoprotein that binds to integrins, fibrin, collagen and proteoglycans and allows for dynamic

movement; it is an insoluble matrix where its monomers are linked together by disulfide bonds.

While laminin, a major extracellular matrix protein of the basal lamina aides in cell adhesion and

Signal Peptide
(1-22)
2.64 kDa

MKITGKCSVL
LLSLALSIPYMT

N-terminus
(23-334)
34.12 kDa

FSDARIDKNKRRIAQINRQVTQNRNLIIKNNR
QIVVTKRTQASLQSEINDLNFQINKLQKEYNVLE
QRYIELLKAIGANEKDIKASISKIQSSNAEIKVNK
DEYNTKIRNYDLIRRARNINQNSGLVGARRDKL
RHDAHAILDLQINKIKGIETYKKGVEQDKIKVE
GIKQKNQSEATKVMSARNDLENKKKELNTAV
ARKNKAVAELRDLQNKLRKENITIEKNNSKLA
SERNRLEAQINAIIRQNAIRQQQLERQRQLALRR
KQQQGSAKHENINVSQEPINVVKGTGSLIYPIRG
RVVVGFGTEKVQ

Q G
335 336

C-terminus
(337-430)
11.98 kDa

GLKSKGIEILGTLGQS
VSAADTGTVIYSGSLG
GLGRVVIISHGSLITVY
GNLASIRVSRNEAVRK
GQSIGTLGRDSETKPTL
YFEVRHGVNIVNPMSYL

Figure 3.9: Schematic representation of site specific degradation. Peg 1 autodegrades itself at the N terminus of the 12 kDa peptide. Cleavage occurs between Q_{335} and G_{336}. Full amino acid sequence of signal peptide, N-terminus and C-terminus are noted below.

provides structural scaffolding. Laminin can also bind to collagen, integrins and other cellular domains.

In addition to testing ECM components; we hypothesized that since this protease had homology to putative zinc endopeptidases that it must require zinc for activation. Therefore the substrates and Peg1 were incubated with varying amounts of salt concentrations. Fibronectin and laminin assays were run on SDS PAGE to determine if Peg1 was capable of complete degradation. Fibronectin and laminin substrates were collected from human tissue in attempt to recreate the in vivo substrates for Peg1. Fibronectin produced a band at 220kDa on the SDS PAGE and while it appeared that Peg1 was able to degrade fibronectin, complete degradation was never seen. While lanes 3 and 5 on the SDS PAGE gel appear to have less fibronectin (Figure 3.10), this might be due to salt concentrations effecting the migration of proteins into the gel. The fibronectin sample located in lane 4 was incubated with 5 mM $ZnCl_2$ and 2mM $CaCl_2$ while lane 2 was incubated with three times more $ZnCl_2$ and five times more $CaCl_2$ concentrations. The results indicate that less protein migrated into the gel which can be due to the salt concentration in the samples. Laminin produced two major bands at 50kDa and 130-160kDa. Although the results of the SDS PAGE could indicate that the protease degrades the 130-160 kDa peptide, once again it appears that the salt concentrations affected the migration of the protein into the gels (Figure 3.11). Furthermore, some of the samples remained in the wells. Therefore it was concluded that Peg1 cannot degrade fibronectin or laminin.

Coagulase potential of Peg1

Several microorganisms are capable of producing coagulase enzymes to convert fibrinogen to fibrin which results in blood clotting, like *S.aureus*. This conversion is viewed as a
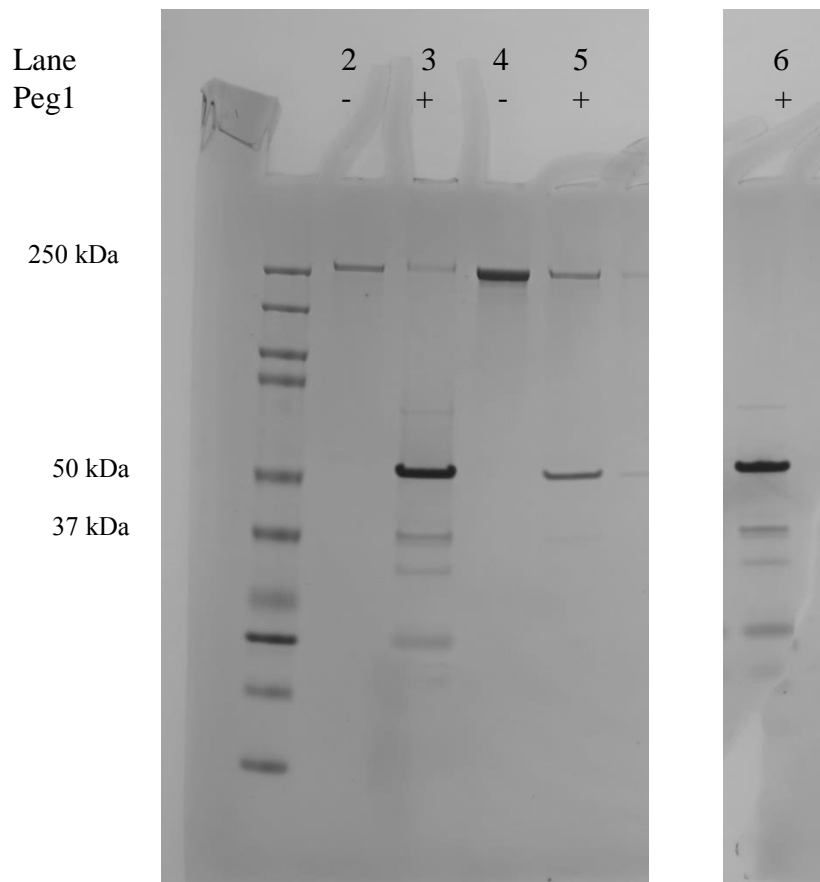
Figure 3.10: SDS PAGE fibronectin degradation assay. Imperial stained SDS- PAGE. Lane 2: Fibronectin+15mMZnCl$_2$+10mM CaCl$_2$, Lane 3: Fibronectin+ 15mM ZnCl$_2$+ Peg1 , Lane 4: Fibronectin+5mMZnCl$_2$+2mM CaCl$_2$, Lane 5: Fibronectin+5mMZnCl$_2$+ (1:5) Peg1, Lane 6: Peg1 alone. Degradation of Fibronectin is not seen.
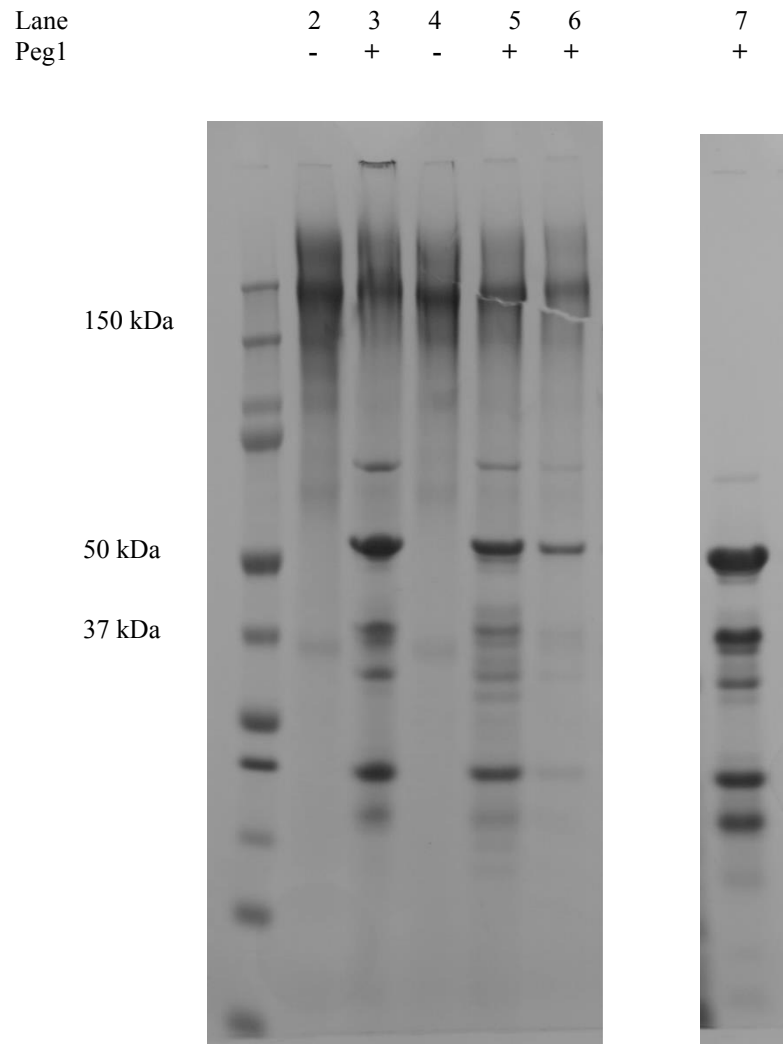
Figure 3.11: SDS PAGE laminin degradation assay. Lane 2: Laminin+15mMZnCl$_2$+10mM CaCl$_2$, Lane 3: Laminin+ 15mM ZnCl$_2$+ Peg1 , Lane 4: Laminin+5mMZnCl$_2$+2mM CaCl$_2$, Lane 5: Laminin+5mMZnCl$_2$+Peg1, Lane 6: Laminin + 5mMZnCl2+ (1:5) Peg1, Lane 7: Peg1 alone. Peg1 is unable to degrade laminin.

virulence factor since it coats the bacterial surface with fibrin and therefore can protect the

bacterial cell from phagocytosis (Tortora et al., 2013). Peg1 was incubated with rabbit

fibrinogen; however, clumping never occurred and therefore Peg1 was not identified as a

coagulase. This experiment was done to determine if Sn35 utilizes Peg1 to remain undetected

from the immune system while traversing the fetal membranes.

Analysis of cytotoxicity of Peg1 to ME-180s

Previous studies indicated that *Sneathia amnii* is cytotoxic to ME-180 cells which are

cervical epithelial carcinoma cells. It is thought that the cytotoxic activity may help the bacteria

ascend into the intrauterine cavity. While Peg1's peptides were analyzed through Edman

degradation, a cytotoxicity assay was performed based upon *Bacteriodes fragilis*'s zinc

endopeptidase's capability to disrupt tight junctions in epithelial cell monolayers. Hypothesizing

that Peg1 could have similar enzymatic capabilities; ME-180 cells were incubated with Peg1 and

$ZnCl_2$. Results indicated that there was a significant increase in cell rounding compared to cells

just treated with $ZnCl_2$ (Figure 3.12). While obvious cytotoxicity was not seen, the monolayers

incubated with Peg1 and $ZnCl_2$ sloughed off.

Analysis of biofilm formation capability of Peg1

Previous attempts to uncover Peg1's protease capabilities indicated that Peg1 did not

have true degradation of substrates utilized in this study. Therefore, it was hypothesized that

Peg1 could be a ligand involved in adhesion and biofilm formation. Collagen type IV, which is

located in the fetal membranes, was utilized to determine whether Peg1 could bind to collagen

type IV and allow *S.amnii* to produce biofilms. Live *S.amnii* was incubated in collagen type IV
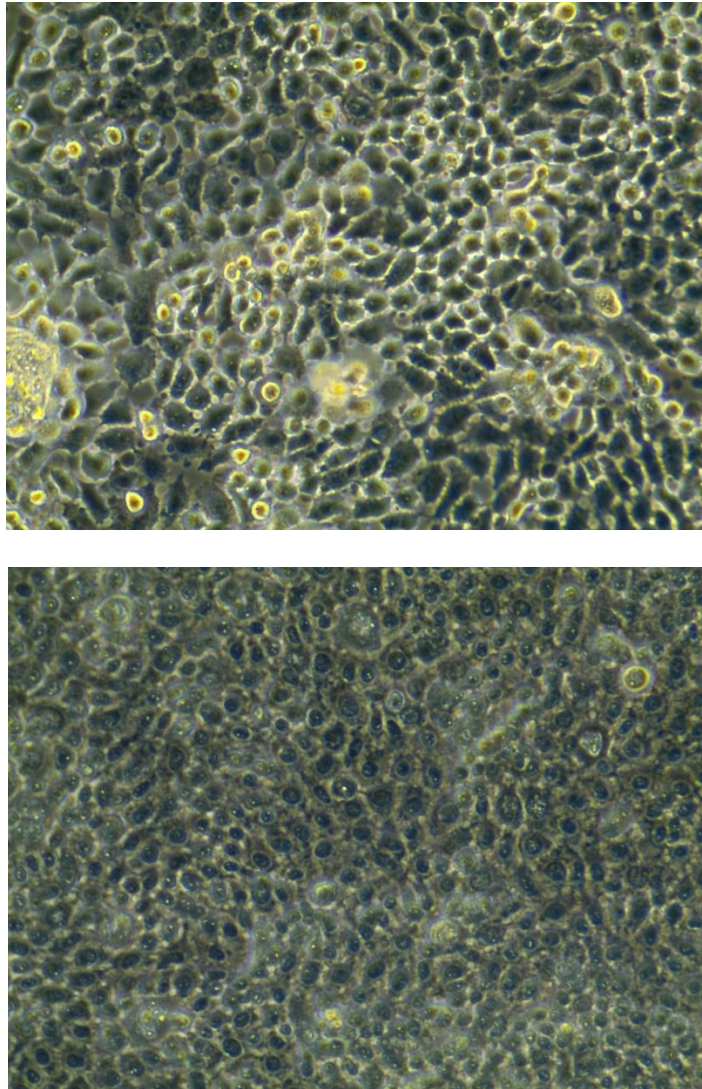
Figure 3.12: Cytotoxic effect of Peg1 on ME-180s. Monolayer exposed to $ZnCl_2$, top image, remained intact and cells had little to no rounding. Monolayer exposed to Peg1 and $ZnCl_2$, bottom image, came apart and sloughed off. An increase in cell rounding was observed when cells were exposed to Peg1.

strip wells with or without the addition of cleaved purified Peg1. After, the wells were stained with safranin to visually evaluate biofilm formation. No biofilm formation occurred in the presence or absence of Peg1.

Bacteriolytic Activity

Production of a bacteriolysin is a virulence factor for the bacterial cell against other species. While autolysins are produced during binary fission to break down the bacteria's own peptidoglycan layer and allow for two cells to form from one cell. We hypothesized that Peg1 could be either a bacteriolysin or an autolysin since proteases, like proteinase K, can break down *Micrococcus luteus*. M23 peptidase family also contains endopeptidases that lyse bacterial cell wall peptidoglycans. The cleaved purified protease was exposed to *Micrococcus luteus* and a zymogram assay was performed. A zone of clearing was produced and therefore it was concluded that Peg1 appeared to have bacteriolytic activity. (Figure 3.13). When the cleaved purified protease was incubated with Sn35 to analyze autolysin potential via OD_{600nm} measurement it was concluded that Peg1 was not capable of autolysin activity since there was not a significant decrease in OD_{600nm} measurement.

Collagen degradation

Collagen, an integral protein of connective tissue, is found in the ECM, skin, tendons, ligaments and other tissues. Collagen exists as procollagen before it is cleaved by proteases to allow for assembly into collagen; collagen type IV is the only type of collagen to be found in the basement membrane which is found in the fetal membranes. It had previously appeared that Sn35 had collagenase activity and could partially degrade collagen type V. To analyze if Peg1

Proteinase K
Peg 1   +
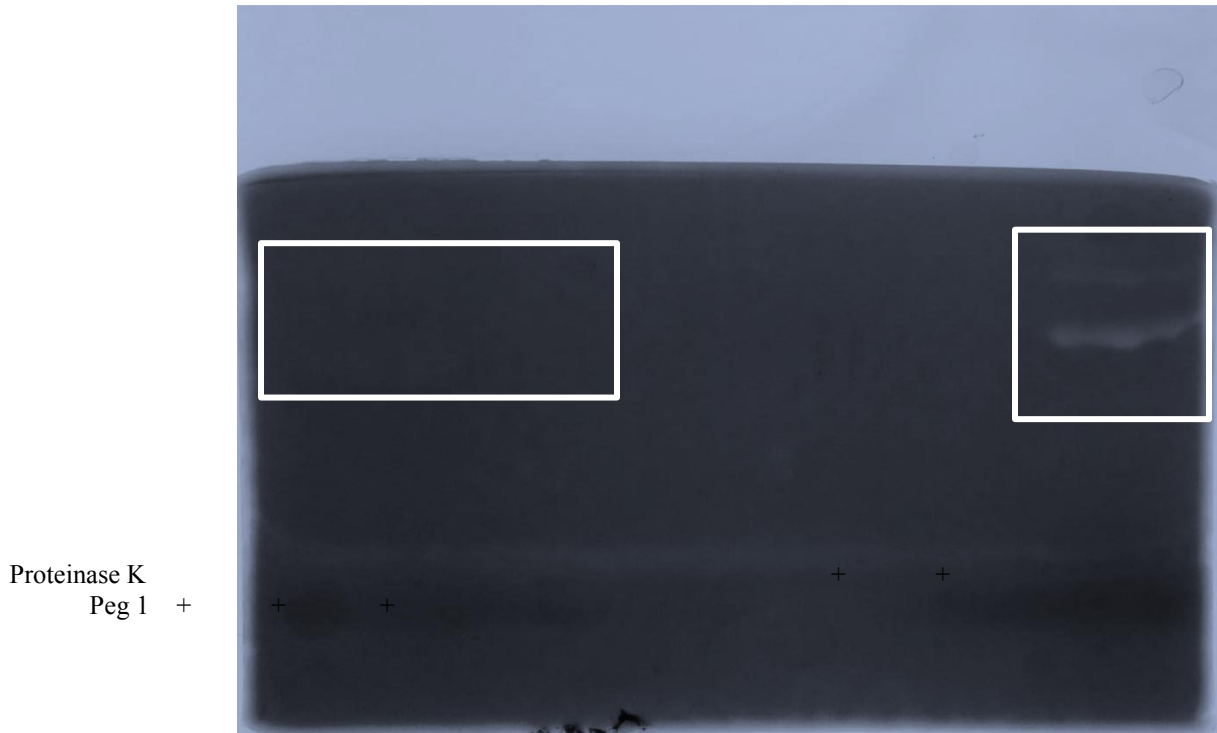
Figure 3.13: Bacteriolytic effect of Peg1 on *Micrococcus lutues*. *M. luteus* embedded SDS

was run and imperial stained to indicate whether or not Peg1 was able to lyse other bacterial

species. A zone of clearing was observed in lanes 2-4 where Peg1 was loaded. Therefore Peg1

appears to have bacteriolytic activity.

was able to degrade collagen, collagen type IV and I were biotinylated and incubated with cleave purified Peg1. Western analysis indicated that Peg1 appears to have the same effect on collagen type IV as live bacteria do (Figure 3.14). Peg1 appears to have collagenase activity.

Analysis of Peg1 antisera production

Bioinformatic analysis of PSORTb identified Peg1 as a lipoprotein located on the outer membrane of the bacterial cell wall. Additionally, PSORTb identified that without the 12kDa fragment the protein had no signal. Therefore cleaved purified protein was used for polyclonal antisera production to further help characterize Peg1. To confirm PSORTb results, live Sn35 was probed with anti-sera; however, western analysis indicated that Peg1 was not found on the outermembrane (Figure 3.15) and instead antisera detected the protein in the cytoplasm. While we hypothesized that the protease was expressed on the outermembrane and PSORTb indicated the same results, the results from western analysis cemented that this protease is not expressed on the outermembrane. To better understand the protease using antisera, a temporal expression assay was done to identify when the putative protease is expressed. The growth curve assay indicated that Peg1 is expressed throughout exponential phase, late exponential phase and stationary phase; hours 4 through 48(Figure 3.16).

Discussion, Conclusion, and Future Perspectives

Almost nothing is known about the process through which *S.amnii* causes preterm birth, even though this newly discovered bacterium is associated with serious health complications for

Figure 3.14: Role of Peg1 in collagen degrdation. Lanes 1 and 2 are live Sn35 incubated with collagen type IV, lanes 3 and 4 are PBS incubated with collagen type IV, lanes 5 and 6 are Peg1 incubated with collagen type IV. Peg1 appears to have collagenase activity, to the same effect that live Sn35 does.
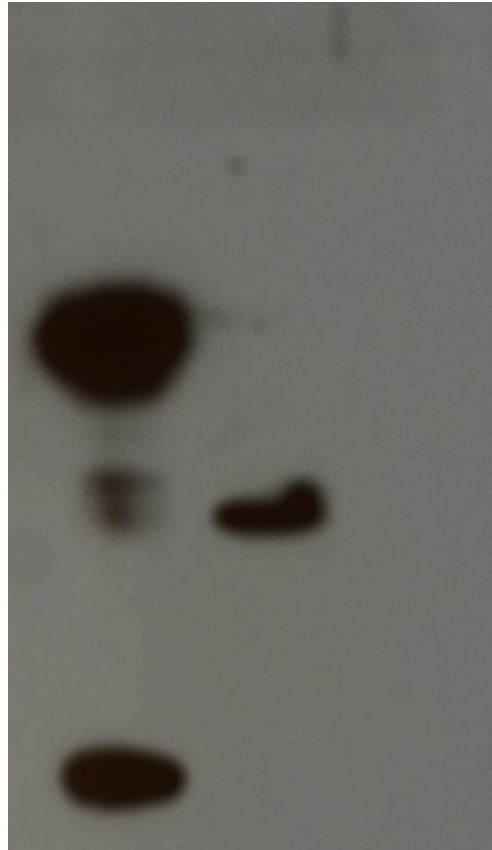
Figure 3.15: Western analysis of Peg1 expression location. Lane 1: molecular weight marker, Lane 2: cytosolic proteins of Sn35, Lane 3: outermembrane protein of Sn35. Peg1 is visualized in the cytoplasm of Sn35 about 75 kDa.
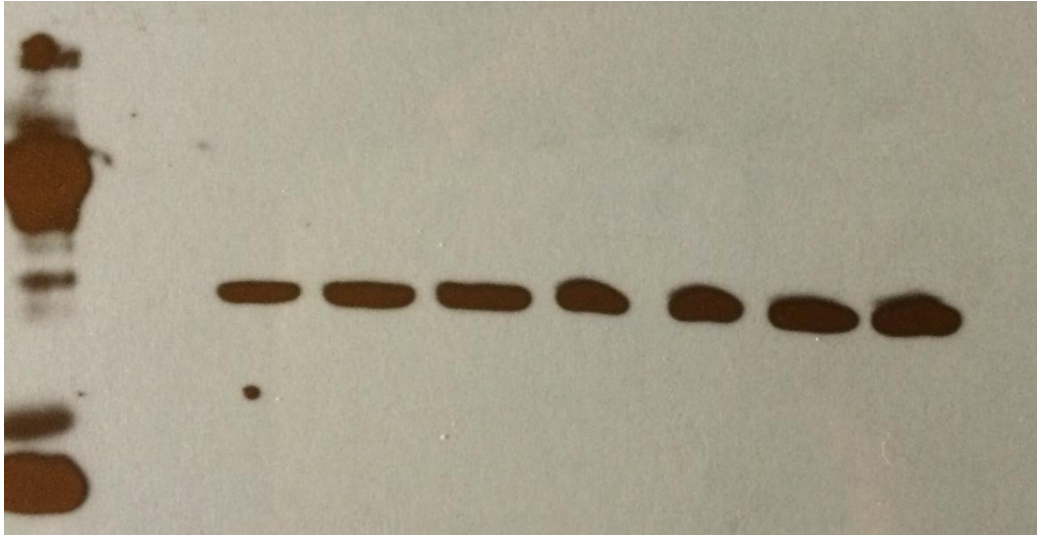
Figure 3.16: Temporal expression of Peg1. Lane 1: ladder, Lane 2: empty, Lanes 3-9 are cell lysates taken at 4,6,8,12,16,24,48 hours respectively. Peg1 expression was found throughout exponential, late exponential and stationary phase. Peg1 is visualized ~75 kDa.

both mother and neonate. We have hypothesized that *S.amnii* has virulence determinants that allow for the bacterium to traverse the fetal membranes and invade the amniotic cavity. The goal of this project was to identify a potential virulence factor that Sn35 utilizes to traverse the fetal membranes and cause preterm birth. We sought to first better understand traversal of *S.amnii* through the fetal membranes and then to explore potential virulence determinants that aided in the degradation of the extracellular matrix. The initial study focused on bacterial adhesion and ascension through the chorion layer to support MIAC. We have developed a method that simulates MIAC *in vitro*, and indicated that Sn35 is able to invade and traverse the chorion layer. These observations are significant because it supports bacterial ascension and indicates that *S.amnii* utilizes virulence determinants to ascend. The lack of detectable bacteria below the placental tissue indicates that further work is needed to develop a MIAC assay that can stay viable longer than 24 hours to observe microbial traversal. While Sn35 did not produce a strong ligand: ligand interaction with VK2 cell line, this indicates that further work is necessary to identify what cell tissue type Sn35 targets for cell adhesion.

The virulence determinants of *S.amnii* remain elusive. Analysis of its genome indicated promising virulence determinants that could enlighten its ability to traverse the fetal membranes and cause preterm birth. We hypothesized that the putative zinc endopeptidase, Peg1, would degrade substrates found in the fetal membranes due to its homology to matrix mellatoproteinases. We attempted to discover Peg1's degradation properties by incubating the cleaved purified protein with various substrates found in the extracellular matrix, like fibronectin and laminin; however, results from each experiment either did not support the hypothesis of degradation. Peg 1, under the conditions tested, was not capable of degrading casein, mucin, gelatin, or fibrinogen. Furthermore, Peg1 lacked biofilm formation capability using collagen type

IV as the substrate.   However, when Peg1 was incubated with collagen type IV, Peg1 appeared to have the same effect on collagen as the live bacteria. It appears that Peg1 has collagenase activity. Peg1 also appeared to have bacteriolytic activity against *Micrococcus luteus* which is characteristic of the M23 peptidase family. Antisera production of Peg1 identified the protease as being cytosolic, disproving PSORTb bioinformatic analysis that placed Peg1 on the outermembrane. Peg1 does appear to have protease activity, however, the conditions in which the protease is secreted where not found. Further work needs to be devoted to understand the conditions in which Peg1 is secreted.

Peg1 appeared to autodegrade and we determined the cleavage site by Edman degradation. Peg1 required the addition of $CaCl_2$ in order to remain an intact peptide of 46.1 kDa. If $CaCl_2$ was not supplemented to the thrombin reaction, Peg1, underwent autocatalytic cleavage. This autocatalytic degradation resulted in two distinct peptides; a peptide sized 34.12 kDa while the other 11.98 kDa. Further identification of the autocatalytic event was examined through N-terminal Edman degradation revealing that site specific cleavage occurred between Glutamine $_{(335)}$ and Glycine $_{(336)}$. Additionally, the cleavage at a glycine residue is often seen in M23 peptidase family, since it is the preferred sight of cleavage.

Given the clinical complications that *Sneathia amnii* possesses as an opportunistic pathogen responsible for an array of infections in numerous body sites in both mother and neonate, research into the virulence determinants could have a significant impact of our understanding in their role in BV, MIAC, PPROM and preterm birth. The mucus plug and fetal membranes are supposed to remain impermeable until the end of gestation; however, *S. amnii* can be found in the uterine cavity, fetal membranes, and amniotic fluid during gestation. The work in this study revealed the ability of Sn35 to traverse the chorion layer of the fetal

membrane. Additionally it appears that Peg1 has collagenase and bacteriolytic activity that could be responsible for MIAC; however, conditions in which Peg1 is secreted were not identified. Therefore, the contributions of this study to our understanding of Sn35's ability to cause preterm birth may help understand its other virulence determinants.

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Literature Cited

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Vita

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Fettweis JM and Brooks JP et.al. "Differences in vaginal microbiome in African American women versus women of European Ancestry."*Microbiology*. 2014.

Abstracts and Presentations:

American Society of Microbiology General Conference May 2015. Characterization of a Putative Protease Expressed by *Sneathia amnii*. Poster.

American Society of Microbiology Virginia Branch Annual Meeting, November 2014. Characterization of a Putative Protease Expressed by *Sneathia amnii*. Presentation.

VCU Institute for Women's Health 10th Annual Women's Health Research, April 2014. The Characterization of a Putative Collagenase Expressed by *Sneathia amnii*. Poster.

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