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The Characterization of a Putative Virulence Factor Expressed By Sneathia amnii

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THE CHARACTERIZATION OF A PUTATIVE VIRULENCE FACTOR

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

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List of Abbreviations

°C	degrees Celsius
µg	microgram
µL	microliter
AF	amniotic fluid
Amp	ampicillin
Amp^R	ampicillin resistance
B. pertussis	*Bordetella pertussis*
BHI	brain-heart infusion
bp	base pair
BSA	bovine serum albumin
BV	bacterial vaginosis
CFU	colony forming unit
Cm	chloramphenicol
Cm^R	chloramphenicol resistance
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
g	gram
gDNA	genomic DNA
HAEC	human amniotic epithelial cell
HS	human serum
IPTG	isopropyl β-D-1-thiogalactopyranoside
IVF	in vitro fertilization
JEG-3	human chorionic trophoblasts
kb	kilobase pairs
kDa	kilodalton
L	liter
LB	Luria-Bertani
M	molar
max	maximum
Mb	megabase
Me-180	human cervical carcinoma cells
mg	milligram
MIAC	microbial invasion of the amniotic cavity
mL	milliliter
mM	millimolar

MW	molecular weight
MWCO	molecular weight cut off
nm	nanometer
OD	optical density
PBS	phosphate buffered saline
PBST	PBS supplemented with 0.05% Tween 20
PCR	polymerase chain reaction
PPROM	preterm premature rupture of membranes
PVDF	polyvinylidene fluoride
qPCR	quantitative PCR
rpm	revolutions per minute
S. amnii	*Sneathia amnii*
sBHI	supplemented brain-heart infusion
SDS	sodium dodecyl sulfate
Sn35	*Sneathia amnii* strain 35
SOC	super optimal broth with catabolite repression
STD	sexually transmitted disease
TAE	tris-acetate-EDTA buffer
UV	ultraviolet
V	volt
VK2	human vaginal keratinocytes

Abstract

THE CHARACTERIZATION OF A PUTATIVE VIRULENCE FACTOR EXPRESSED BY *SNEATHIA AMNII*

By Amy L. Sanford
B.S., Virginia Polytechnic Institute and State University

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

Virginia Commonwealth University
2015

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Preterm birth, defined at birth before 37 weeks gestation, affects millions of newborns worldwide every year. Preterm birth is a leading cause of infant morbidity and mortality. One major cause of preterm birth is preterm premature rupture of membranes (PPROM), which can be triggered by bacterial infection and inflammation. A bacterial species that has been implicated in preterm birth and other obstetric complications is *Sneathia amnii*. The goals of this study were to observe cytopathogenic effects caused by *S. amnii* strain Sn35 and identify putative virulence factors causing those effects. Sn35 was able to adhere to, invade, and damage/kill various host cell lines. We

characterized these virulence attributes. A putative virulence determinant was identified, and a fragment of the protein was expressed for polyclonal antiserum production. Antiserum was used to characterize the expression and subcellular localization of the protein in Sn35. However, antiserum was unable to prevent cytopathogenic effects.

Introduction

I. Bacterial Vaginosis

Bacterial vaginosis (BV) is the most common vaginal disorder in women of reproductive age. A national survey conducted from 2001-2004 estimated that BV affects roughly 29% of women ages 14-49 in the United States[1]. The development of BV is correlated with an imbalance of bacteria in the vagina; this imbalance is characterized by a decrease in healthy vaginal microorganisms and an increase in pathogenic microorganisms.

Lactobacillus species are usually the predominant bacteria found in healthy vaginal microflora[2]. As their name implies, lactobacilli are lactic acid-producing bacteria; lactic acid in the vagina helps to keep the pH at an acidic level. The acidity of the vagina is important, as it prevents many pathogenic microorganisms from colonizing and causing infections. Some lactobacilli can also produce hydrogen peroxide, which is toxic to pathogenic bacteria. The protectiveness of lactobacilli varies by species; *Lactobacillus crispatus*, for example, is known to be a protective species, due to the fact that most strains can produce hydrogen peroxide[3]. Conversely, *Lactobacillus iners* is considered a less protective species, as most strains do not produce hydrogen peroxide[4]. Reasons for the decrease in healthy vaginal bacteria have not yet been identified; however, studies have shown that colonization by less protective

Lactobacillus species are related to positive cases of BV[5]. There are several pathogenic microorganisms associated with BV, a predominant one being *Gardnerella vaginalis*. It was originally believed that *G. vaginalis* was the etiological cause of BV. However, not all women who test positive for BV are colonized with *G. vaginalis*[6], and colonization with *G. vaginalis* does not always lead to BV. Therefore, BV is now considered a complex, polymicrobial disorder.

A multitude of risk factors for developing BV have been identified through various studies; these factors include but are not limited to vaginal douching[7], new or multiple sexual partners[8], the use of intrauterine contraceptive devices (IUDs)[9], smoking[8], and stressful life events[10]. Once developed, BV dramatically increases the risks of several complications, such as the susceptibility to HIV and other STIs[11] and infection following surgical procedures, such as hysterectomies or abortions[12, 13]. If a pregnant woman develops BV, the risk of pregnancy complications, such as preterm birth and low birth weight, also increases[14].

BV can present as a symptomatic or asymptomatic disorder, although roughly half of all BV cases are asymptomatic[15]. Symptoms can include abnormal vaginal discharge or odor, painful urination, and/or general vaginal pain. Diagnosis of BV can occur via two methods; the first method, the Amsel criteria, utilizes 4 different characteristics for a positive BV diagnosis. The first characteristic is the presence of a white or gray, thin, homogenous vaginal discharge. The second is the detection of clue cells via microscopy; clue cells are vaginal epithelial cells that have a distinct stippled appearance due to being covered with bacteria. The third characteristic is a vaginal fluid pH above 4.5, as the pH of a healthy vagina is under 4.5[16]. The fourth and last

characteristic is the presence of an amine odor upon adding 10% potassium hydroxide solution. In order to confirm a positive BV diagnosis, 3 out of the 4 criteria mentioned must be present.

The second method for diagnosing BV, the Nugent criteria, involves using a Gram-stained vaginal smear to score the types and amounts of bacteria present. The scoring system is used to quantify the amount of long Gram-positive rods versus shorter Gram-negative rods and cocci. The decrease in Gram-positive rods, such as *Lactobacillus* species, is scored from 0 to 4 (with a score of 4 equaling the highest decrease in Gram-positive rods). The presence of small gram-variable and Gram-negative rods, such as *G. vaginalis, Prevotella* species, etc., is scored from 0 to 4. The presence of curved Gram-negative rods, such as *Mobiluncus* species, is scored 0 to 2[17]. Scores of 7 to 10 are consistent with cases of bacterial vaginosis.

For the treatment of nonpregnant woman, the CDC currently recommends an antibiotic regimen of 500 mg metronidazole 2x a day for a week, 0.75% metronidazole gel (5 g) once a day for 5 days, or 2% clindamycin cream (5 g) once a day for a week[18]. Treatment is recommended for symptomatic pregnant women, but antibiotic regimens are altered from those of nonpregnant women[18].

As mentioned previously, studies have estimated that BV affects 29% of women in the United States[1]. African American women have a three-fold higher risk of bacterial vaginosis compared to Caucasian women (10-20% in Caucasian women, 30-50% in African American women); estimates can vary based upon the populations studied[19, 20]. In addition to the increased likelihood of developing BV, African American women are also twice as likely to experience preterm birth and have a three-fold higher risk of very

preterm birth[21, 22]. In a recent study, DNA extraction and 16S rRNA gene analysis of mid-vaginal isolates obtained from pregnant African American women and pregnant women of European ancestry revealed very significant differences in each group's vaginal microbiome[23]. While European ancestry women are predominantly colonized with *Lactobacillus* species, the vaginal microbiomes of African American women are more diverse. Several bacterial taxa, such as *Gardnerella, Mycoplasma,* and *Sneathia*, were found more frequently in the microbiomes of African American women. These taxa are also associated with cases of preterm birth and microbial invasion of the amniotic cavity.

II. Preterm Birth

Preterm birth, defined as birth before 37 completed weeks of pregnancy, affects an estimated 15 million newborns worldwide every year[24]. Preterm birth can be caused by a number of factors, including premature rupturing of the amniotic sac and release of amniotic fluid. While rupturing of these membranes can occur shortly before term labor and is termed premature rupture of membranes (PROM), rupture prior to 37 weeks gestation is defined as preterm PROM (PPROM). One-third of all preterm births are associated with PPROM[25].

Preterm birth is a leading cause of infant morbidity and mortality worldwide[26]. Infants born prematurely may suffer from a myriad of problems, including breathing and feeding difficulties[27, 28], hearing and vision impairments[29], and developmental delays[30]; studies have also associated preterm birth with cerebral palsy[31]. Complications of preterm birth vary based on gestational age; preterm births are defined as extremely

preterm (<28 weeks), very preterm (28 to <32 weeks), or moderate to late preterm (32 to <37 weeks)[32], with the worst complications associated with younger gestational age.

Many risk factors have been correlated with experiencing preterm birth. While environmental factors such as stress[33] and socioeconomic status[34] have been associated with preterm labor, physical factors also play a significant role. Having previously experienced a premature birth[35], multiple gestations (carrying twins, triplets, etc.)[36], irregularities in reproductive organs (such as a short cervix or shortening of the cervix early in pregnancy)[37], being underweight or overweight before pregnancy[38, 39], and conception using in vitro fertilization (IVF)[40] are physical factors that may increase the risk of preterm birth. As previously mentioned, race has also been correlated to preterm birth risk. Of the factors listed, experiencing previous preterm births is the greatest risk for a new preterm birth[35].

Two additional factors have been associated with the development of PPROM. The first, weakening of fetal membranes, is another normal process that occurs at the onset of labor. However, if it occurs preterm, it can contribute to a premature delivery. Fetal membranes can be weakened due to collagen degradation by matrix metalloproteinases (MMPs). Various types of collagen are present in placental membranes. Studies have found that amniotic fluid isolated from cases of PPROM have higher levels of MMPs[41, 42]. The second factor, infection, has become a major target of interest. Bacterial infections and inflammation triggered by infections could contribute to weakened membranes and development of PPROM. Some bacterial factors, like lipopolysaccharides (LPS), have the ability to increase MMP expression[43]. Furthermore,

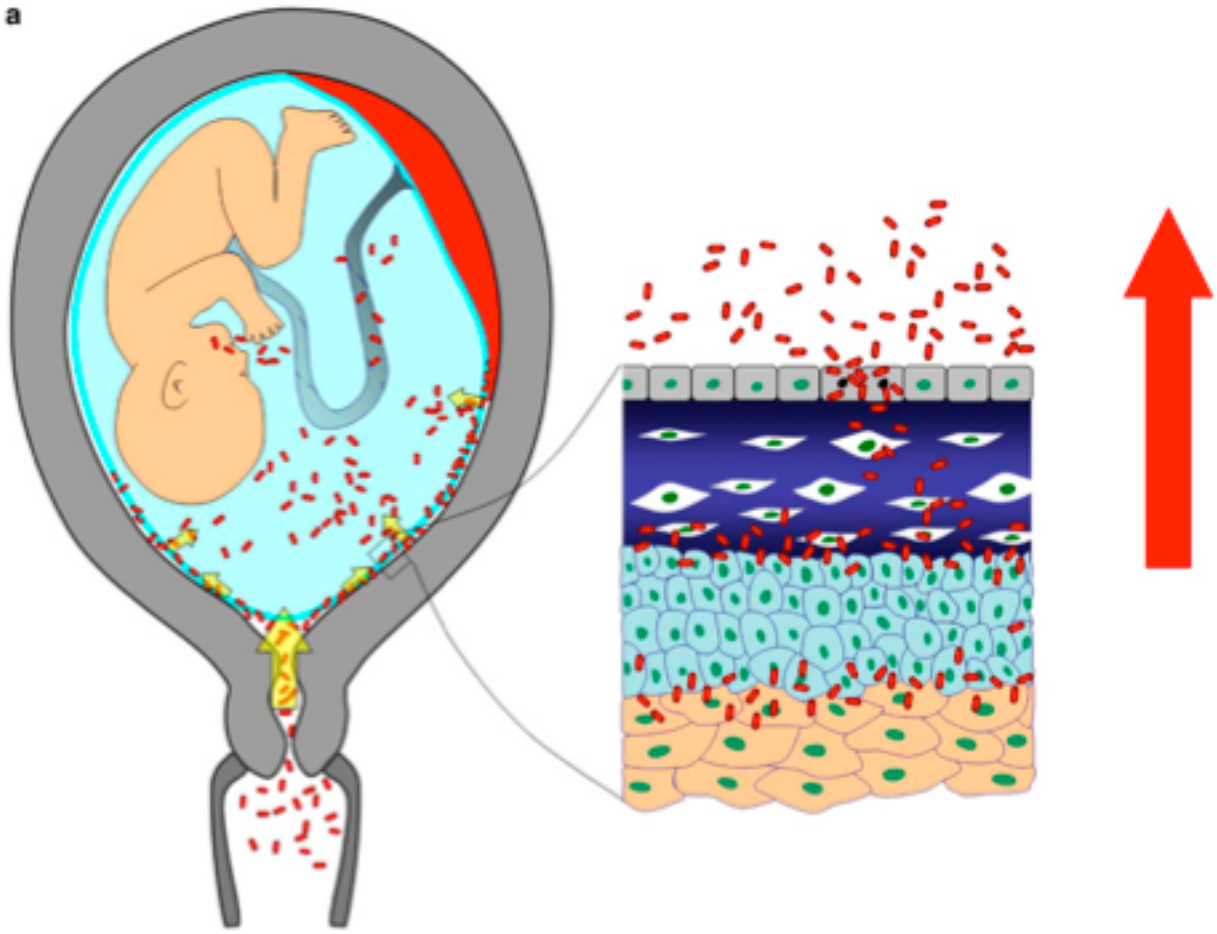
bacteria have been isolated from amniotic fluid obtained from PPROM[44], indicating that the bacteria are able to ascend into and invade the amniotic cavity.

III. Microbial Invasion of the Amniotic Cavity

Microbial invasion of the amniotic cavity (MIAC) is defined by a culture of amniotic fluid (AF) that tests positive for the presence of bacteria. While less than 1% of AF samples isolated from women at term tested positive for bacteria, 12.8% of AF samples isolated from patients experiencing preterm birth with intact membranes gave positive results[45]. The same study discovered that 32.4% of AF samples obtained from women experiencing PPROM tested positive for bacteria. Rates may be falsely low due to failure to cultivate certain fastidious organisms.

Little is known about how bacteria gain access to the amniotic fluid; it is currently thought that vaginal bacteria traverse the cervical mucus plug and enter the uterine cavity[46]. This is a difficult route, as mucus plugs form specifically to act as a barrier between the vagina and the uterine cavity. Furthermore, the mucus plug contains a number of antimicrobial agents that aid in blocking bacterial infection[47]. After getting past the mucus plug, bacteria can remain in the uterine cavity, traverse into the placental membranes, causing chorioamnionitis, or completely pass though the placental membranes and access the amniotic fluid (**Figure 1)**. Bacteria can trigger intraamniotic inflammation, leading to an increase in a number of factors such as IL-6[48], TNF-α[49], or MMP-8[50]. These inflammatory markers, specifically MMP-8, can degrade collagen present in the placental membranes, weakening them. The inflammatory

Figure 1. Model of microbial invasion of the amniotic cavity (MIAC). This is the currently accepted model for how microbes are able to access and invade the amniotic environment. It is thought that bacteria (shown in red) ascend through the cervical mucus plug and into the uterine cavity. Once in the uterus, they can invade the chorion layer of the placenta (indicated by the tan cells), and cause chorioamnionitis. Furthermore, they can travel through the fetal membranes (indicated by the gray cells) and access the amniotic fluid (which would be located above the gray cells).



Reprinted by permission from Macmillan Publishers Ltd: Nature – Laboratory investigations. Kim, M. J., et al., Widespread microbial invasion of the chorioamniotic membranes is a consequence and not a cause of intra-amniotic infection. *Laboratory investigation; a journal of technical methods and pathology* 2009, *89* (8), 924-36.

http://www.nature.com/labinvest/journal/v89/n8/full/labinvest200949a.html

response can also trigger uterine muscle contractions. Collectively, these consequences can lead to PPROM and preterm birth[51].

Several microorganisms have been associated with MIAC, PPROM, and preterm birth. *Gardnerella vaginalis*, *Mycoplasma hominis, Fusobacterium* species, *Ureaplasma* species, and *Sneathia* species have all been detected in PCR-based studies of MIAC[52, 53]. Many studies utilize non-culture, DNA-based methods, as many microbial species have yet to be cultivated[54]. DNA-based methods are not able to distinguish between live and dead microorganisms, but can detect microorganisms that culture-based methods might not[55].

IV. *Sneathia amnii*

Sneathia amnii is a Gram-negative anaerobic bacterium, and is a member of the Fusobacteriaceae family. Formerly grouped with the genus *Leptotrichia*, *Sneathia* species have been isolated as part of normal reproductive microflora and in association with obstetric complications[56, 57]. *Sneathia* has been associated with bacterial vaginosis, preterm labor, and postpartum bacteremia, and has been associated with STDs. It is also one of the most common genera found in amniotic fluid. A novel species, *Sneathia amnii*, was isolated and characterized by the Vaginal Human Microbiome Project at Virginia Commonwealth University. The specific strain was isolated from a mid-vaginal sample obtained from an African American woman experiencing symptoms of preterm labor at 26 weeks gestation; this type strain was designated Sn35[58].

Sn35 possesses a genome that is 1.34 Mbp in size; to date, this is the smallest genome in the Fusobacteriaceae family. The GC content of the genome is roughly 28%,

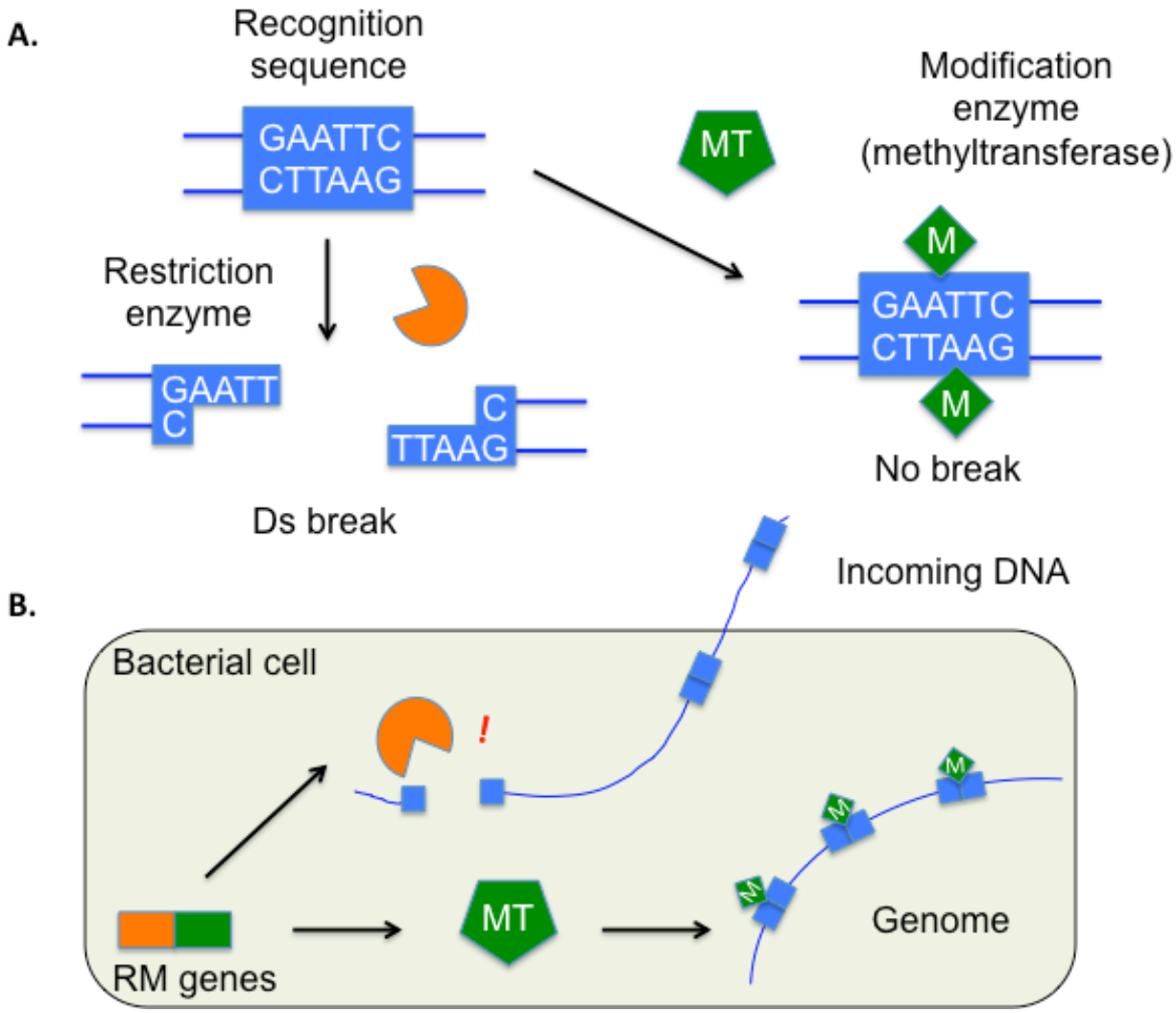
and an estimated 1,282 putative protein-encoding genes exist within the genome[58]. Analysis of the genome identified genes for glucose, maltose, glycogen, and glucosamine metabolism; however, Sn35 is unable to ferment mucin, starch, fructose, galactose, mannose, or sucrose. While Sn35 does not encode enzymes to synthesize the majority of amino acids, it appears to thrive via utilization of host factors[58].

Antibiotic resistance of this strain was also studied. As is true for most Gram-negative organisms, Sn35 is highly resistant to nafcillin. However, Sn35 tests sensitive to vancomycin, which is contrary to most Gram-negative organisms. Sn35 is also sensitive to metronidazole, which is commonly used to treat bacterial vaginosis[58].

Several genes of interest were identified during analysis of the Sn35 genome. A putative DNA restriction-modification system was discovered; this system was predicted to be acting as a Type II system (**Figure 2)**. Additionally, several genes were predicted to encode proteins present in Type II protein secretion systems. A large protein with homology to hemagglutinin domains and hemolysins was identified, yet hemagglutination of human red blood cells was not detected[58]. Sn35 exhibits weak hemolytic activity, but requires the presence of human blood to grow on agar plates. Human serum is required for growth in liquid culture. Attempts to use Brucella sheep's blood agar or fetal bovine serum for Sn35 growth were unsuccessful[58]; these results demonstrate the specificity of Sn35 for its human host. This could be due to human-specific growth factors present in blood and serum.

Although very little is known about this organism, due to its implications in both bacterial vaginosis and preterm birth, *S. amnii* strain Sn35 appears to be a great focus for further research in the context of reproductive and neonatal health. Furthermore, its

Figure 2. Putative Type II restriction-modification system expressed by *S. amnii* strain Sn35. A) Orange represents the restriction enzyme, while green represents both the methyltransferase (MT) and the methylation (M) present on DNA. B) The methyltransferase will methylate genomic DNA, while the restriction enzyme can recognize foreign DNA and attack unmethylated sites. Figure adapted from Ishikawa et al., 2010[59].


A.
Recognition sequence
GAATTC
CTTAAG
Restriction enzyme
GAATT
C
C
TTAAG
Ds break
MT
Modification enzyme (methyltransferase)
M
GAATTC
CTTAAG
M
No break
B.
Incoming DNA
Bacterial cell
!
MT
RM genes
Genome
M
M
M

specificity for human blood products strongly implies its exclusive niche in humans. Its contributions to MIAC, PPROM, and preterm birth, as well as vaginal health as a whole, need to be further elucidated.

V. Research Objectives

As previously mentioned, *Sneathia* is one of the most common bacterial genera found in amniotic fluid; in addition, *Sneathia* has been implicated in a number of obstetric complications. Furthermore, previous work has demonstrated that *S. amnii* strain Sn35 is both cytotoxic and adherent to Me-180 human cervical cells[58]. These results suggest both the production of virulence factors by *S. amnii* and a possible mechanism of pathogenicity. Therefore, the goal of this study is to further characterize the pathogenicity of Sn35 in preterm birth. Three objectives were established for this study:

1. Determine pathogenic effects that Sn35 has on a variety of immortalized and primary cell lines. It was hypothesized that in order to invade the amniotic cavity and exist in amniotic fluid, bacteria must be able to damage and/or traverse one or more host cell barriers present during pregnancy. This was addressed by performing various host cell assays in the presence of Sn35 to determine pathogenic effects.

2. Identify the putative virulence factor responsible for the observed pathogenic effects. It was hypothesized that in order to exert a pathogenic effect on host cells, Sn35 must be producing a virulence factor. This was addressed by performing bioinformatic analyses to determine whether Sn35 encoded genes with similarity to known virulence

factors.

3. Characterize the function of the putative virulence factor. It was hypothesized that the virulence factor could be acting as a cytotoxin or adhesin, allowing for either adherence/invasion of host cells or cell damage/death. This was addressed by attempting to clone and express the putative virulence factor in *E. coli*; later, polyclonal antiserum was used to better characterize the protein.

Materials and Methods

Bacterial Strains and Culture Conditions

The bacterial strains and plasmids used in this study are listed in **Table 1**. *Sneathia amnii* strain Sn35 was grown and maintained anaerobically on supplemented brain heart infusion (sBHI) plates containing 1.5% (w/v) BactoAgar (BD, Franklin Lakes, NJ), 3.7% (w/v) BHI (Thermo Scientific, Waltham, MA), 1% (w/v) gelatin, 1% (w/v) yeast extract, 0.1% (w/v) starch, 0.1% (w/v) dextrose, and 10% whole human blood (BioChemed Services, Winchester, VA).

Participants were recruited from outpatient clinics at the Virginia Commonwealth University Medical Center from 2009-2013 as part of the Human Vaginal Microbiome project. The Institutional Review Boards for Human Subjects Research at VCU (Panel B) and the Virginia Department of Health reviewed and approved this study. Strain Sn35 was obtained through this study. Liquid cultures of Sn35 were grown anaerobically in sBHI broth containing 10% pooled human serum (HS) (MP Biomedicals, Santa Ana, CA). All cultures were grown under anaerobic conditions in a BacBasic anaerobic incubator (ShelLab, Cornelius, OR) containing a gas mix of 5.0% carbon dioxide, 5.0% hydrogen, and nitrogen as the balance gas.

sBHI + chocolate agar plates were used for recovery of bacteria during adherence and gentamicin protection assays. Whole human blood was added to

Table 1. Plasmids and bacterial strains

Plasmids	Description	Source
pCR4-TOPO-AS01	pCR4-TOPO vector with TvbA fragment AS01 cloned in	This study
pET32xT – AS01	pET32xT vector with TvbA fragment AS01 cloned in	This study

***S. amnii* Strains**	**Description**	**Source**
Sn35	Mid-vaginal isolate obtained from patient experiencing preterm birth at 26 weeks gestation	Harwich et al. (2012)

***E. coli* Strains**	**Description**	**Source**
CH3 Blue	Chemically competent cells derived from *E. coli* K12, cloning strain	Bioline (Tauton, MA)
BL21-CodonPlus(DE3)-RIL	Electrocompetent cells derived from *E. coli* BL21, recombinant protein expression strain (Cm^R)	Stratagene (La Jolla, CA)
BL21 + pET32xT + AS01	BL21-CodonPlus(DE3)-RIL cells containing TvbA peptide fragment (AS01) cloned into pET32xT (Amp^R, Cm^R)	This study

autoclaved sBHI agar to achieve a final concentration of 10%. The mixture was placed at 80°C for 30 minutes prior to pouring in order to achieve whole blood lysis. sBHI + chocolate agar plates were made with a concentration of 1% (w/v) BactoAgar.

E. coli strains were grown aerobically in LB at 37°C and 200 rpm. Appropriate antibiotics were added at the following concentrations: 100 µg/mL ampicillin (Amp) for *E. coli*, and 35 µg/mL chloramphenicol (Cm) for *E. coli*.

Agarose Gels

1% agarose gels were prepared by dissolving agarose in 1X tris-acetate-EDTA (TAE) buffer. Ethidium bromide was added to the gel at a final concentration of 1 µg/mL before pouring. 5X DNA loading buffer blue (Bioline, Taunton, MA) was added to the DNA samples to achieve a 1X final concentration. Gels were run at 124 volts and visualized under ultraviolet (UV) light. DNA size was determined by comparison against the Hyperladder series of molecular weight marker (Bioline, Taunton, MA).

Cell Lines

JEG-3 human chorionic trophoblasts (ATCC, Manassas, VA) were cultured at 37°C in 5% CO_2 in minimum essential media (EMEM) (Quality Biological, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 1 IU mL^{-1} penicillin/streptomycin (Life Technologies, Carlsbad, CA). VK2/E6E7 human vaginal keratinocytes (ATCC, Manassas, VA) were cultured at 37°C in 5% CO_2 in keratinocyte-SFM (Life Technologies, Carlsbad, CA) supplemented with human recombinant Epidermal Growth Factor 1-53, Bovine Pituitary Extract, and 1 IU mL^{-1} penicillin/streptomycin (Life

Technologies, Carlsbad, CA). Primary human amniotic epithelial cells (HAECs) were isolated from healthy, full-term placentas obtained via C-section (MCV, Richmond, VA). Cells were cultured at 37°C in 5% CO_2 in DMEM/F12 (1:1) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1 IU mL^{-1} penicillin/streptomycin (Life Technologies, Carlsbad, CA).

Trypan Blue Cytotoxicity Assay

Twenty-four-well polystyrene plates (CellTreat, Shirley, MA) were seeded with respective cell lines. Once a monolayer of cells had formed, growth medium was removed, the host cell monolayer was washed twice with sterile 1X PBS, and fresh growth medium was added. Bacterial cultures were grown overnight in 2 to 3 mL of SBHI/HS, and diluted to an OD_{600nm} of 0.1. Bacterial cultures were grown overnight in 2 to 3 mL of SBHI/HS, and diluted to an OD_{600nm} of 0.1. Bacterial cells were resuspended in cell growth medium, and 100 µL of the suspension was added to each well. After a 4 hour incubation with bacteria, host cell wells were washed 3 to 4 times with sterile 1X PBS. A 1:1 ratio solution of trypan blue (Thermo Scientific, Waltham, MA): sterile 1X PBS was added to the cell wells for 4 minutes. The trypan blue solution was removed, and images of each well were taken with an EVOS AMEX-100 microscope (Life Technologies, Carlsbad, CA).

MTT Cytotoxicity Assay

Twenty-four-well polystyrene plates (CellTreat, Shirley, MA) were seeded and incubated as previously described. Growth medium was removed, the host cell

monolayer was washed twice with sterile 1X PBS, and fresh growth medium was added. Bacterial cultures were grown overnight in 2 to 3 mL of SBHI/HS, and diluted to an OD_{600nm} of 0.1. Bacterial cells were resuspended in cell growth medium, and 100 µL of the suspension was added to each well. The plates were centrifuged at 500 rpm for 1 minute to maximize contact between the bacteria and the host cells. After a 4 hour incubation with bacteria, host cell wells were washed 3 to 4 times with sterile 1X PBS and fresh growth medium was added. MTT (EMD Millipore, Billerica, MA) was added to each well, and the plates were centrifuged using the settings previously mentioned to maximize contact between the MTT reagent and the cells. The plates were incubated for 2 hours at 37°C in 5% CO_2. After incubation, MTT stop solution was added according to the manufacturer's instructions. Cells were transferred to a 96-well plate (CellTreat, Shirley, MA) for A_{570} and A_{630} readings using a plate reader (BioTek, Winooski, VT). Absorbance values were detected with a 1:1 mix of MTT reagent and stop solution alone; therefore, these values were subtracted from the experimental values obtained. Untreated (control) cell viability was normalized to 100% for each MTT assay.

Bacterial Adherence Assay

Twenty-four-well polystyrene plates (CellTreat, Shirley, MA) were seeded and incubated as previously described. Growth medium was removed, the host cell monolayer was washed twice with sterile 1X PBS, and fresh growth medium was added. Bacterial cultures were grown overnight in 2 to 3 mL of SBHI/HS, and diluted to an OD_{600nm} of 0.1. Bacterial cells were resuspended in cell growth medium, and 100 µL

of the suspension was added to each well. After a 1 hour incubation with bacteria, host cell wells were washed 3 to 4 times with sterile 1X PBS. Water was added to each well in order to lyse the host cells. The bottoms of the wells were scraped vigorously with a pipet tip, and all contents were collected and placed in a 1.5 mL microcentrifuge tube. The contents were serially diluted and plated onto sBHI + chocolate agar plates. Alternatively, after washing 3 to 4 times with sterile 1X PBS, DNA from each well was extracted using a PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA).

Gentamicin Protection Assay

Twenty-four-well plates (CellTreat, Shirley, MA) were seeded and incubated as previously described. Assays were performed as described previously for bacterial adherence assays, except bacterial cultures were diluted to an OD_{600nm} of 0.001. After a 1 hour incubation with bacteria, host cell wells were washed 3 to 4 times with sterile 1X PBS and antibiotic-free cell media was added. Gentamicin was added to each well at a final concentration of 100 µg/mL. Cells were incubated at 37°C in 5% CO_2 for 1 hour, then washed 2 to 3 times with sterile 1X PBS. Water was added to each well in order to lyse the host cells. The bottoms of the wells were scraped vigorously with a pipet tip, and all contents were collected and placed in a 1.5 mL microcentrifuge tube. The contents were serially diluted and plated onto sBHI + chocolate agar plates.

Adherence/Invasion Assay Utilizing Confocal Microscopy

Confocal assays were performed as previously described[60] with slight modifications. Individual glass bottom plates (MatTek, Ashland, MA) were seeded with

respective cell lines. Once a monolayer of cells had formed, growth medium was removed, the cell monolayer was washed twice with sterile 1X PBS, and stained with 2.5 µL/mL per plate of Vybrant DiI (Invitrogen, Grand Island, NY) membrane stain (Invitrogen, Grand Island, NY) for 15 minutes at 37°C in 5% CO_2. After incubation, cells were washed twice with sterile 1X PBS to remove excess stain.

Bacterial cultures were grown overnight in 2 to 3 mL of SBHI/HS, and diluted to an OD_{600nm} of 0.150. 1.4 mL of the culture was centrifuged to collect the cells. Bacterial cells were washed twice with sterile 1X PBS and stained with 5 µL of BacLight green bacterial stain (Invitrogen, Grand Island, NY) in 1 mL 1X PBS. Bacterial cells were incubated with the stain for 15 minutes at 37°C in 5% CO_2, then washed twice with 1X PBS to remove excess stain. 100 µL of bacteria in 1 mL 1X PBS were added per individual plate of host cells. Plates were incubated for 15 minutes at 37°C in 5% CO_2. Cell monolayers were washed twice with sterile 1X PBS to remove nonadherent bacteria. Plates were analyzed by confocal microscopy using a Zeiss LSM 710 Confocal Microscope. Microscopy was performed at the VCU – Dept. of Anatomy and Neurobiology Microscopy Facility, supported, in part, by funding from NIH-NINDS Center Core Grant 5 P30 NS047463 and, in part, by funding from NIH-NCI Cancer Center Support Grant P30 CA016059.

Quantitative Polymerase Chain Reaction (qPCR)

DNA isolated from adherence/invasion assays was used to determine the relative amount of adhesive/invasive bacteria using 16S primers. The reaction mix was composed of 5 µL of undiluted DNA, 10 pmol of both the forward and reverse primers,

Table 2. Primer List

Primer Name	Sequence
AgSn35Fwd	5’ ATGATGGGATCCGCAGAAAATATTTCAAAAAC 3’
AgSn35Rev	5’ ATGATGCTCGAGTTGACCAGAGTTAGCTTCTACTTTAC 3’
SnRTFwd (for qPCR)	5’ TGATCCAGCAATTCTGTGTG 3’
SnRTRev (for qPCR)	5’ TAGGCAAGCCTATGGTTGAG 3’

5.5 µL sterile water, and 12.5 µL iTaq Universal SYBR Green Supermix (Bioline, Tauton, MA). qPCR was performed in an iQ5 Multicolor Realtime PCR Detection system (BioRad, Hercules, CA) following the programmed steps: 1 cycle at 94°C for 2 minutes, 45 cycles of 94°C for 15 seconds, 52°C for 15 seconds, 71°C for 15 seconds while collecting real time-PCR readings, and 31 cycles of 65°C for 10 seconds while collecting melting curve recordings. Data was recorded in cycle threshold (Ct) for each sample in technical triplicate, averaged, and normalized by calculating $2^{(-Ct)}$. Ratios were determined between input control values and experimental values to determine differences in amount of bacteria.

In silico Prediction of Antigenic Regions

The amino acid sequence of TvbA was submitted to an antigenic peptide predictor, which predicted the segments within the protein sequence likely to be antigenic using the methods of Kolaskar and Tongaonkar[61].

Polymerase chain reaction (PCR) and Product Purification

Reaction mixtures were generally 50 µL total volume composed of 10 µL 5X Phire buffer (Thermo Scientific, Waltham, MA), 1 µL 10 mM dNTPs (2.5 mM of each), 10 pmol of both the forward and reverse primers, DNA template, and 1 µL Phire II polymerase in sterile water. For reactions using gDNA as template, the template was diluted 1:10 and 1 µL was used as template. Reaction conditions were programmed into a thermal cycler machine as: hot start at 105°C, initial denaturation at 98°C for 1 minute, followed by 35 cycles of 98°C for 30 seconds, 54°C for 30 seconds, 72°C for 45

seconds, and a round of final extension at 72°C for 5 minutes. Resulting PCR products were purified after agarose gel electrophoresis using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA), following the manufacturer's instructions.

Plasmid DNA Purification and Manipulation

Plasmid purifications were performed using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, using overnight cultures of *E. coli* single colony transformants in 3 mL LB containing the appropriate antibiotic. All restriction enzymes, specific buffers, and BSA used for restriction digests of plasmid DNA or PCR products were purchased from New England Biolabs (Ipswich, MA) and used according to the respective manufacturer's recommendations. After restriction digest, the 5' phosphoryl groups were removed using Antarctic Phosphatase and supplied buffer (New England Biolabs, Ipswich, MA) according to manufacturer's instructions. Ligation of DNA fragment inserts into digested plasmids was accomplished using Ready-To-Go T4 DNA Ligase (Amersham Biosciences, Piscataway, NJ).

Transformation of Chemically Competent *E. coli*

One vial of CH3-Blue chemically competent *E. coli* cells (Bioline, Taunton, MA) was thawed on ice; 3 µL of the plasmid ligation reaction were added and incubated on ice for 20 minutes. The tube was transferred to a 42°C waterbath for 30 seconds; 250 µL of SOC media (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgSO_4$, 10mM $MgCl_2$, 20mM glucose) was immediately added to the tube

after removal from the waterbath. The cells recovered at 37°C for 1 hour, and were then plated on LB plates with the appropriate antibiotic selection.

Transformation of Electrocompetent *E. coli*

One vial of electrocompetent *E. coli* was thawed on ice; 4 µL of the plasmid ligation reaction were added and incubated on ice for 10 minutes. The mixture was transferred to a chilled electroporation cuvette with a gap length of 0.1 cm and pulsed once using the Micropulser machine (BioRad, Hercules CA) preset *E. coli* setting 1. Immediately following electroporation, 250 µL of SOC media was added and the entire mixture was transferred to a 1.5 mL microcentrifuge tube. The cells recovered at 37°C for 1 hour, then plated on LB plates containing the appropriate antibiotic; plates were incubated overnight at 37°C.

Construction of Expression Plasmid

The primer set AgSn35Fwd (BamHI site) and AgSn35Rev (XhoI site) were used to PCR amplify the TvbA peptide AS01 from Sn35 gDNA with directional restriction sites on either end of the PCR product. After gel electrophoresis and purification by gel extraction, the PCR product was “A-tailed” by incubation with Taq DNA polymerase, which adds an adenine to the 3’ end of DNA fragments. The 15 µL reaction was composed of 10 µL PCR product, 1 µL 10 mM dNTPs, 2 µL sterile water, 1.5 µL Taq buffer, and 0.5 µL Taq; this reaction was incubated at 72°C for 15 minutes. The A-tailed PCR product was ligated into the pCR4-TOPO vector (Life Technologies, Carlsbad, CA), which is a linear vector containing single, overhanging, 3’ deoxythymidine residues. The 3 µL ligation reaction was composed of 2 µL of the previously A-tailed

PCR product, 0.5 µL salt solution provided with the vector, and 0.5 µL of the pCR4-TOPO vector. This reaction was incubated at room temperature for 20 minutes. All 3 µL of the ligation reaction was used for transformation of CH3-Blue chemically competent *E. coli* cells, as described previously. Transformants were selected for on LB agar plates containing ampicillin. The plasmid construct was isolated and purified using the previously mentioned plasmid miniprep kit, and sequence fidelity of the gene insert was confirmed by nucleotide sequencing.

The pCR4-TOPO-AS01 plasmid construct was digested with BamHI and XhoI (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. The isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible plasmid pET32xT (generously supplied by Dr. David Williams, VCU, Richmond, VA) was also digested with BamHI and XhoI. The digested PCR product was ligated into the digested plasmid using DNA ligase, as described previously. The plasmid construct was used for transformation of CH3-Blue chemically competent *E. coli* cells, as described previously. Transformants were selected for on LB agar plates containing ampicillin. The plasmid construct was isolated and purified using the previously mentioned plasmid miniprep kit. The pET32xT-AS01 plasmid construct was used to transform electrocompetent *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA), as described previously. Transformants were selected for on LB agar plates containing ampicillin and chloramphenicol.

Polyacrylamide Gel Electrophoresis

Gels were run using the NuPAGE SDS-PAGE system (Life Technologies, Carlsbad, CA). Manufacturer's instructions were followed with slight modifications. Sample mixes consisted of 30 µL sample, 10 µL 4X Novex NuPAGE LDS Sample Buffer (Life Technologies, Carlsbad, CA), and 4 µL 10X NuPAGE Sample Reducing Buffer (Invitrogen, Grand Island, NY). Samples were placed in boiling water for 10 minutes, lightly sonicated, and loaded into the wells of a NuPage 4-12% Bis-Tris 1.5 mm gel (Invitrogen, Grand Island, NY). The gel was electrophoresed in 1X NuPAGE Running Buffer for 35 minutes at 200 V. Protein size was determined by comparison against the Precision Plus Protein Kaleidoscope standard (BioRad, Hercules, CA). Lanes were visualized by staining the gel with Imperial Protein Stain (Thermo Scientific, Waltham, MA) overnight, and destaining with water until bands were visible.

Recombinant Peptide Expression

To induce the expression of recombinant AS01, a streak of BL21+pET32xT-AS01 colonies was used to inoculate 270 mL of LB containing ampicillin and chloramphenicol. The culture was incubated with shaking overnight at 37°C. 120 mL of the overnight culture was transferred to 2 L of LB containing ampicillin, and individual 60 mL aliquots of the overnight culture was transferred to 2 flasks of 1 L of LB containing ampicillin. The cultures were then incubated shaking at 30°C for 3 hours. A 1 mL aliquot of each culture was removed and saved prior to induction with 1 mM IPTG for 3 hours at 30°C. Following protein induction, another 1 mL aliquot was removed and saved. The remaining culture volume was centrifuged to collect the bacterial cells. Pellets were

washed one time in sterile 1X PBS, and frozen at -80°C. The pre- and post-induction samples were run on a polyacrylamide gel, stained, and visualized in order to confirm proper protein expression.

Recombinant Peptide Purification

Recombinant protein purification was conducted under denaturing conditions following the Probond Purification System (Life Technologies, Carlsbad, CA) with modifications. Each induced BL21+pET32xT-AS01 pellet was resuspended in 40 mL of prewarmed guanidinium lysis buffer containing one dissolved EDTA-free protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN). Resuspended bacterial cells were rocked at room temperature for 10 minutes, and then sonicated 6 times for 30 seconds each. Cell debris was removed by centrifugation, and the supernatant was filter sterilized using a syringe capped with a pre-filer and a 0.45 µm pore filter. 10 mL of HisPur Cobalt Resin (Thermo Scientific, Waltham, MA) was washed once with sterile water and once with denaturing binding buffer. The filtered protein supernatant was applied to the beads in a 50 mL conical tube, and the tube was rocked at room temperature for 20 minutes. A purification column was then prepared with the protein supernatant + bead mixture. The column was washed once with denaturing binding buffer, four times with denaturing wash buffer, and one time with denaturing elution buffer containing no imidazole. Recombinant AS01 was eluted from the column with 5 mL denaturing elution buffer + 500 mM imidazole; 1 mL was added and collected at a time. All fractions were pooled, concentrated, and loaded into a 3.5K MWCO Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, Waltham, MA) to be dialyzed overnight in 3L

of 10 mM Tris, pH 8.0 and 0.1% Triton X-100 stirring at 4°C. The dialyzed peptide was removed from the dialysis cassette and total peptide concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). In order to determine the location of recombinant AS01 throughout the purification process, fractions of all washes and elutions were run on a polyacrylamide gel, stained, and visualized.

Western Analysis

A 0.45 µm PVDF transfer membrane (Thermo Scientific, Waltham, MA) was soaked in an 80% methanol/20% ethanol solution, then dipped in 1X NuPage transfer buffer (Invitrogen, Grand Island, NY) containing 10% methanol. Transfer paper and sponges were also soaked in transfer buffer. After gel electrophoresis, the gel was prepared for electrophoretic transfer by rinsing in deionized water and then placing against the PVDF membrane. Transfer papers and sponges were used to maintain tight contact between the gel and membrane. Transfers were run in 1X NuPage transfer buffer at 45V for 90 minutes at room temperature. Membranes were blocked overnight at 4°C in 5% skim milk diluted in 1X PBS, then probed with appropriate concentrations of respective primary antibodies in 1X PBS + 1-1.5% blocking solution + 0.05% Tween-20 (Fisher Scientific, Pittsburg, PA) for 1 hour at room temperature, while rocking. Membranes were washed three times for 10 minutes with 1X PBST (PBS + 0.05% Tween-20), then probed with 1:50,000-diluted goat anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Invitrogen, Grand Island, NY) in 1X PBST for 45 minutes at room temperature, while rocking. Bands were visualized with the ECL Plus

western blotting detection system (GE Healthcare, Buckinghamshire, UK). Blots were incubated in the chemiluminescence developing reagent for 30 seconds, covered in plastic wrap, exposed to X-ray film, and developed in an X-O-Mat.

Rabbit Polyclonal Antiserum Production

Recombinant AS01 was expressed and purified using methods previously described, and sent to New England Peptides. Prior to immunization with recombinant AS01, 5 mL of pre-immunized serum was collected from the rabbit (New Zealand White – SPF). The rabbit was immunized with recombinant AS01 3 separate times. Boosts were administered on days 14 and 28, while bleeds were collected on days 35 and 40. Upon receipt of post-immunized serum, serum was aliquoted into cryovials and stored at -80°C.

Determining Location of TvbA Expression

A 5 mL overnight culture of Sn35 grown in SBHI+HS under anaerobic conditions was centrifuged to obtain a cell pellet; the supernatant was concentrated, and the cell pellet was resuspended and lysed in lysis buffer. Samples were subjected to SDS-PAGE and Western analysis as described previously; the primary antiserum dilution used in this experiment was 1:2500.

Temporal Protein Expression Assay

An overnight culture of Sn35 grown under anaerobic conditions in SBHI+HS was subcultured at a 1:50 dilution into fresh SBHI+HS and grown at 37°C under anaerobic

conditions. 1 mL samples were collected at 4, 6, 8, 12, 16, 24, and 48 hours. Cell pellets were obtained by centrifugation at 16,000 rpm for 5 minutes at room temperature; after storage at -80°C, pellets were resuspended in varying amounts of lysis buffer using pellet size as a rough estimation of equal protein amounts. Samples were subjected to SDS-PAGE and Western analysis as described previously; the primary antiserum dilution used in this assay was 1:500.

Utilizing Antiserum in Inhibition Studies

Inhibition assays were performed as previously described, with modifications. Bacteria were incubated with varying concentrations of pre-immunized serum or post-immunized serum for 30 minutes at 37°C in 5% CO_2. Treated bacteria were then added to cell monolayers and changes in cytopathogenicity were observed as previously described.

Results

I. Sn35 is Cytopathogenic to Host Cells

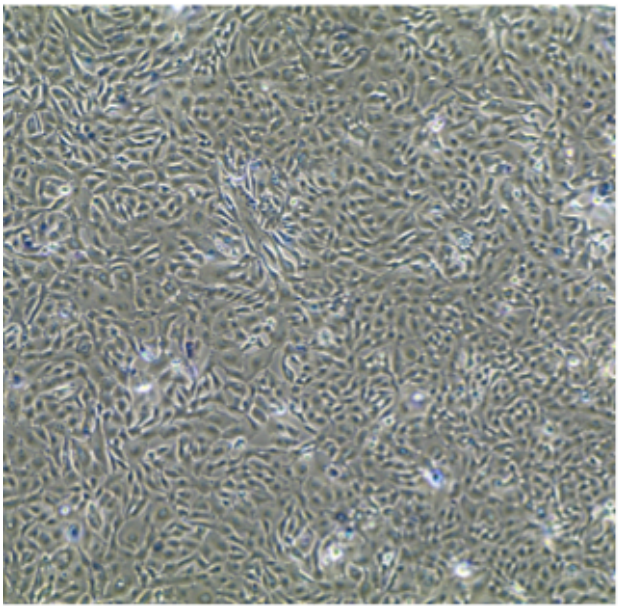
As previously discussed, it has been shown that Sn35 exerts cytotoxic effects on Me-180 human cervical carcinoma cells, as observed by cell rounding and loss of adherence[58]. Therefore, the first focus of this study was to identify additional cytopathogenic effects caused by Sn35; for this focus, a variety of immortalized cell lines were used. Immortalized cell lines were chosen due to their isolation from the female reproductive system and their relevance to the study. Primary human amniotic epithelial cells (HAECs) were also isolated and used in the study.

A. Cytotoxicity to HAECs

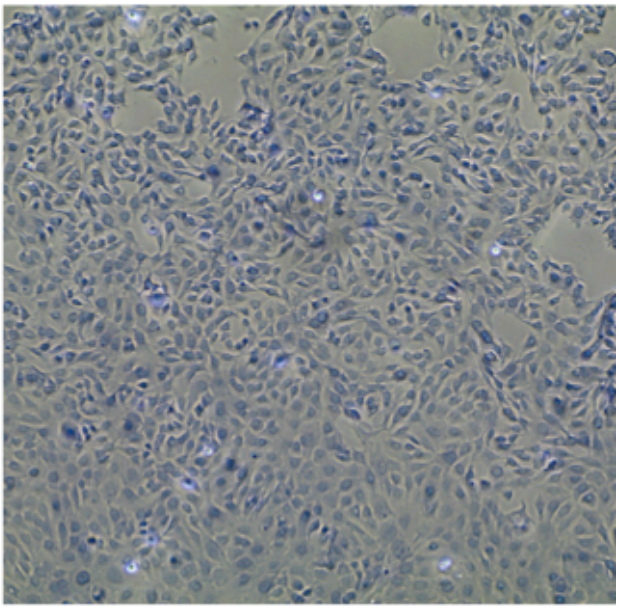
Sn35 was tested for its ability to induce loss of cell integrity (measured via a trypan blue assay). 1.2×10^7 bacteria were added to a confluent monolayer of HAECs. Following a 4 hour incubation, the monolayer was washed and incubated with trypan blue for 4 minutes. The stain was removed and microscopic images were recorded. As shown in **Figure 3**, epithelial cells treated with Sn35 are stained blue, indicating cell damage and/or death due to incubation with Sn35. The trypan blue assay was repeated using a 3 hour incubation time with similar results.

Figure 3. Loss of cell integrity of HAECs due to incubation with Sn35. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria were standardized to an OD_{600nm} = 0.1, and added to HAECs for 4 hours. Cells were washed after incubation, and trypan blue was added for 4 minutes to determine reduction in cell integrity. Light microscopy images were taken after the trypan blue incubation. Experiments were also repeated using a 3 hour bacteria-host cell incubation.

Control after 4 hours



Treated w/ Sn35 after 4 hours



B. Cytotoxicity to JEG-3 cells

In addition to primary cells, we analyzed the ability of Sn35 to induce loss of cell integrity and viability (measured via qualitative trypan blue assays and quantitative MTT assays) in immortalized cell lines. 1.2×10^7 bacteria were added to a confluent monolayer of JEG-3 human chorionic trophoblasts. Trypan blue and MTT assays were performed as described previously. **Figure 4** shows trypan blue cell integrity results for JEG-3 cells. Treatment with Sn35 for 4 hours appears to inflict cell damage and/or death, and allows for the trypan blue stain to traverse cell membranes. The trypan blue assay was repeated using a 3 hour incubation time with similar results.

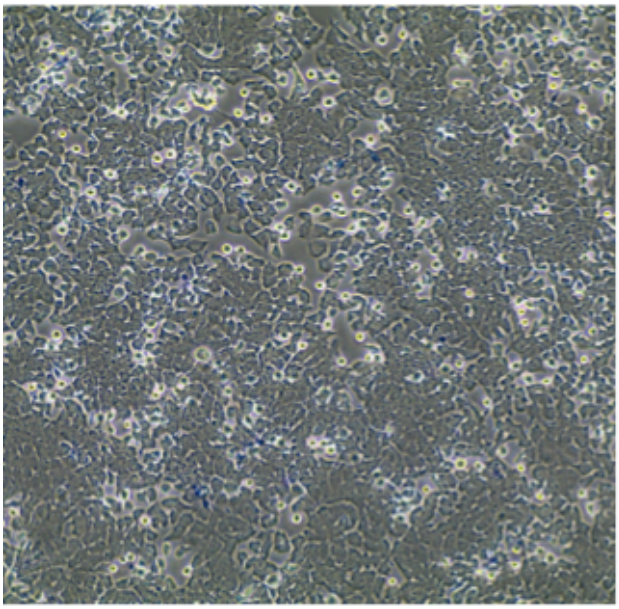
Figure 5 shows results representative of 2 biologic replicates of the MTT viability assay for Sn35-induced cytotoxicity. After a 4 hour incubation of JEG-3 cells with Sn35, the OD_{570nm} readings, a measure of cell viability, had decreased to 25.9% relative to the untreated controls. Those cells treated with 100% methanol for 4 hours had a decreased viability of 9.7% relative to untreated controls. The MTT assay was repeated with similar results.

C. Cytotoxicity to VK2 cells

The VK2 human vaginal keratinocyte cell line was also used to study cytotoxicity; both trypan blue assays and MTT assays were performed as described above. Consistent with trypan blue results from the JEG-3 cell line, exposure to Sn35 for 4 hours appeared to induce cell damage and/or death in the VK2 cell line (**Figure 6)**. The trypan blue assay was repeated using a 3 hour incubation time with similar results.

Figure 4. Loss of cell integrity of JEG-3 cells due to incubation with Sn35. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria were standardized to an OD_{600nm} = 0.1, and added to JEG-3 cells for 4 hours. Cells were washed after incubation, and trypan blue was added for 4 minutes to determine reduction in cell integrity. Light microscopy images were taken after the trypan blue incubation. Experiments were also repeated using a 3 hour bacteria-host cell incubation.

Control after 4 hours



Treated w/ Sn35 after 4 hours

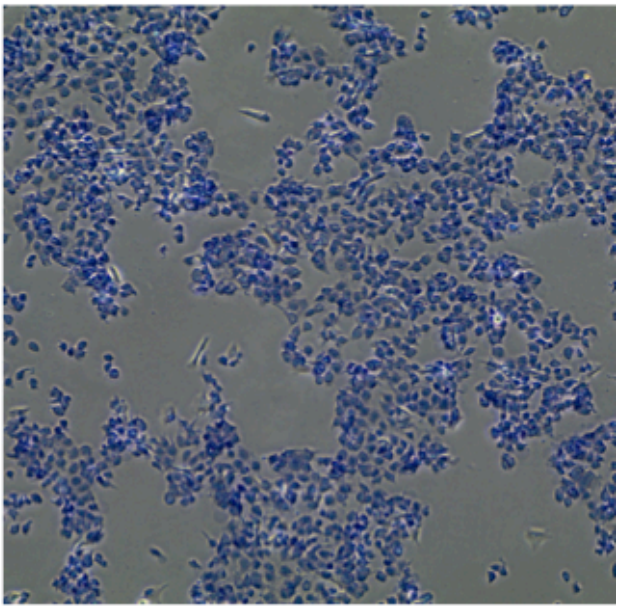


Figure 5. Loss of viability of JEG-3 cells due to incubation with Sn35. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria were standardized to an OD_{600nm} = 0.1, and added to JEG-3 cells for 4 hours. Cells were washed after incubation, and MTT was added to each well for 2 hours. After stop solution was added, A_{570nm} readings were collected via a 96-well plate reader to determine cell viability. Statistical comparison (using student t-test) between Sn35-treated cells and the control cells gave a P value of <0.05. Experiments were performed twice with similar results; this is a representative graph of viability.

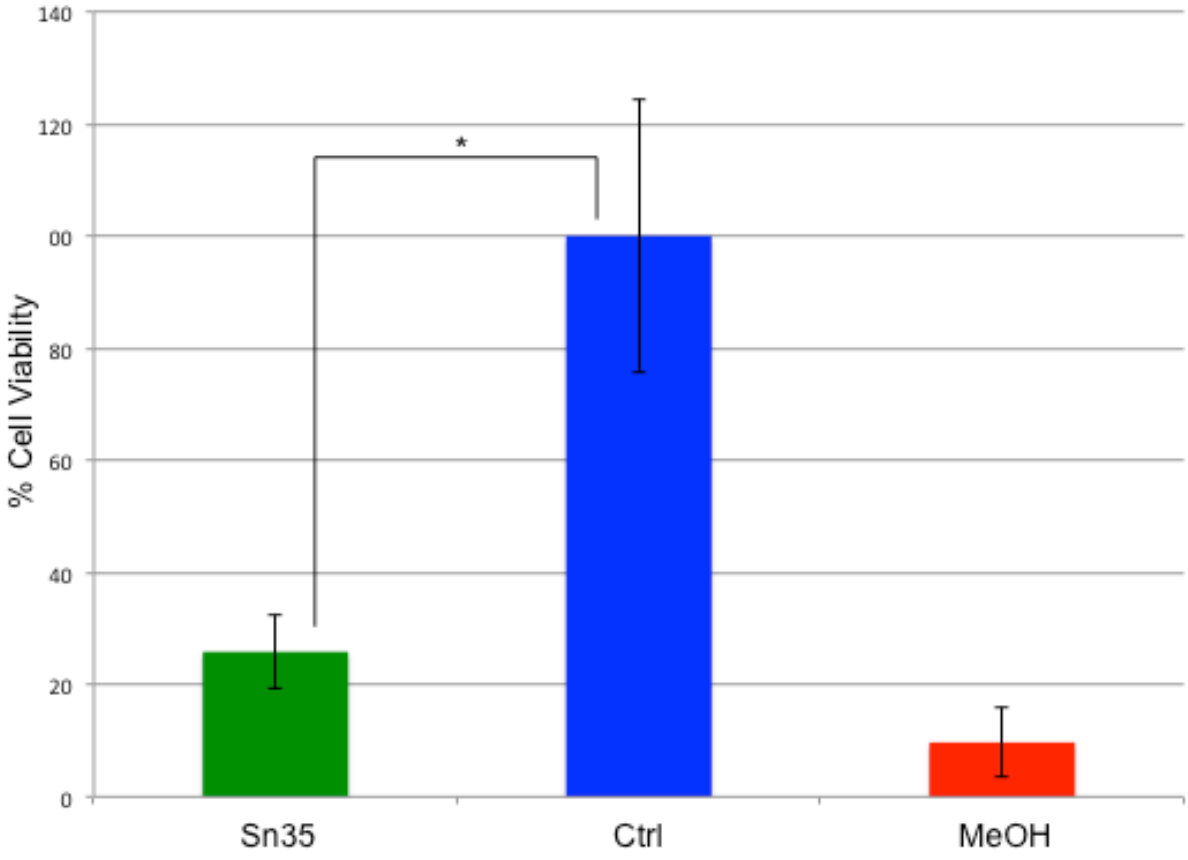
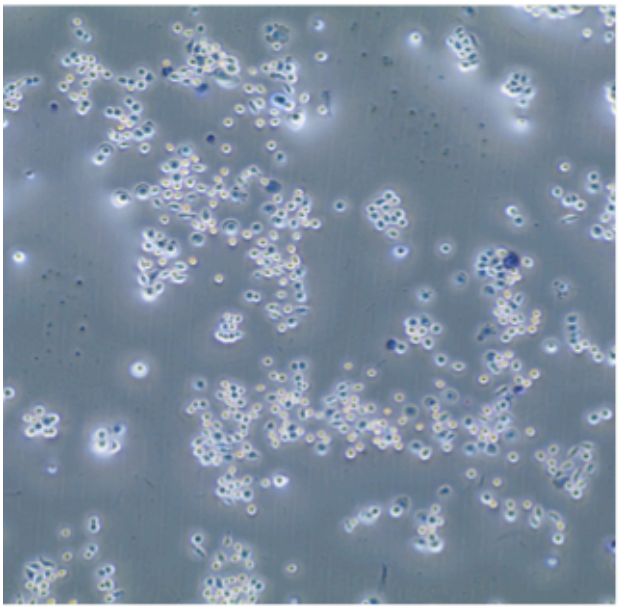

140
120
00
80
60
40
20
0
% Cell Viability
*
Sn35
Ctrl
MeOH

Figure 6. Loss of cell integrity of VK2 cells due to incubation with Sn35. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria were standardized to an OD_{600nm} = 0.1, and added to VK2 cells for 4 hours. Cells were washed after incubation, and trypan blue was added for 4 minutes to determine reduction in cell integrity. Light microscopy images were taken after the trypan blue incubation. Experiments were also repeated using a 3 hour bacteria-host cell incubation.

Control after 4 hours



Treated w/ Sn35 after 4 hours

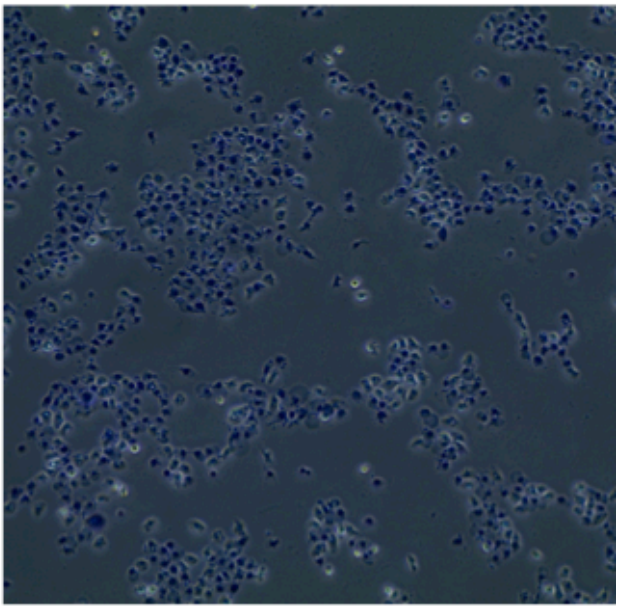


Figure 7 shows results representative of 2 biologic replicates of the MTT viability assay for Sn35-induced cytotoxicity. After a 4 hour incubation of VK2 cells with Sn35, the OD_{570nm} readings had decreased to 63.5% relative to the untreated controls. Those cells treated with 100% methanol for 4 hours had a decreased viability of 7.3% relative to untreated controls**.** The MTT assay was repeated with similar results.

II. Adherence and Invasion of Host Cells

Previous studies have also demonstrated adherence of Sn35 to the Me-180 cell line[58]. Sn35 was therefore tested for its ability to adhere to the cell lines previously described. In addition, Sn35 invasion of host cells was also studied, as some bacterial species invade host cells after adhering to their surfaces.

A. Adherence and Invasion of HAECs

Confocal microscopy was used to determine if Sn35 was capable of adhering and/or invading HAECs. Epithelial cells were stained with VyBrant DiI membrane stain, while bacterial cells were stained with BacLight Green bacterial stain. Cells and bacteria were incubated together for 15 minutes, and then cell monolayers were washed and imaged with a Zeiss LSM 710 Confocal Microscope. **Figure 8** indicates that Sn35 is able to invade HAECs, as demonstrated by the fluorescence of the bacteria within the

Figure 7. Loss of viability of VK2 cells due to incubation with Sn35. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria were standardized to an OD_{600nm} = 0.1, and added to VK2 cells for 4 hours. Cells were washed after incubation, and MTT was added to each well for 2 hours. After stop solution was added, A_{570nm} readings were collected via a 96-well plate reader to determine cell viability. Statistical comparison (using student t-test) between Sn35-treated cells and the control cells gave a P value of <0.05. Experiments were performed twice with similar results; this is a representative graph of viability.

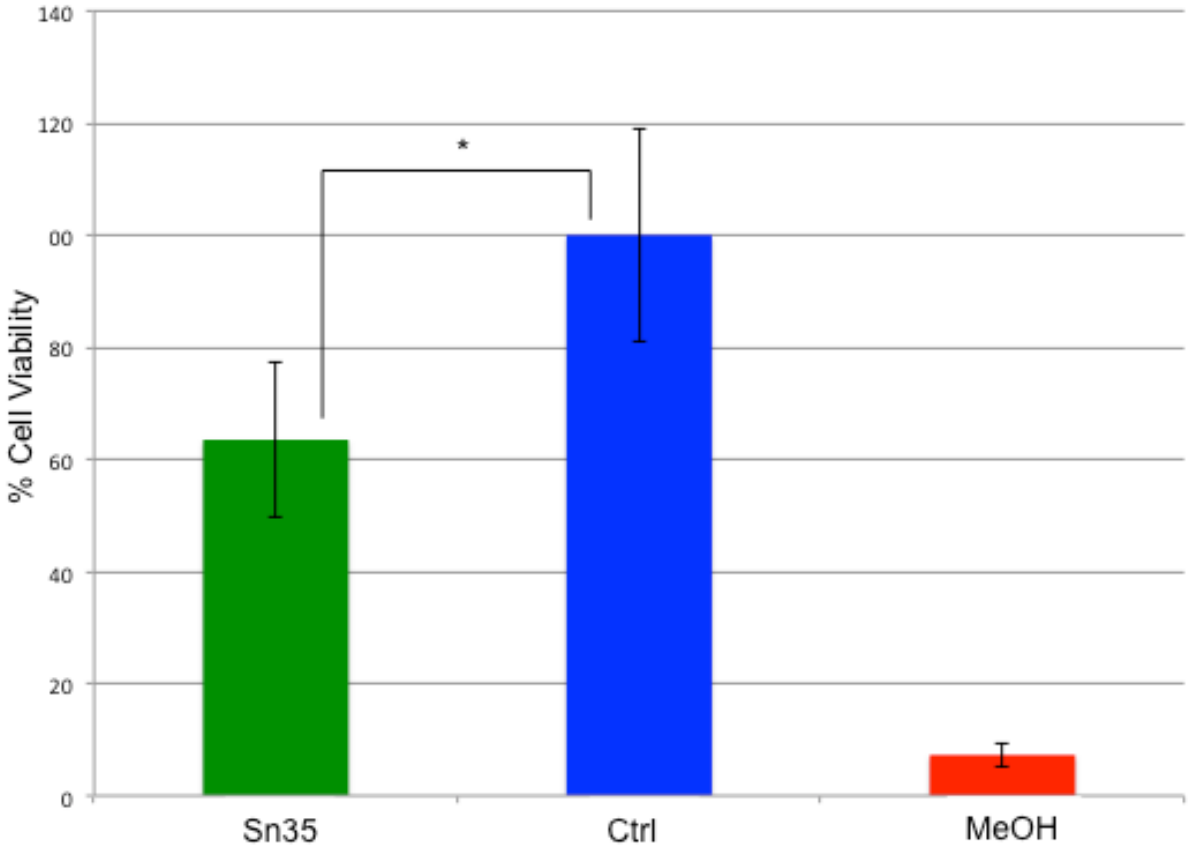
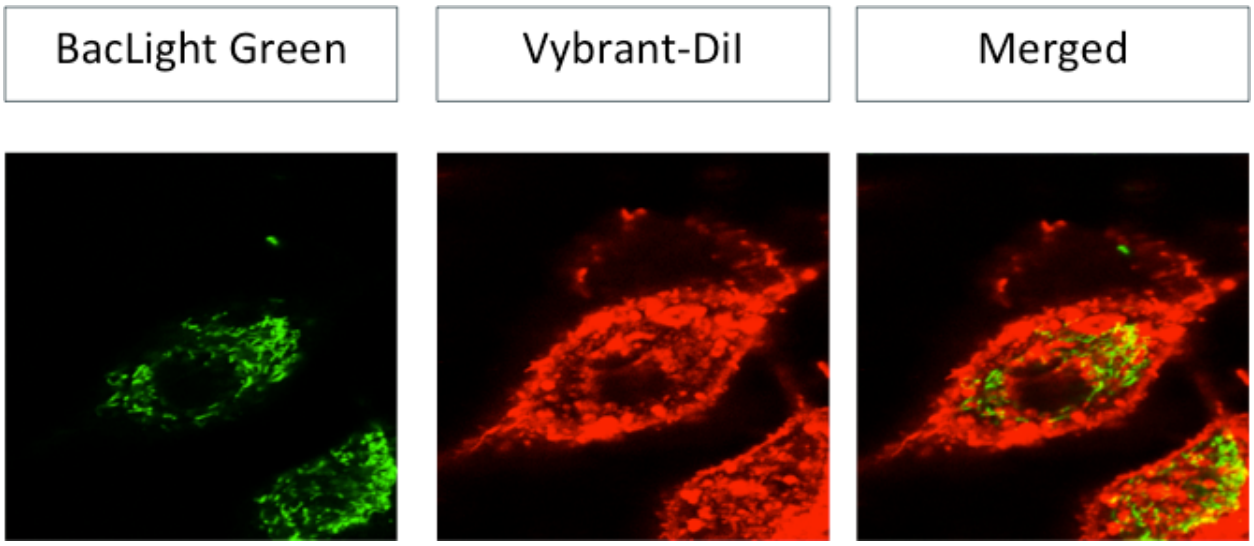

140
120
00
80
60
40
20
0
% Cell Viability
*
Sn35
Ctrl
MeOH

Figure 8. Invasion of Sn35 into HAECs. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria (green) were standardized to an OD_{600nm} = 0.150, and added to HAECs (red) for 15 minutes. Cells were washed after bacterial incubation to remove any nonadherent bacteria. Adherence was observed by confocal microscopy. Experiments were performed twice, with similar results.


BacLight Green
Vybrant-DiI
Merged

epithelial cell. A Z-stack was also performed to confirm the presence of Sn35 within the cell (**Figure 9**). The confocal assay was performed two times with similar results.

B. Adherence and Invasion of JEG-3 cells

Confocal assays were performed on the JEG-3 cell line as described above. **Figure 10** shows that Sn35 appears to some interaction with this cell line; however, the interaction is not as pronounced as that of Sn35 and HAECs. This assay was performed two times with similar results.

In addition to confocal assays, several other adherence and invasion assays were performed with the JEG-3 cell line. qPCR was used to determine the amount of bacteria that adhered and invaded JEG-3 cells during a 1 hour incubation. **Figure 11** demonstrates that 4.2% of Sn35 added to each well was recovered by qPCR relative to the amount of bacterial input. The qPCR assay was performed two times with inconsistent results. Plating assays were also performed to obtain adherence and invasion percentages. **Figure 12** demonstrates that, through plating recovery experiments, 1.7% of bacteria were able to adhere to JEG-3 cells relative to the bacterial input, while 0.3% of bacteria were able to invade the JEG-3 cells. Plating assays were performed two times with inconsistent results.

C. Adherence and Invasion of VK2 cells

Confocal, adherence, and invasion assays were performed on the VK2 cell line as described above. **Figure 13** demonstrates that Sn35 is able to invade VK2 cells, as shown by confocal microscopy. This assay was performed twice with similar results.

Figure 9. Z-stack of Sn35 invasion into HAECs. Successive slices of different focal planes were used to confirm invasion of bacteria into the cells.

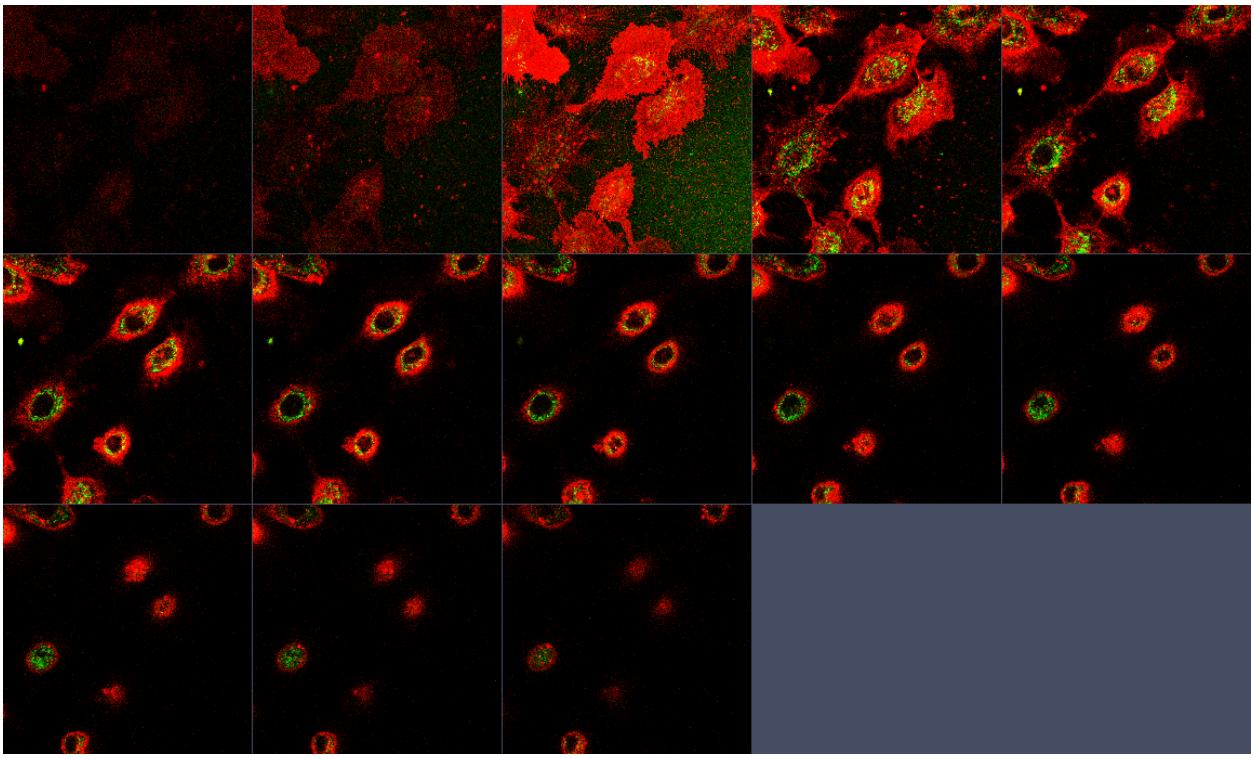


Figure 10. Invasion of Sn35 into JEG-3 cells. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria (green) were standardized to an OD_{600nm} = 0.150, and added to JEG-3 cells (red) for 15 minutes. Cells were washed after bacterial incubation to remove any nonadherent bacteria. Adherence was observed by confocal microscopy. Experiments were performed twice, with similar results.

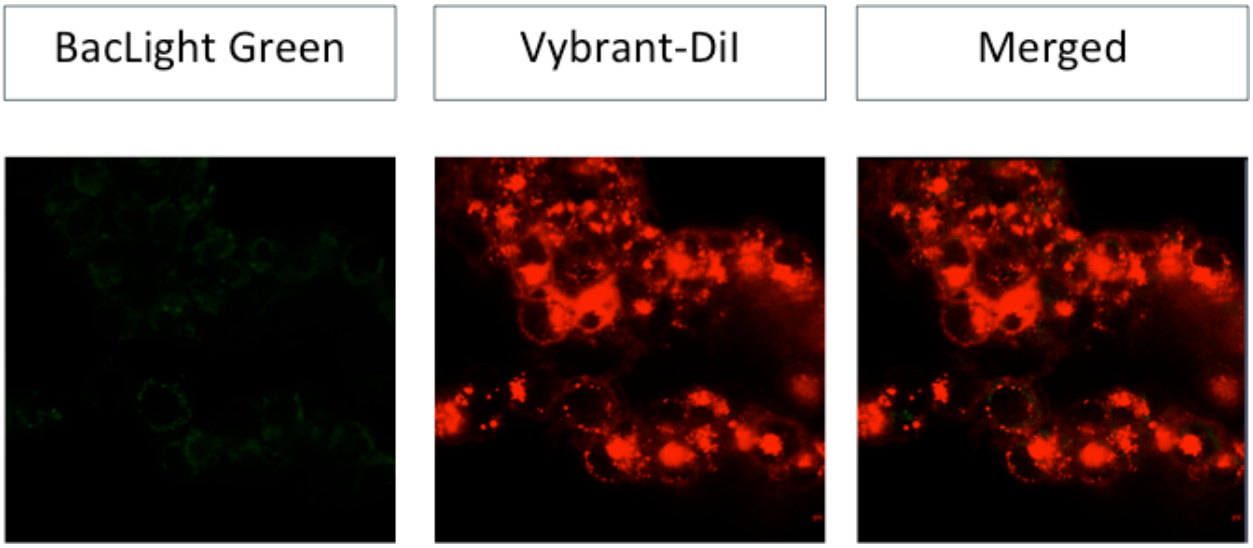

BacLight Green
Vybrant-DiI
Merged

Figure 11. qPCR-based recovery of Sn35 after incubation with JEG-3 cells.

Bacteria were grown anaerobically in SBHI + HS overnight. The culture was standardized to OD_{600nm} = 0.1, and incubated with JEG-3 cells for 1 hour. DNA was extracted and subjected to qPCR with Sn35-specific 16S primers.

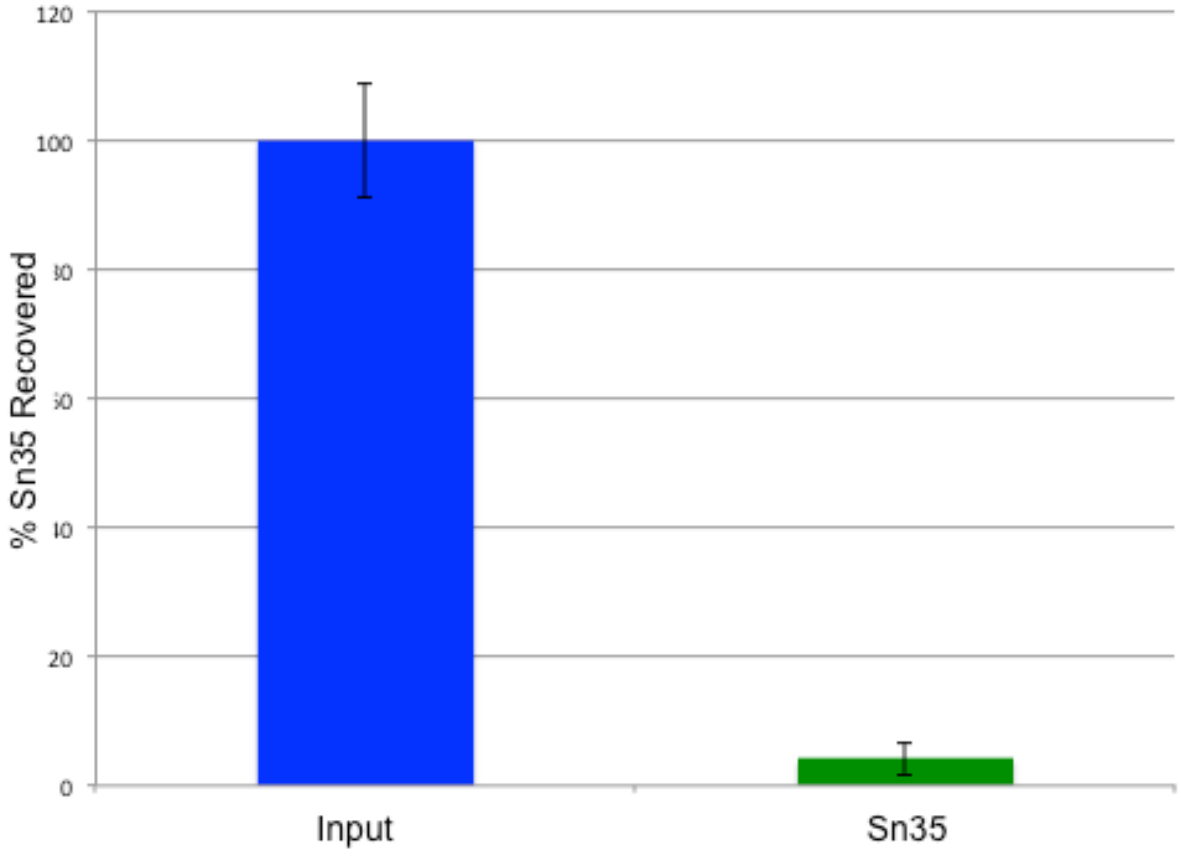

120
100
80
60
40
20
0
% Sn35 Recovered
Input
Sn35

Figure 12. Adherence and invasion of JEG-3 cells by Sn35. An overnight Sn35 culture was standardized to OD_{600nm} = 0.1 for adherence studies and 0.001 for gentamicin protection studies. Cells were incubated for an hour with bacteria; gentamicin assays were incubated with the antibiotic for an additional hour. Percent adherence and invasion were calculated from CFUs plated on SBHI + chocolate agar.

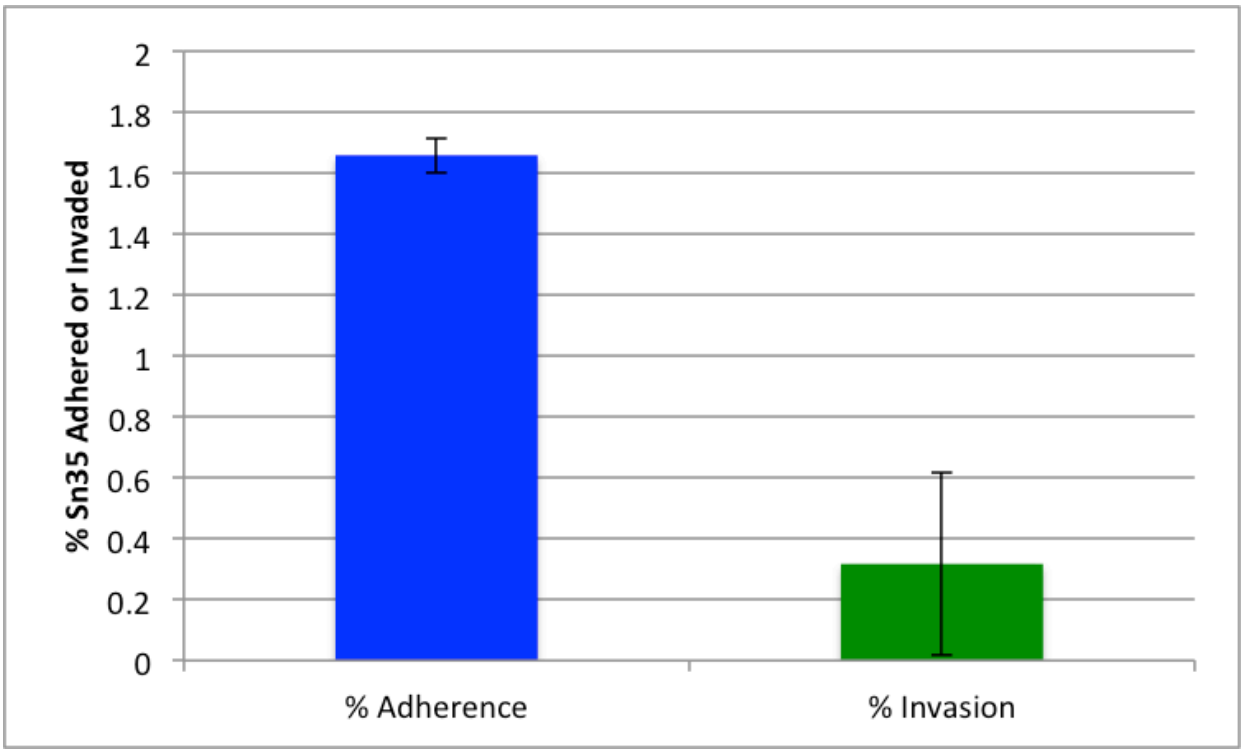

% Sn35 Adhered or Invaded
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
% Adherence
% Invasion

Figure 13. Invasion of Sn35 into VK2 cells. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria (green) were standardized to an OD_{600nm} = 0.150, and added to VK2 cells (red) for 15 minutes. Cells were washed after bacterial incubation to remove any nonadherent bacteria. Adherence was observed by confocal microscopy. Experiments were performed twice, with similar results.

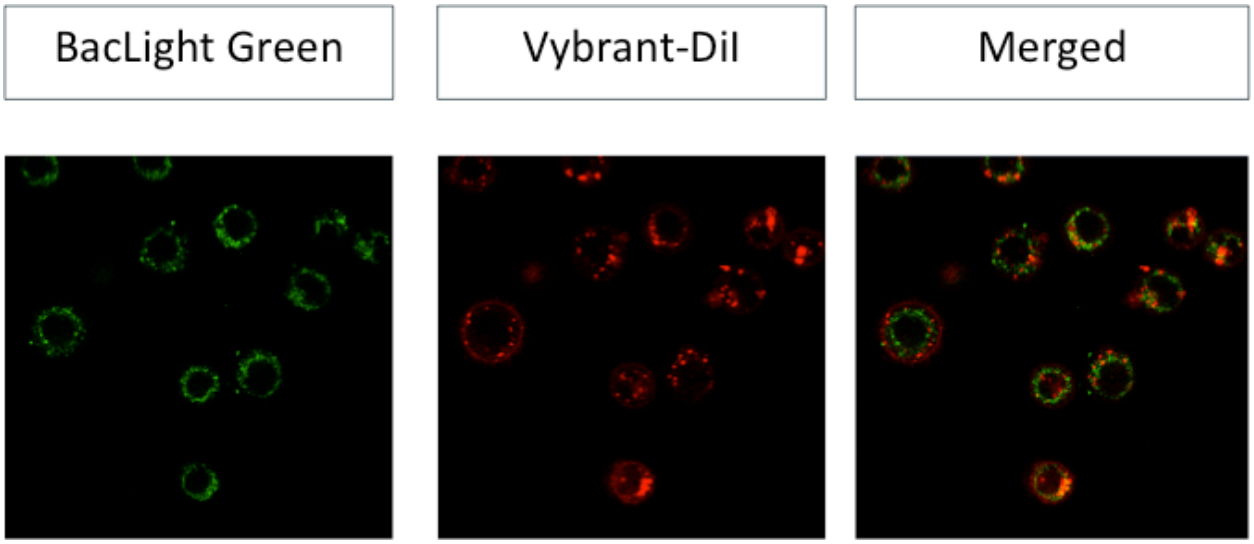

BacLight Green
Vybrant-DiI
Merged

Figure 14 demonstrates that 7.3% of Sn35 added to each well was recovered by qPCR relative to the amount of bacterial input. As with the JEG-3 cell line, the qPCR assay was performed two times with inconsistent results. **Figure 15** demonstrates that, through plating recovery experiments, 1.4% of bacteria were able to adhere to VK2 cells relative to the bacterial input, while 0% of bacteria were able to invade the VK2 cells. Plating assays were performed two times with inconclusive results.

III. Identification of Putative Virulence Gene in Sn35

Bioinformatic analysis of the Sn35 genome revealed a putative virulence gene with homology to a number of haemagluttinin repeats and haemagglutination activity domains; homology to a filamentous haemagglutinin family domain was also identified. One well-studied filamentous haemagglutinin, FHA, is expressed by *Bordetella pertussis.* FHA is the best-studied adhesin produced by *B. pertussis*, and requires a single specific accessory protein, FhaC, for secretion. FHA and FhaC work together as components of a two-partner Type Vb two-partner secretion system (**Figure 16**). FHA begins as a precursor with an N-terminal signal peptide that is recognized by the Sec pathway machinery. Once through this machinery, FhaC both aids in the folding of FHA and mediates the secretion of the mature FHA protein by forming a pore in the outer membrane. Because a potential virulence factor was identified, additional analyses were performed to find a putative transporter gene in the Sn35 genome. Upstream of the previously identified putative virulence gene, a gene with homology to

Figure 14. qPCR-based recovery of Sn35 after incubation with VK2 cells. Bacteria were grown anaerobically in SBHI + HS overnight. The culture was standardized to OD_{600nm} = 0.1, and incubated with VK2 cells for 1 hour. DNA was extracted and subjected to qPCR with Sn35-specific 16S primers.

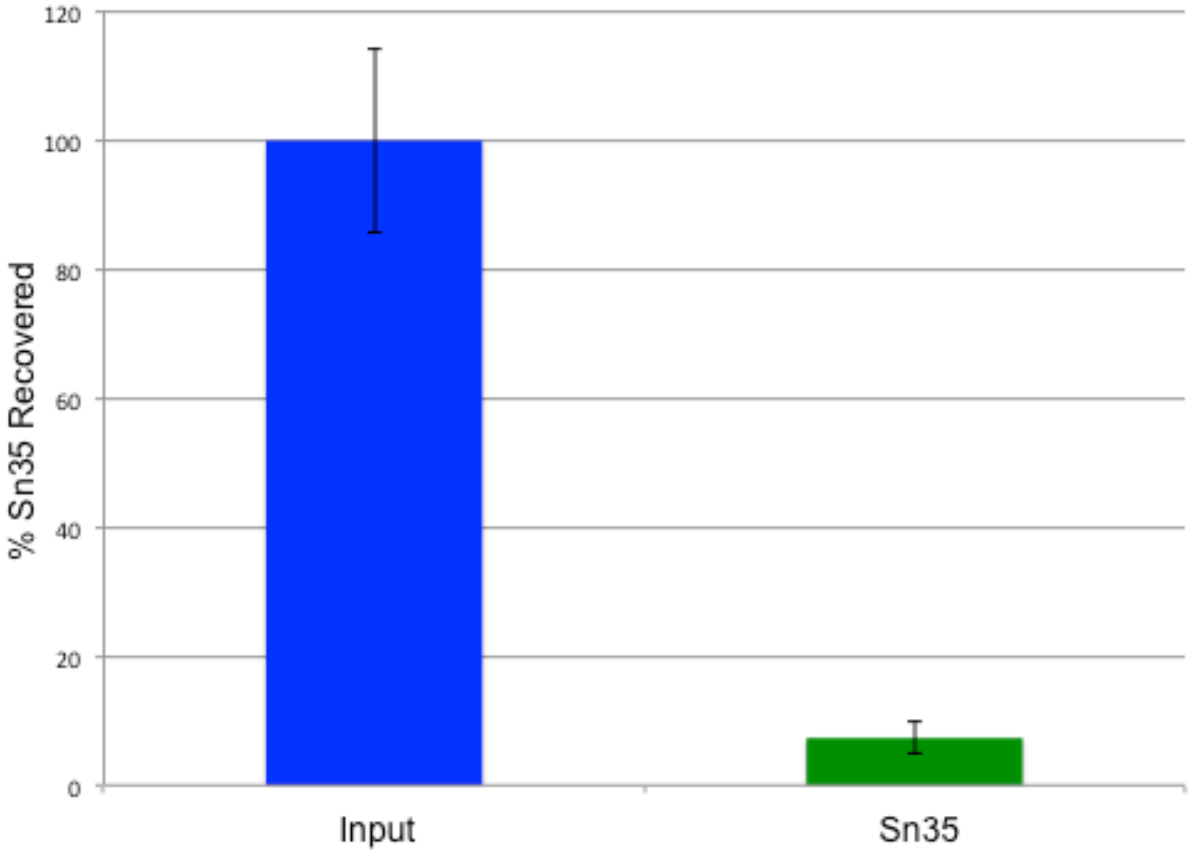

120
100
80
60
40
20
0
% Sn35 Recovered
Input
Sn35

Figure 15. Adherence and invasion of VK2 cells by Sn35. An overnight Sn35 culture was standardized to OD_{600nm} = 0.1 for adherence studies and 0.001 for gentamicin protection studies. Cells were incubated for an hour with bacteria; gentamicin assays were incubated with the antibiotic for an additional hour. Percent adherence and invasion were calculated from CFUs plated on SBHI + chocolate agar.

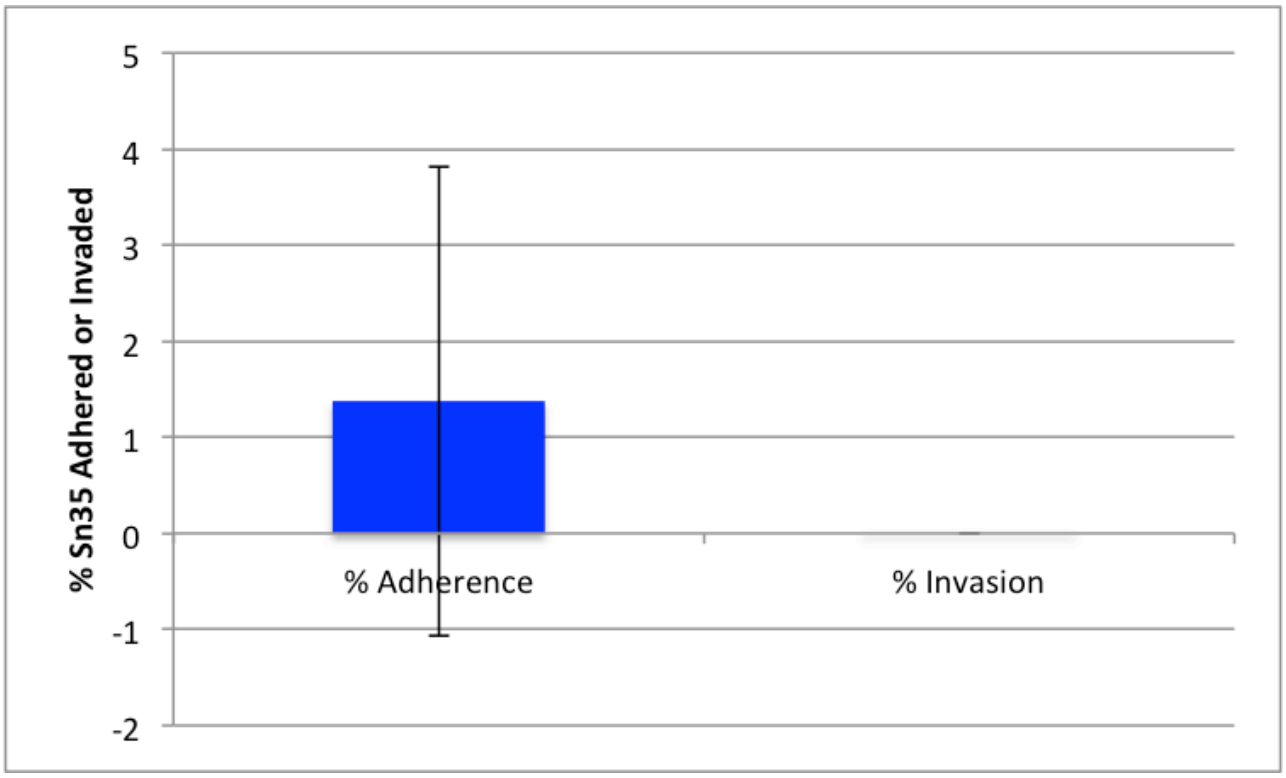
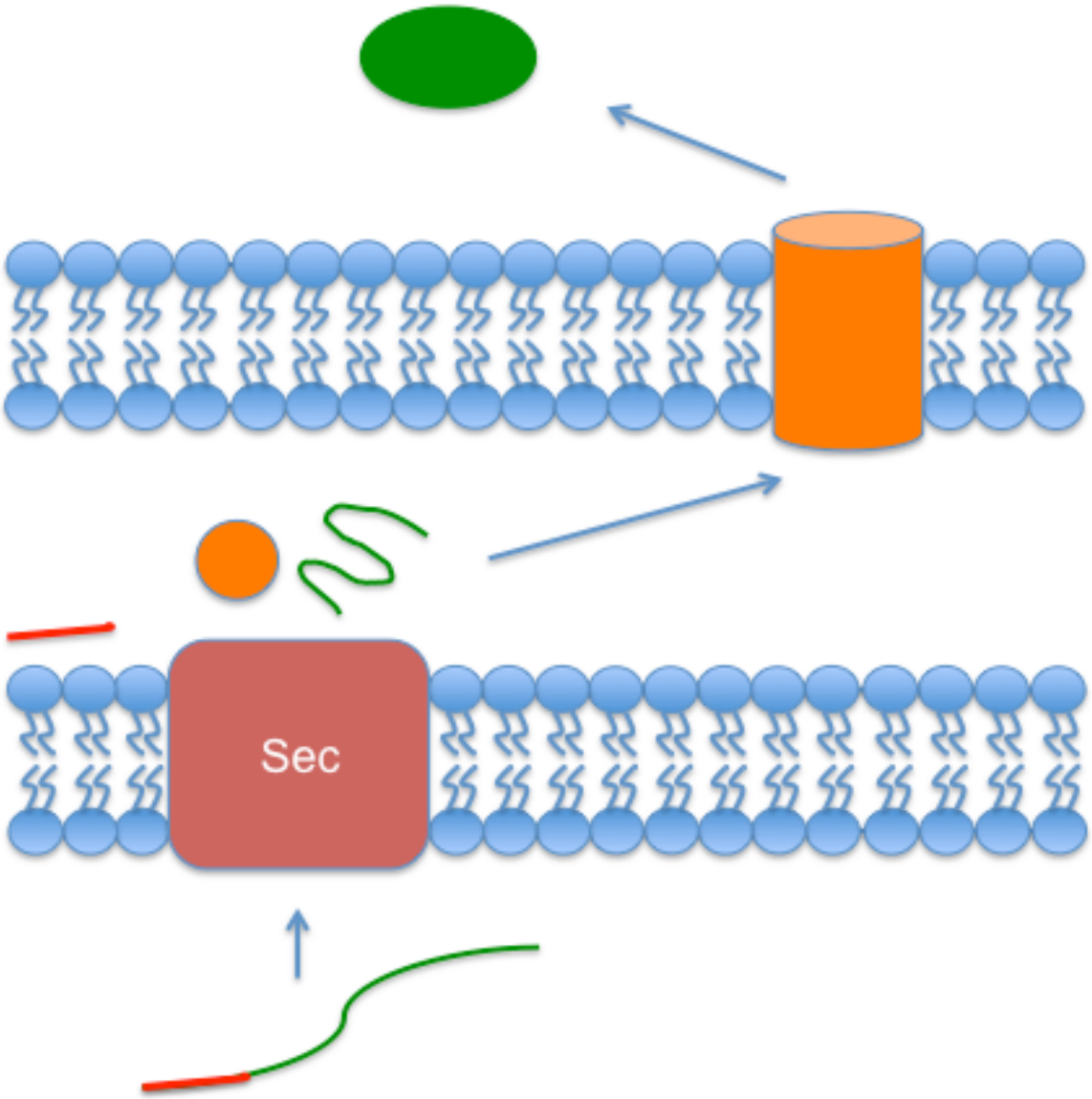

% Sn35 Adhered or Invaded
5
4
3
2
1
0
-1
-2
% Adherence
% Invasion

Figure 16. Type Vb two-partner secretion system expressed by *Bordetella pertussis*, and putatively expressed by Sn35. The secreted protein is represented in green, and red represents the signal peptide cleaved off of the protein after translocation through the Sec pathway (shown in maroon). The transporter protein is represented in orange, which helps to fold the protein into its functional conformation and aids in its secretion through the outer membrane. Figure adapted from Thanassi et al., 2000[62].


Sec

FhaC was identified. This was not surprising, as genes encoding Type Vb two-partner systems are organized into operons, where the transporter gene usually precedes the virulence gene[63]. Because many virulence factors are secreted by two-partner secretion systems, the putative genes in Sn35 became the targets of this study. The putative virulence protein was designated TvbA, for putative **T**ype **Vb** protein **A**; the putative transporter protein was designated TvbB, for putative **T**ype **Vb** protein **B**. TvbA is approximately 1715 amino acids and 184.55 kDa in size, and TvbB is approximately 441 amino acids and 50.63 kDa in size.

IV. Polyclonal Antiserum Production Using TvbA Peptide

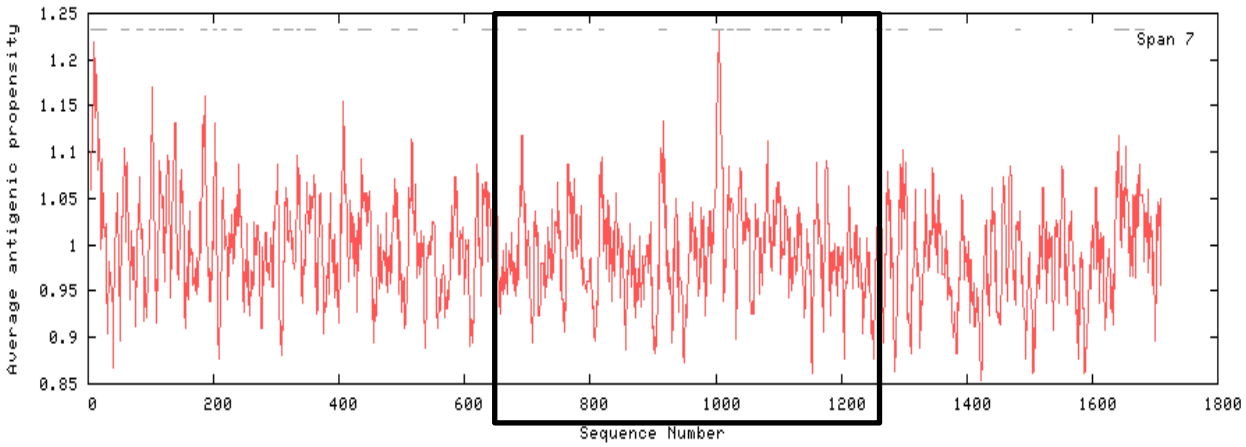
In order to study the newly identified TvbA, attempts to clone and express the full-length, functional protein were made. However, these were unsuccessful; one difficulty encountered was the complexity of the putative two-partner system. If TvbA does utilize a two-partner secretion system, the putative transporter TvbB would be absolutely necessary for the proper folding and secretion of the putative virulence protein. Therefore, both proteins would need to be cloned and expressed together in order to produce functional TvbA. The two-gene operon is 6.483 kb in size. Purified TvbA would also be difficult to obtain; inserting a purification tag between the two secretory components might disturb secretion, as the N-terminus of the protein encodes a signal sequence prior to processing. Additionally, previous studies have demonstrated that virulence components of two-partner systems can contain C-terminal domains that are removed during translocation[64]. If further processing occurs at the C-terminus of

TvbA, a C-terminal purification tag would be cleaved off and rendered useless. For these reasons, an alternate tactic was developed. Upstream and downstream fragments of TvbA were amplified via PCR, fused to a chloramphenicol resistance cassette, and cloned into a suicide vector for use in generating a TvbA gene interruption via allelic exchange. Currently, this approach has also been unsuccessful. As previously discussed, a genetic system has not been established for Sn35, making modification difficult. Due to the inability to work with recombinant full-length TvbA or purify the protein, polyclonal antiserum to a fragment of TvbA was produced.

A. Identification of Antigenic Epitopes within TvbA

In order to identify the regions of TvbA with the highest antigenic propensity, the amino acid sequence of the full-length protein was submitted to an online antigenic peptide predictor, which compares the occurrence of amino acid residues in submitted protein sequence to those found in experimentally determined epitopes. According to the prediction algorithm used, if the average antigenic propensity for the whole protein is above 1.0, all residues with a propensity of above 1.0 are potentially antigenic. If the average is below 1.0, all residues with a propensity above the average of the total protein are potentially antigenic[61]. The average antigenic propensity for TvbA is 0.9937, according to this algorithm. The results obtained from the predictor are shown in **Figure 17**.

Figure 17. *In silico* prediction of antigenic epitopes within TvbA. Average antigenic propensities generated for TvbA demonstrated a number of antigenic epitopes in the middle of the protein. The peptide targeted for antiserum production is boxed.


Average antigenic propensity
1.25
1.2
1.15
1.1
1.05
1
0.95
0.9
0.85
0
200
400
600
800
1000
1200
1400
1600
1800
Sequence Number
Span 7

While epitope prediction tools are helpful, they only provide estimations. When choosing a peptide for antiserum production, the N- or C-terminal of the full-length protein is typically used, as those parts of the protein are usually exposed[65]. However, as previously mentioned, using the termini of TvbA as antiserum targets would be risky. Therefore, the middle of TvbA was targeted for eventual polyclonal antiserum production. It was thought that by selecting a larger peptide, the polyclonal antiserum would be able to detect multiple epitopes present in TvbA. This is ideal for our current studies, as the conformation of functional TvbA is currently not known. The target peptide was 60.50 kDa in size without purification tags; the vector tags added ~20 kDa to the final peptide size. This peptide fragment is referred to as AS01. The boxed area in **Figure 17** indicates the targeted peptide region.

B. Expression and Purification of Recombinant AS01

AS01 was amplified by PCR as previously described (**Figure 18**). AS01 was cloned into the pCR4-TOPO vector, digested out using restriction enzymes, and subsequently subcloned into digested pET32xT (**Figure 19**). Presence of the fragment was confirmed via sequencing and restriction digest (**Figure 20**). After transformation into *E. coli* cells and verification of transformants, recombinant AS01 was expressed with samples collected both pre- and post-induction. Whole cell lysates obtained pre-induction and post-induction were subjected to SDS-PAGE and visualized as previously described. Expression of the TvbA fragment was confirmed by the presence of an ~80 kDa product after induction with 1 mM IPTG (**Figure 21).** Expressed recombinant AS01

Figure 18. PCR amplification of AS01 DNA. AS01-specific primers were used to amplify AS01 off of the Sn35 genome. Lane 1 contains Hyperladder 1kb, and Lane 4 contains AS01 (1680 bp in size). PCR products were run on 1% agarose DNA gel.

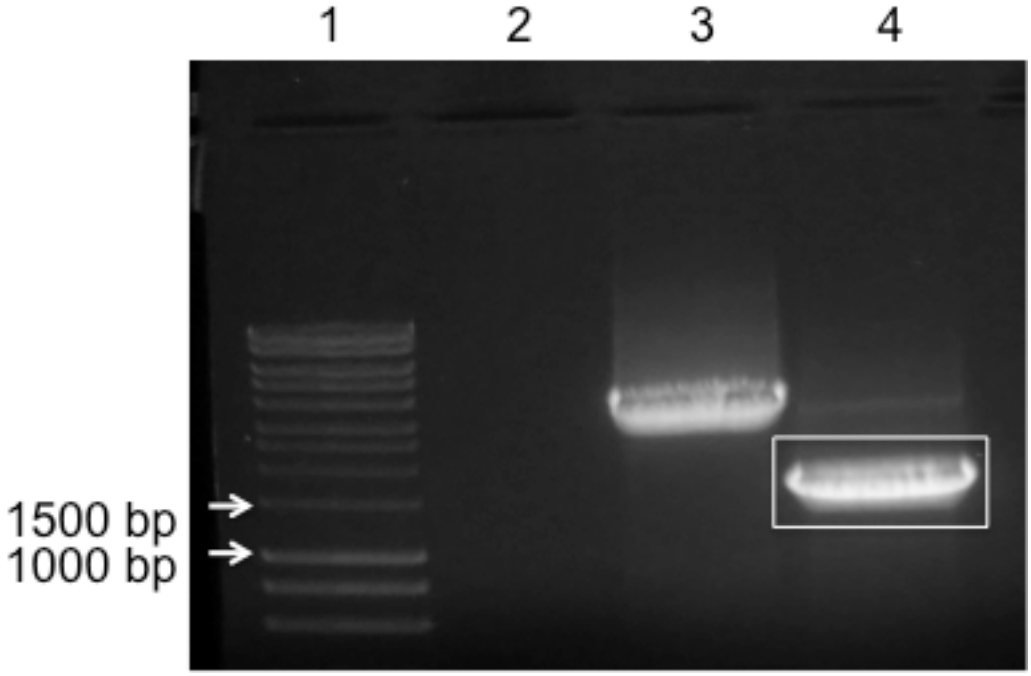

1
2
3
4
1500 bp
1000 bp

Figure 19. Schematic representation of the pET32xT-AS01 plasmid. The AS01 peptide is shown in green. The peptide is tagged with 2 6xHis tags: one at the 5' end of the insert and one at the 3' end.

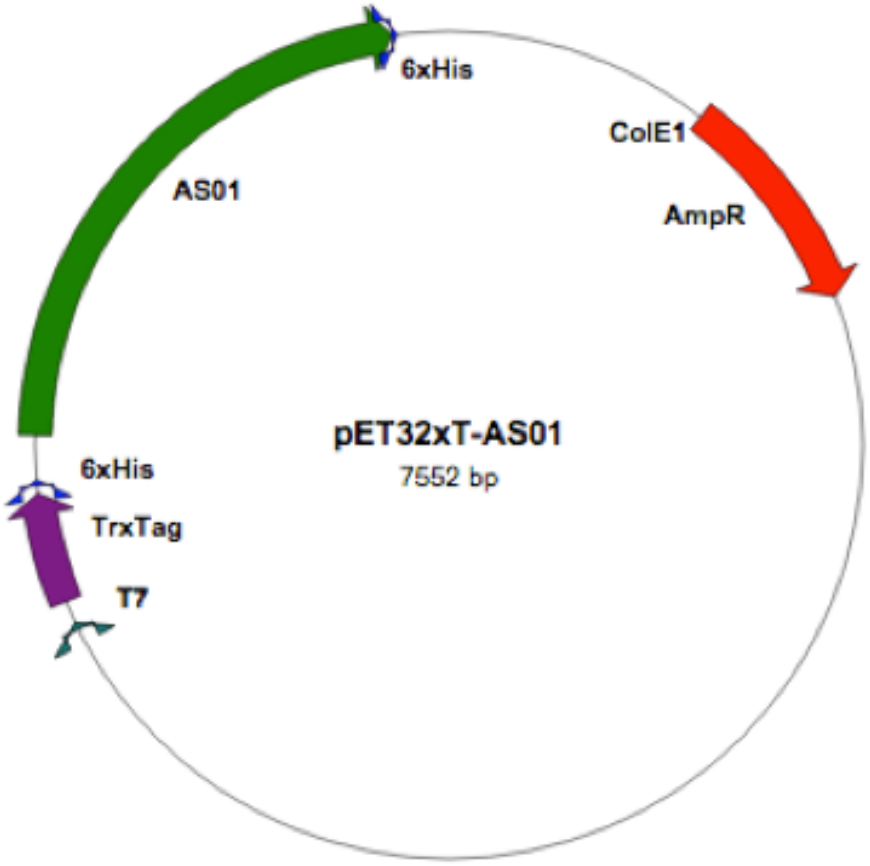

6xHis
ColE1
AS01
AmpR
pET32xT-AS01
7552 bp
6xHis
TrxTag
T7

Figure 20. Restriction digest confirmation of pET32xT-AS01 candidates in chemically competent *E. coli* cells. Lane 1 contains Hyperladder 1kb, Lane 2 is empty, and Lanes 3-11 are candidates digested with XbaI. All candidates (except the one in Lane 8) exhibit the expected banding pattern (6547 bp and 1005 bp). Candidate 1 (Lane 3) was used in this study.

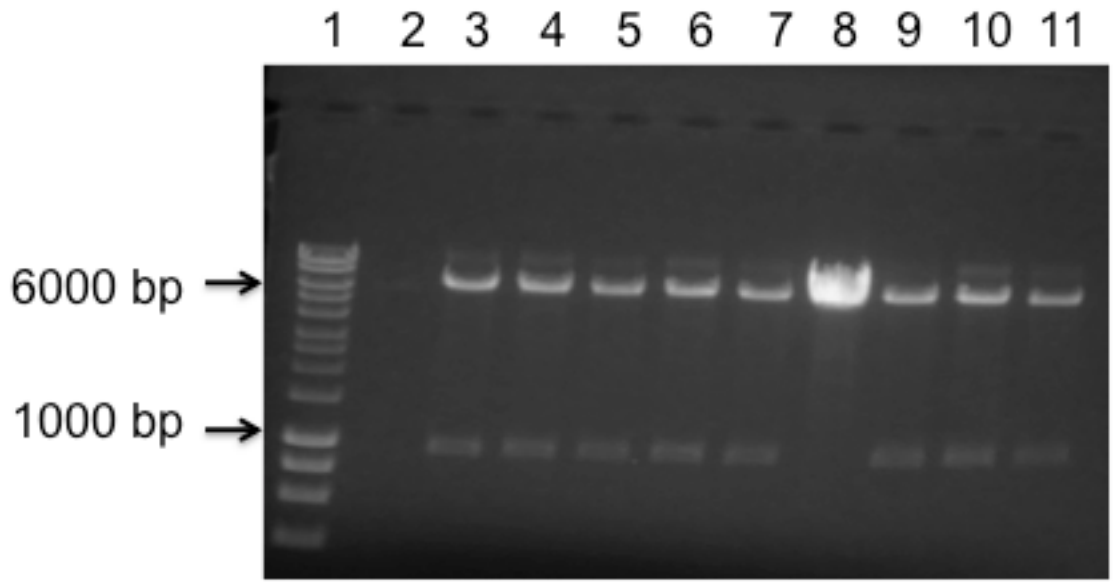
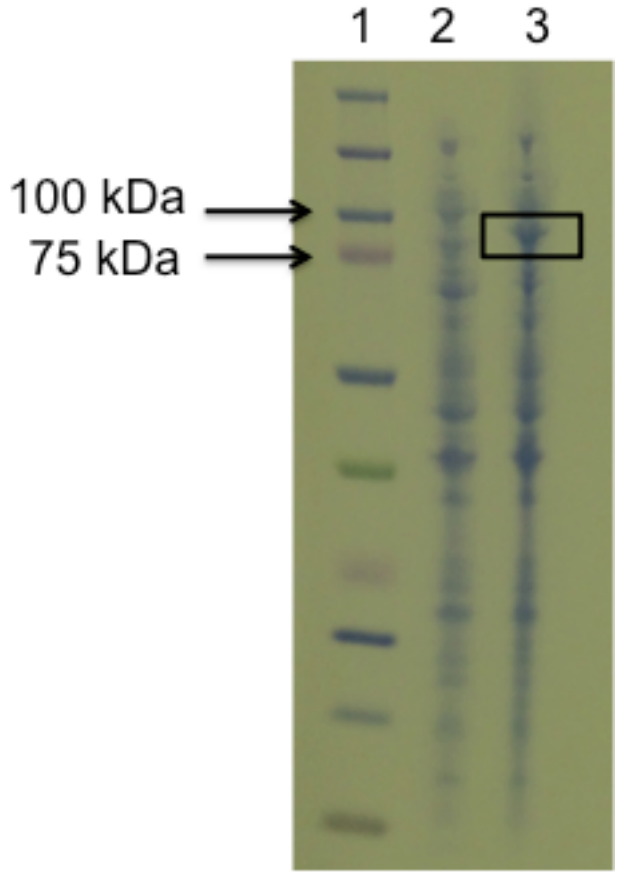

1 2 3 4 5 6 7 8 9 10 11
6000 bp
1000 bp

Figure 21. Confirmation of induced AS01 with 1mM IPTG at 30°C. An SDS-PAGE gel was Imperial-stained and used to confirm the presence of AS01 after IPTG induction (Lane 3, boxed band). Lane 1 contains protein ladder. Lane 2 contains a pre-induced culture sample.


1
2
3
100 kDa
75 kDa

was purified via column purification utilizing cobalt resin (**Figure 22**). After dialysis, the purified peptide was sent to New England Peptides for polyclonal antiserum generation.

C. Characterizing the Expression of TvbA in Sn35 Using AS01 Antiserum

AS01 antiserum was first used to identify the localization of TvbA expression in Sn35. To determine whether TvbA is associated with the bacterial cell surface or secreted into bacterial media, an overnight culture of Sn35 was centrifuged and separated. Fractions of cell pellets and cell supernatants were subjected to SDS-PAGE and Western analysis as previously described. TvbA was detected in the cell pellet fraction, as depicted by **Figure 23,** suggesting that the protein is not secreted into bacterial media. According to the genome sequence of Sn35, TvbA should be 184.55 kDa in size. However, the bands detected appear to be much bigger than that. It's possible that TvbA is modified in some way; it's also possible that the protein is dimerizing.

To further characterize the expression of TvbA in a temporal context, samples of an actively growing Sn35 culture were collected and subjected to SDS-PAGE and Western analysis. TvbA appears to be expressed throughout exponential, late exponential, and stationary phases (**Figure 24**). Again, the protein is detected at a size above 250 kDa. However, a smaller band that appears to sit at ~180 kDa appears over time. This could further support the hypothesis that TvbA is dimerizing.

Figure 22. Purification of recombinant AS01. Fractions obtained from cobalt resin column purification were run on an SDS-PAGE gel and Imperial-stained. Lane 1 contains protein ladder. Lane 2 contains a sample of culture lysate. Lane 3 contains flow through after initial application of culture lysate to the resin, and Lane 4 contains a single wash with denaturing binding buffer. Lanes 5 and 6 contain the first and last washes with denaturing wash buffer. Lane 7 contains one wash with denaturing elution buffer containing no imidazole. Lanes 8 and 9 contain the eluted AS01 peptide with denaturing elution buffer and 500mM imidazole (boxed), and Lane 10 indicates cobalt resin remaining in the column.

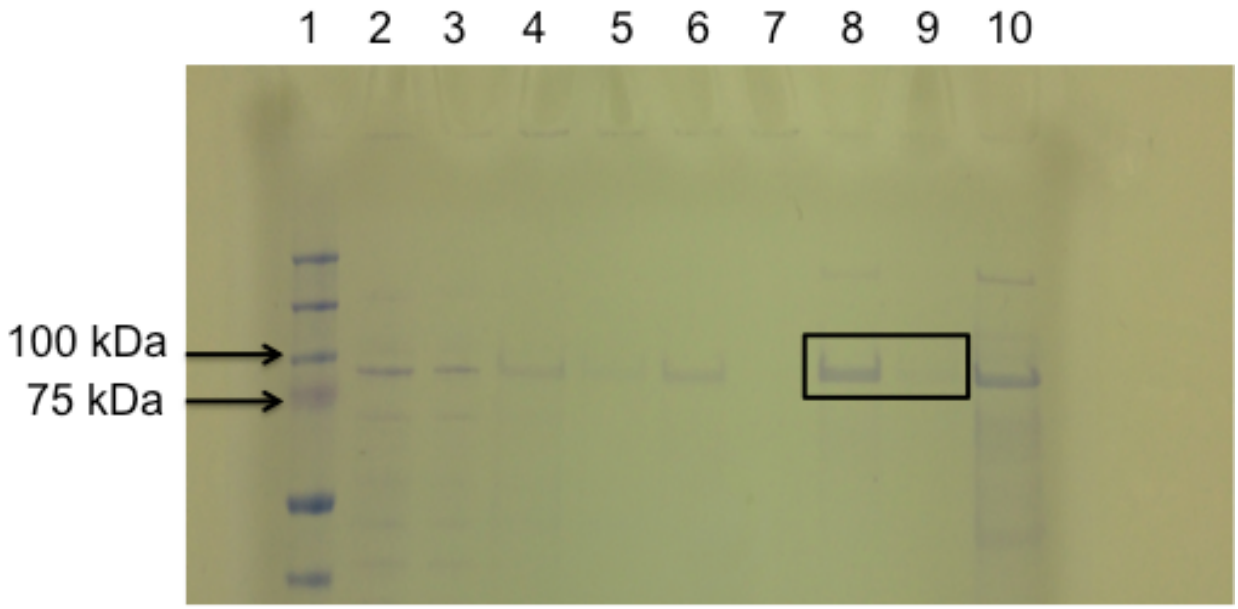

1 2 3 4 5 6 7 8 9 10
100 kDa
75 kDa

Figure 23. Characterization of the subcellular location of TvbA in Sn35. Western analysis using TvbA antiserum demonstrated that TvbA is localized in the cell pellet (Lane 3) and outer membrane proteins (Lane 4), as indicated by the boxed bands. Lane 2 shows concentrated supernatant; TvbA was not present in that fraction. Lane 1 contains protein ladder.

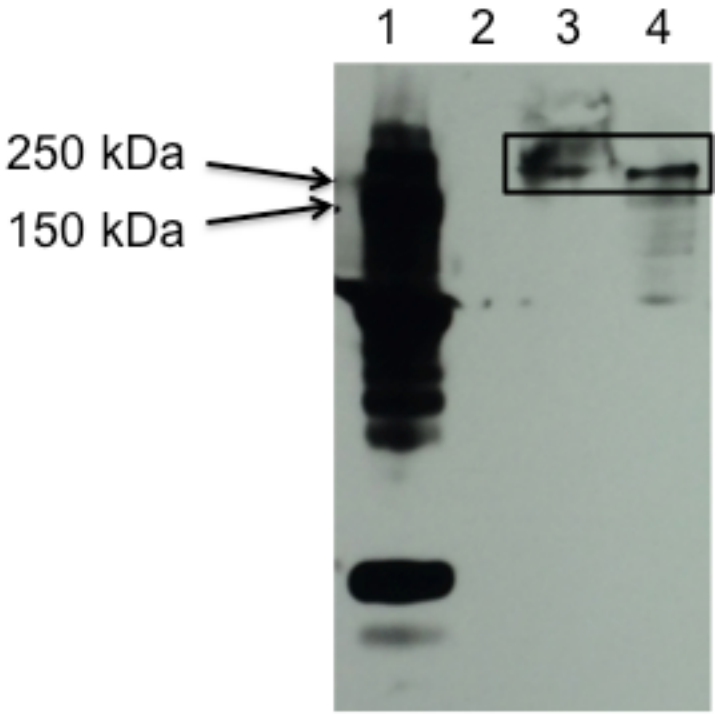
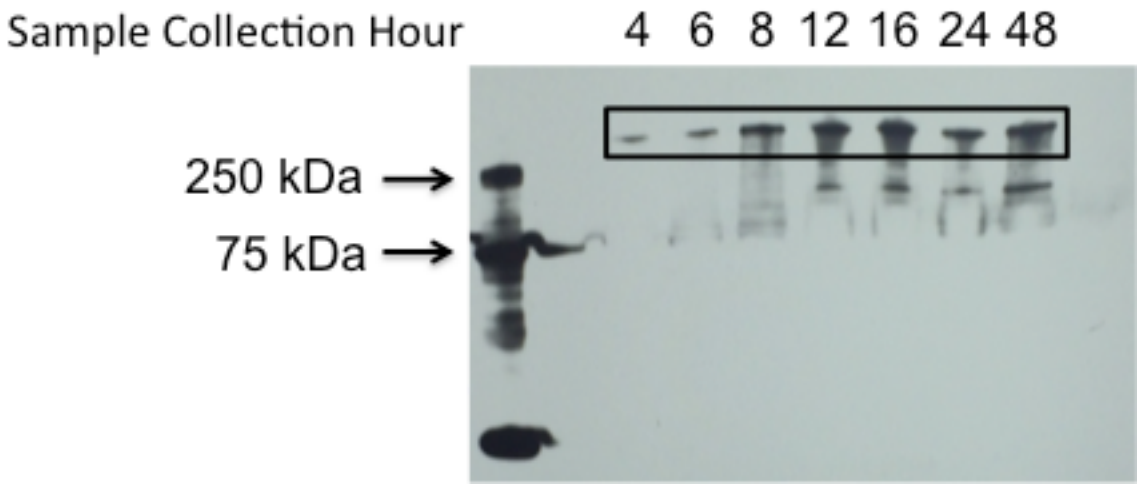

1
2
3
4
250 kDa
150 kDa

Figure 24. Temporal expression of TvbA by Sn35. TvbA is expressed through exponential, late exponential, and stationary phase. TvbA appears to be detected above 250 kDa, as indicated by the boxed bands. Lane 1 contains protein ladder.


Sample Collection Hour
4 6 8 12 16 24 48
250 kDa
75 kDa

D. Inhibiting Cytotoxicity, Adherence, and/or Invasion Using AS01 Antiserum

Following successful detection of TvbA using antiserum, inhibition studies of cytotoxicity, adherence, and invasion were performed. Trypan blue assays were performed on the JEG-3 cell line to determine the inhibition effects of antiserum. Sn35 incubated with a 1:100 dilution of antiserum was still able to induce cell damage and/or death to the host cells (**Figure 25**). Incubation with pre-immunized serum also had no effect on cytotoxicity (data not shown). Assays were repeated with antiserum dilutions of 1:1000 and similar results were observed.

Furthermore, confocal assays were performed on the VK2 cell line to determine if antiserum blocked the adherence and/or invasion of host cells. Sn35 incubated with a 1:100 dilution of antiserum was still able to invade the VK2 cells, as shown in **Figure 26**. Again, incubation with pre-immunized serum had no effect on invasion (data not shown). Assays were repeated with antiserum dilutions of 1:1000, and similar results were observed.

Figure 25. Inhibition of Sn35 cytotoxicity to JEG-3 cells. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria were standardized to an OD_{600nm} = 0.1, and incubated with a 1:100 dilution of antiserum for 30 minutes at 37C. Bacteria were added to JEG-3 cells for 4 hours. Cells were washed after incubation, and trypan blue was added for 4 minutes to determine cell viability. Light microscopy images were taken after the trypan blue incubation. This experiment was repeated with a 1:1000 dilution of antiserum, with similar results.

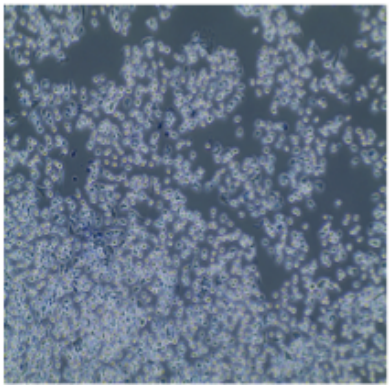
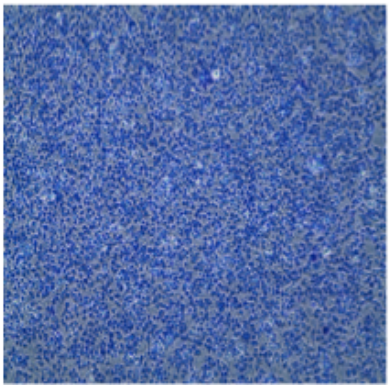
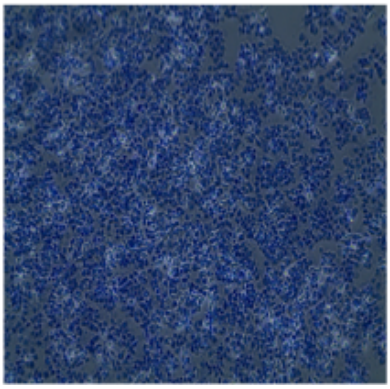
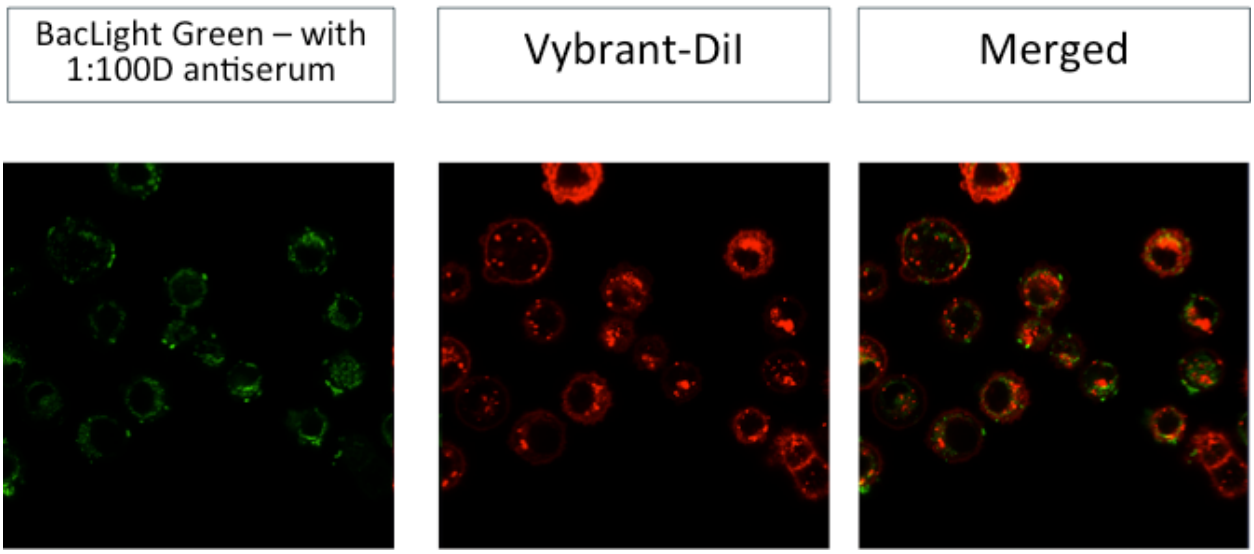
Control Cells	1:100D Antiserum	Sn35
		

Figure 26. Inhibition of Sn35 adherence to VK2 cells. Bacteria were grown in SBHI + HS anaerobically overnight. Bacteria (green) were standardized to an OD_{600nm} = 0.150, and incubated with a 1:100 dilution of antiserum for 30 minutes at 37C. Bacteria were added to VK2 cells (red) for 15 minutes. Cells were washed after bacterial incubation to remove any nonadherent bacteria. Adherence was observed by confocal microscopy. This experiment was repeated with a 1:1000 dilution of antiserum, with similar results.


BacLight Green – with 1:100D antiserum
Vybrant-DiI
Merged

Discussion

Although *Sneathia* has been implicated in a number of obstetric complications and is one of the most common bacterial species isolated from amniotic fluid, the specific role of *S. amnii* strain Sn35 in preterm births caused by MIAC is still unclear. Through this study, the pathogenic potential of Sn35 was further characterized by assessing its effects on host cells, as well as its production of a putative virulence determinant.

The first research focus of this study was on the pathogenicity of Sn35 and its effect on various cell lines. It was hypothesized that Sn35 damaged and/or traversed host cell barriers formed during pregnancy, allowing it to invade the amniotic cavity. This hypothesis was confirmed, as our data demonstrates the ability of Sn35 to adhere, invade, and kill host cells.

In the current study, MIAC is modeled using immortalized and primary cell lines. While these models are able to provide some insight on bacterial-host cell interactions, they leave much to be desired. At this time, the specific cell target of Sn35 is unknown. Because fetal membranes are multi-layered and contain a variety of cell types, it's possible that the host cells upon which Sn35 acts were not tested in this study. Primary HAECs were thought to be the best model for this focus; however, isolation and maintenance of these cells was difficult.

Another obstacle to overcome is the translation of studies *in vitro* to infection *in vivo*. The placenta is designed to withstand a growing fetus for 9 months; even in cases of very preterm birth, such as the circumstance under which Sn35 was isolated, membranes have been in place for several weeks. Currently, it is not known how long it takes for Sn35 to access the amniotic cavity *in vivo*. We assume that this process is gradual, and most likely doesn't occur in the short time periods that we have tested *in vitro*. Relevant studies have utilized mice, rabbit, and sheep models to study preterm birth and therapeutic targets aimed at improving neonatal outcome[66]. The ideal animal model for human pregnancy is the nonhuman primate, as their placentation and hormonal events occurring during pregnancy most closely resemble those of humans[66]. While animal models were not used for this study, further characterization of Sn35 in the context of preterm birth would benefit from their use.

The second objective concentrated on the identification of putative virulence determinants that could cause the observed pathogenic effects seen with Sn35-treated host cells. It was hypothesized that some virulence factor must be produced in order for cytopathogenicity to occur. Bioinformatic analyses identified a protein within Sn35 that had homology to haemagluttinin domains/repeats and adhesins. A putative Type Vb two-partner secretion system was identified as being the possible mechanism by which the putative virulence factor is secreted; a putative transporter gene was identified directly upstream of the putative virulence gene, indicating that the two genes are components of a two-partner secretion system.

The third objective focused on characterizing the function of the putative virulence factor. It was hypothesized that the putative virulence factor was acting as a

cytotoxin or adhesin. At this time, this hypothesis has yet to be confirmed, as antiserum-treated bacteria were still able to invade host cells and induce cell damage and/or death. This could be due to a number of reasons; one plausible explanation is that TvbA is acting as neither a cytotoxin nor an adhesin. According to bioinformatic analyses, this is not likely, as the majority of known protein sequences that produce significant alignment with TvbA are adhesins or toxins.

A second explanation is that the polyclonal antiserum is not specific for many of the epitopes of native TvbA. The AS01 fragment used for polyclonal antiserum production may have a different conformation compared to native TvbA; this would result in the generation of antibodies specific to epitopes found on the fragment, but not the native protein. One additional explanation is that the antibodies specific for native TvbA epitopes could be blocked from binding due to steric hindrance. It's possible that the antibodies within the antiserum are too bulky to bind to their specific epitopes. Additional experiments with the polyclonal antiserum, to include antibody fragmentation, will need to be performed to confirm or disprove this hypothesis.

Surprisingly, native TvbA was detected at ~250 kDa or above in all Western analyses. Currently, the genome sequence of Sn35 places the protein size at ~180 kDa. It's possible that several repeats exist within the TvbA sequence, and misassemblies during genome sequencing caused the collapsing of these repeats. Post-translational modification, such as glycosylation or phosphorylation, would also explain the increase in protein size. An increase of ~60 kDa or higher, as observed in our analyses, could indicate modifications at multiple amino acids. Finally, TvbA could be forming a dimer.

This would explain the presence of smaller bands (~180 kDa) seen during temporal expression studies.

Collectively, the results of this study signify the need for further research into *S. amnii* strain Sn35 and the role it plays in preterm birth. Although Sn35 is not solely responsible for eliciting MIAC and PPROM, there is no doubt that it possesses the pathogenicity potential to contribute to these obstetric complications. The presence of Sn35 in vaginal and AF samples acquired from preterm births makes the organism one worth studying. While the findings presented in this work have further built upon the current understanding of Sn35, they have only scratched the surface. Therefore, having a better understanding of this organism will, in turn, result in a more complete knowledge of how MIAC, PPROM, and, ultimately, preterm birth develop.

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Abstracts and Presentations:

A. Sanford, M. Harwich, G. Buck, the Vaginal Microbiome Consortium, K. Jefferson. Characterization of hypothetical virulence factors in *Sneathia amnii* that may be involved in preterm birth. 115th General Meeting of the American Society for Microbiology, New Orleans, LA. 2015. (Poster)

A. Sanford, M. Harwich, the Vaginal Microbiome Consortium, K. Jefferson. Characterization of a putative virulence factor in *Sneathia amnii.* 10th Annual Women's Health Research Day, Richmond, VA. (Poster)