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Characterization of adherence, cytotoxicity and biofilm formation by Gardnerella vaginalis

Jennifer Patterson
Virginia Commonwealth University

School of Medicine
Virginia Commonwealth University

This is to certify that the dissertation prepared by Jennifer Louise Patterson entitled "CHARACTERIZATION OFADHERENCE, CYTOTOXICITY AND BIOFILM FORMATION BY *GARDNERELLA VAGINALIS*" has been approved by his or her committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy

Kimberly K. Jefferson, Ph.D., Director of Dissertation, School of Medicine

Philippe H. Girerd, M.D., School of Medicine

Todd O. Kitten, Ph.D., School of Medicine

Cynthia N. Cornelissen, Ph.D., School of Medicine

Dennis E. Ohman, Ph.D., Chair, Department of Microbiology and Immunology

Jerome F. Strauss, III, M.D., Ph.D., Dean, School of Medicine

F. Douglas Boudinot, Ph.D., Dean of the Graduate School

Date

CHARACTERIZATION OF ADHERENCE, CYTOTOXICITY AND BIOFILM

FORMATION BY *GARDNERELLA VAGINALIS*

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

JENNIFER LOUISE PATTERSON
B.S., Concord University, 2005

Director: Kimberly K. Jefferson, Ph.D.
Assistant Professor, Department of Microbiology and Immunology
Virginia Commonwealth University
Richmond, Virginia

Virginia Commonwealth University
Richmond, Virginia
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Dedication

I would like to thank my parents, Bill and Cindy Patterson, for instilling in me a love of science. I would like to thank my sister, Jasmine, for always being there for me and fostering a competitive streak that pushed me beyond my boundaries. Finally, I would like to thank the rest of my family and friends, especially my grandmothers Joan Zeiter and Ramona Patterson and family friend Evan Clyburn, for always believing in me. Without all of you, I would not be who I am today.

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Abbreviations

µg/ml ..... microgram/milliliter
µl ..... microliter
ATCC ..... American Type Tissue Collection
ATP ..... adenosine triphosphate
BAP ..... biofilm-associated protein
BapL ..... Bap-Like
BHI ..... brain heart infusion media
BHIG ..... brain heart infusion media + 1% glucose
bp ..... base pair
BPS ..... bromocresol purple starch agar
BSA ..... bovine serum albumin
BV ..... bacterial vaginosis
CBD ..... chitin-binding domain
CDC ..... Center's for Disease Control and Prevention
cDNA ..... complementary DNA
CDM ..... chemically defined medium
CFU ..... colony forming units
CO_2 ..... carbon dioxide
ConA ..... Concanavalin A
CVL ..... cervicovaginal lavage
DIC ..... differential interference contrast microscopy
DNA ..... deoxyribonucleic acid
DspB ..... Dispersin B
DTT ..... dithiothreitol
ECM ..... extracellular matrix proteins
eDNA ..... extracellular DNA
EDTA ..... ethylenediaminetetraacetic acid
EMS ..... ethyl methanesulfonate
FACS ..... fluorescence activated cell sorting
FEP ..... functional equivalent pathogens/pathogroups
Gly ..... Glycine Max
GV ..... *Gardnerella vaginalis*
H_2O_2 ..... hydrogen peroxide
HIV ..... Human immunodeficiency virus
HRP ..... horseradish peroxide
ica ..... intercellular adhesion locus
IL-1 α/β ..... Interleukin-1 alpha/beta
IL-8 ..... Interleukin-8
IPTG ..... Isopropyl β-D-1-thiogalactopyranoside

LB ..... Luria-Bertani broth
M ..... molar
MB ..... megabase
ME180 ..... cervical epithelial cell line
mg ..... milligram
MH ..... Mueller-Hinton broth
MIC ..... minimal inhibitory concentration
ml ..... milliliter
mM ..... millimolar
mRNA ..... messenger RNA
MRS ..... Man, Rogosa and Sharpe broth
MSCRAMM ..... microbial surface components recognizing adhesive matrix molecules
MW ..... molecular weight
MWCO ..... molecular weight cut-off
NaCl ..... sodium chloride
nm ..... nanometer
OD ..... optical density
ORF ..... open reading frame
PBS ..... phosphate buffered saline
PCR ..... polymerase chain reaction
pH ..... potentiometric hydrogen ion concentration
PNAG ..... poly-N-acetylglucosamine
RLU ..... relative light units
RNA ..... Ribonucleic acid
sBHI ..... supplemented brain heart infusion media
sBHIG ..... supplemented brain heart infusion media + 1% glucose
sBHIs ..... supplemented brain heart infusion media + 1% glucose and 8% human serum
SDS ..... sodium dodecyl sulfate
STD ..... sexually transmitted disease
TAE ..... Tris-acetate-EDTA buffer
TetM ..... Tetracycline resistance M determinant
TLR-4 ..... Toll-like receptor 4
TNF α ..... Tumor necrosis factor-alpha
TSB ..... Tryptic Soy broth
VEC ..... vaginal epithelial cell
VCU ..... Virginia Commonwealth University
WBC ..... white blood cell
WGA ..... Wheat germ agglutinin

Abstract

CHARACTERIZATION OF ADHERENCE, CYTOTOXICITY AND BIOFILM FORMATION BY *GARDNERELLA VAGINALIS*

By: Jennifer Louise Patterson, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Major Director: Kimberly Jefferson, Ph.D.
Assistant Professor, Department of Microbiology and Immunology

Worldwide, bacterial vaginosis (BV) is the most common vaginal disorder in women of childbearing age. BV is of major clinical importance due to its ability to significantly affect pregnancy outcome and enhance the transmission and acquisition of HIV. BV is characterized by a dramatic shift in the vaginal microflora; in most BV cases, the predominant bacterial species is *Gardnerella vaginalis*. It has been demonstrated that *G. vaginalis* forms an adherent biofilm on the vaginal epithelium of women with BV. Furthemore, evidence suggests that the high rate of recurrence associated with BV is

related to incomplete eradication of the biofilm. The overall goal of this study was to characterize *G. vaginalis* virulence properties, including biofilm formation, in order to better understand the pathogenesis of BV and to improve available treatment methods.

In an effort to tease apart the uncertain etiology of this disorder, we utilized in vitro assays to compare three virulence properties of *G. vaginalis* relative to other BV-associated anaerobes. Only *G. vaginalis* demonstrated all three virulence properties, including robust biofilm formation. It has been shown that the biofilm phenotype allows its constituent bacteria to be resistant to many negative environmental stimuli. Therefore, we studied the susceptibilities of biofilm vs. planktonic cultures to H_2O_2 and lactic acid. Biofilms tolerated higher concentrations of both chemicals; however, when the biofilm was proteolytically disrupted, sensitivity to the chemicals returned to planktonic levels.

Since our data suggested a critical role for a protein in biofilm formation, a partial genome sequence of *G. vaginalis* was searched for sequence homology to known biofilm adhesins using the tBLASTn program. This revealed an open-reading frame encoding a hypothetical protein with significant homology to the staphylococcal Bap protein. Antibody towards a portion of the identified gene product was produced in rabbits by inoculation of a recombinant peptide to an antigenic region of the protein. Antibody inhibition assays against biofilm formation, adherence, initial adherence and aggregation were conducted. Relative expression levels of the biofilm-associated protein were analyzed under different conditions by western blot analysis. Finally, the protein was expressed in heterologous hosts and analyzed for an increase in biofilm formation.

CHAPTER 1

Introduction

Clinical overview

The human vagina is a complex environment, composed of stratified squamous nonkeratinized epithelium and its native bacteria. In normal, healthy women, the vagina is populated mainly by hydrogen peroxide producing lactobacilli that inhibit the growth of other vaginal flora by maintaining the vaginal acidity, competing with other bacterial species for space and nutrients, and producing bacteriocins (Eschenbach 1993; Boskey, Cone et al. 2001; Atassi, Brassart et al. 2006). The mild acidity of the healthy vagina has been shown to correlate with a decreased risk of trichomoniasis, urinary tract infection and infection with genital mycoplasma (Stamey and Timothy 1975; Hanna, Taylor-Robinson et al. 1985). One of the hallmarks of bacterial vaginosis (BV) is a reduction in vaginal acidity.

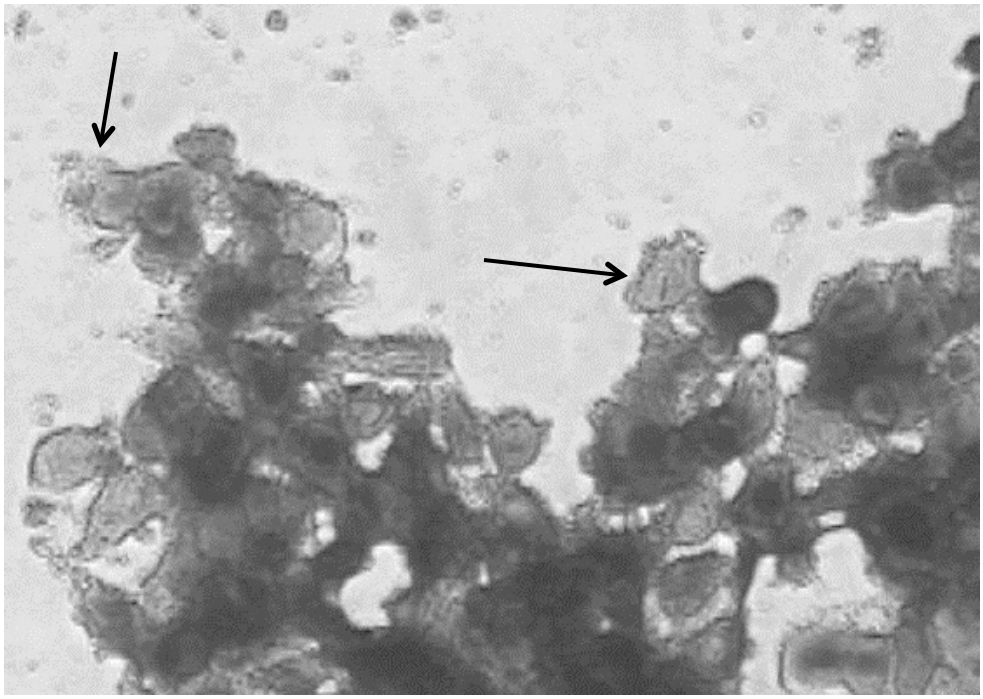
BV is the most prevalent vaginal disorder in women of reproductive age, affecting 10 to 20% of Caucasian women and 30 to 50% of African American women, although, estimates of its prevalence depend on the population studied (Eschenbach 1993; Sobel 2000). BV is frequently observed in women attending sexually transmitted disease (STD) clinics, where the percentage reaches as high as 40%; however, the disease can occur in the absence of sexual intercourse (Sobel 2000). 16-29% of pregnant women are

BV positive, although, it is more often detected in women attending infertility clinics (Sobel 2000).

BV is characterized by a loss of protective lactobacilli, an increase in the vaginal pH to >4.5, and the proliferation of a variety of anaerobic species. An increase in the prevalence and concentration of *Gardnerella vaginalis*, *Mycoplasma hominis*, *Atopobium vaginae*, *Prevotella* sp., and numerous other gram-positive rods, is characteristic of BV (Sobel 2000; Fredricks, Fiedler et al. 2005; Fredricks, Fiedler et al. 2007; Witkin, Linhares et al. 2007; Oakley, Fiedler et al. 2008). Risk factors for BV include sexual activity and number of sexual partners, use of intrauterine devices, douching, antibiotic treatment and infection with sexually transmitted diseases (Sobel 2000).

Symptoms of BV can include a thin, grayish-white vaginal discharge with a foul odor and mild irritation, absence of inflammatory signs and increased vaginal pH; however, many women with BV are asymptomatic (Sobel 2000). The massive overgrowth of anaerobes associated with BV leads to an increased production of proteolytic enzymes, which in turn break down vaginal peptides into amines that are volatile and malodorous. These amines are associated with the foul odor of BV and squamous epithelial cell exfoliation, creating the typical discharge abundant with clue cells. Clue cells, which are seen in **Figure 1**, are vaginal epithelial cells that are coated by bacteria and are pathognomonic for BV (Eschenbach, Hillier et al. 1988). BV is primarily diagnosed by the Amsel criteria, which includes: presence of a thin, white discharge, positive whiff test with addition of 10% potassium hydroxide, an elevated vaginal pH, and the presence of clue cells (Amsel, Totten et al. 1983).

Figure 1. Photomicrograph of a clue cell. ME180 vaginal epithelial cells were allowed to interact with a *G. vaginalis* overnight culture for 10 minutes. The bacteria and cells were placed on a microscopy slide, allowed to dry, flamed, then gram stained. Arrows indicate a rough edge of the VECs where bacteria is attached. This image is representative of what a clue cell from a patient with BV would look like. (magnification 40X)



BV has been consistently associated with adverse pregnancy outcomes such as preterm delivery, low birth weight, postpartum endometritis, as well as other gynecologic complications such as pelvic inflammatory disease and vaginal cuff cellulitis (Gravett, Hummel et al. 1986; Soper, Bump et al. 1990; Sobel 2000; Larsson, Bergstrom et al. 2005; Denney and Culhane 2009; Nelson, Hanlon et al. 2009). Studies by Oleen-Burkey et al. and Hillier et al. have detected increased risks of preterm birth ranging from 2.0 to 6.9 odds ratio directly attributable to BV (Hillier, Nugent et al. 1995; Oleen-Burkey and Hillier 1995); however, patients at high risk for preterm delivery who were treated with oral metronidazole, all showed considerable reduction in the incidence of preterm labor associated with BV (Morales, Schorr et al. 1994; Hauth, Goldenberg et al. 1995; McGregor, French et al. 1995; Camargo, Simoes et al. 2005). Ten trials that evaluated the use of metronidazole for routine operative prophylaxis before abortion and hysterectomy demonstrated a substantial reduction in postoperative infectious complications (Jackson, Ridley et al. 1979; Soper, Bump et al. 1990; Watts, Krohn et al. 1990; Larsson, Platz-Christensen et al. 1991). Because of the increased risk of postoperative infectious complications associated with BV, some specialists suggest screening for BV and treating women before reproductive tract surgeries.

Importantly, BV has been linked to increased acquisition and transmission of HIV (Cohen, Duerr et al. 1995; Sewankambo, Gray et al. 1997; Kapiga, Sam et al. 2007). In a paper by Kapiga et al., they demonstrate a 2-fold higher risk of HIV-1 in women with BV than compared to women with normal flora (Kapiga, Sam et al. 2007). Levels of HIV RNA in female genital mucosal fluid are also increased in women with BV, with women

with the lowest numbers of lactobacilli having 15.8 fold higher endocervical HIV-1 RNA than women with normal lactobacilli levels (Coleman, Hitti et al. 2007). Normal, healthy women with no vaginal disruptions have a low susceptibility to HIV infection because they lack suitable HIV-1 host cells; however, BV may increase a women's risk of HIV acquisition by causing leukocytosis and recruitment of potential HIV-sensitive host cells into the vagina (Anderson and Hill 1991). Many BV-associated bacterial pathogens produce sialidases and other mucin-degrading enzymes that alter the epithelial layer thus weakening the mucosal barrier and increasing the transmission of HIV from infected male partners (Larsson, Bergstrom et al. 2005). Perhaps most importantly, there is a decrease in the number of hydrogen-peroxide producing lactobacilli found in the vagina of women with BV. Hydrogen peroxide is not only toxic to a number of microorganisms, but capable of inactivating viruses such as HIV as well (Klebanoff and Coombs 1991). A reduction in the amount of hydrogen peroxide in the vagina also leads to an increase in vaginal pH and activation of CD4 lymphocytes, or HIV-target cells (Schmid, Markowitz et al. 2000).

The most successful oral therapy for BV remains metronidazole, although clindamycin is also used. The beneficial effect of metronidazole results from its activity against anaerobic bacteria and the susceptibility of *G. vaginalis* to the hydroxymetabolites of the antibiotic. The Centers for Disease Control and Prevention (CDC) recommends 500 mg of metronidazole orally twice a day for 7 days. Topical therapy with 2% clindamycin gel once daily for 7 days or 0.75% metronidazole gel once daily for 5 days was shown to be as effective as oral metronidazole (Ferris, Litaker et al.

1995). Antibiotic therapy can fail in up to 60% of cases (Anukam, Osazuwa et al. 2006) and even when effective, BV frequently recurs, with rates as high as 20% within one month and 80% within 9 months (Hillier and Holmes 1990; Hay 1998; Beigi, Austin et al. 2004).

Although BV is not considered an inflammatory disorder, several cytokines are upregulated. The abnormal vaginal microbiota associated with BV has been shown to increase cytokines like IL-1α/β and IL-8 (Platz-Christensen, Mattsby-Baltzer et al. 1993; Wennerholm, Holm et al. 1998). Cervicovaginal lavage samples (CVL) from women with BV were able to induce expression of TNF-α secretion by primary peripheral-blood mononuclear cells and monocytic cell lines. The CVL samples from women with BV also increased expression of Toll-like Receptor 4 (TLR-4) mRNA expression up to 60-fold in cell lines; this suggests a new mechanism whereby BV can affect HIV transmission (Zariffard, Novak et al. 2005). It has been seen that stimulation through TLR4 is able to increase HIV expression by infected cells (Equils, Faure et al. 2001; Bafica, Scanga et al. 2004); the paper by Zariffard et al. suggests that HIV-infected cells present in the genital tract of women with BV may be directly stimulated through TLR4 to express increased levels of HIV (Zariffard, Novak et al. 2005).

Not only does an altered microbiota and presence of BV lead to changes in the cytokine profile in the vagina, but it also affects the presence of vaginal hydrolytic enzymes and certain immunoglobulins found in the vagina. A paper by Cauci et al. showed that pregnant women with BV have higher concentrations of sialidase and prolidase, two microbial enzymes that affect the vaginal epithelium integrity (Cauci, Hitti

et al. 2002). In addition, pregnant women with BV have lower antibodies to the vaginolysin toxin produced by *G. vaginalis* than women with normal flora (Cauci, Hitti et al. 2002). The presence of higher than normal sialidase and lower amounts of anti-vaginolysin antibodies could potentially hamper the local mucosal immune response by degrading glycoproteins such as antibodies, or by failing to adequately bind circulating toxin (Pilatte, Bignon et al. 1993).

Although BV is characterized by low levels of lactobacilli and increased numbers of anaerobic bacteria, the etiology of BV is poorly understood with no single causative agent isolated. *Gardnerella vaginalis*, however, has been found to be present in up to 95% of cases and is found only rarely and in low numbers in healthy women (Gardner and Dukes 1955). To date, BV is still considered a polymicrobial disorder and the disruption of normal vaginal flora is not disputable; however, a critical question that relates to the sequence of the disruption is whether the loss of protective lactobacilli and an increase in vaginal pH must precede the overgrowth of anaerobic bacteria or whether anaerobes such as *G. vaginalis* colonize the vagina leading to a decrease in lactobacilli and increased pH.

Gardnerella vaginalis

G. vaginalis cells are small, pleomorphic rods, which were once classified as *Haemophilus* sp. by Gardner and Dukes because of their ability to grow on chocolate agar (Gardner and Dukes 1955; Catlin 1992). The tendency to retain the crystal violet dye in the Gram reaction and some other corynebacterium-like features, suggested that

the organism might be better associated with the genus *Corynebacterium*, hence it was referred to *C. vaginale* in later years (Zinnemann and Turner 1963). Finally, after two large taxonomic studies were published in 1980 which analyzed DNA-DNA hybridization and electron microscopy data, the bacteria were classified into a new genus and called *Gardnerella* (Greenwood 1983; Piot and Van Dyck 1983).

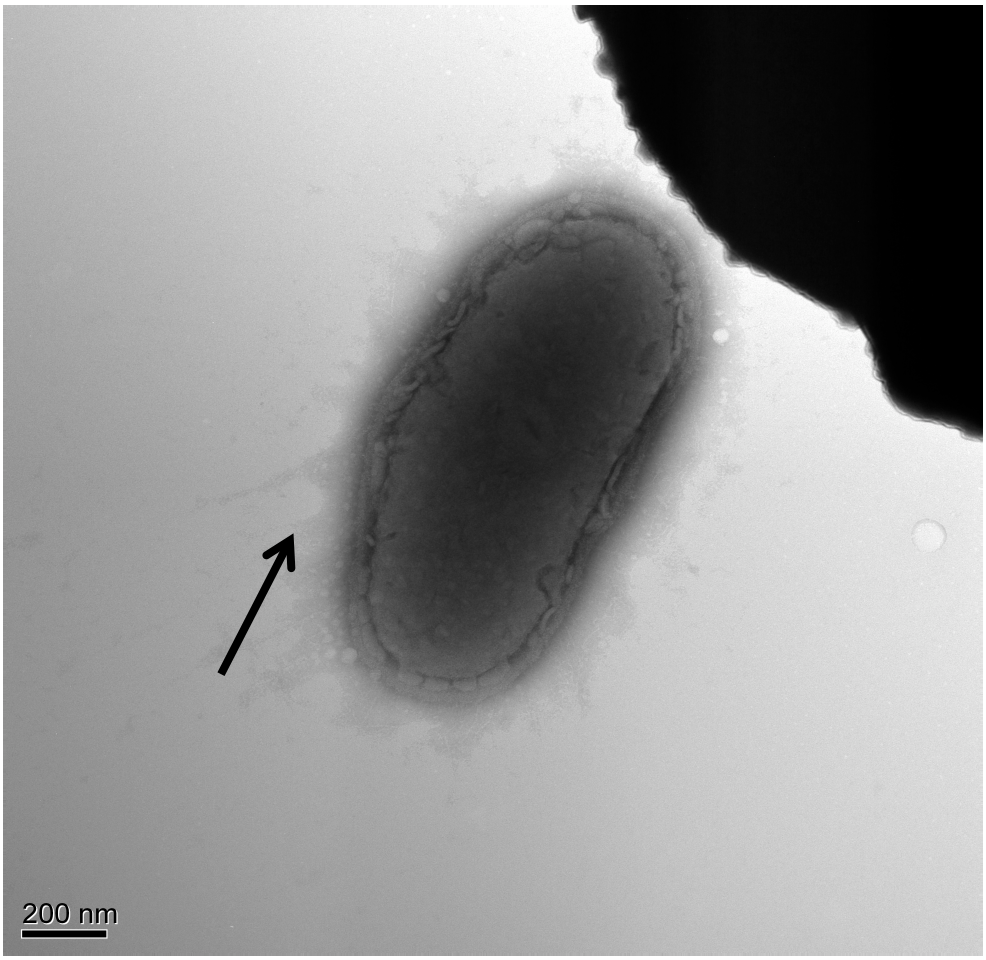
G. vaginalis cells are nonmotile and do not possess flagella, catalase, endospores or typical capsules (Catlin 1992). Pyrosequencing of a strain isolated from a woman without signs of BV (*G. vaginalis* 5-1) found that the genome size was approximately 1.65 MB. Electron microscopy by Reyn et al. proved that *G. vaginalis* has a gram-positive cell wall (Reyn, Birch-Andersen et al. 1966); however, the physiological state of the bacteria affects their morphology and staining reactions (personal observation). The bacterial cells have an angular or palisade arrangement of cells and volutin granules form in *G. vaginalis* during growth in the presence of a fermentable compound, leading to polar granule observation during gram staining (Edmunds 1960). *G. vaginalis* is beta-hemolytic on media containing human or rabbit blood but not on sheep blood agar; this hemolysis is improved in anaerobic incubation (Edmunds 1960; Shaw, Forsyth et al. 1981; Greenwood 1983; Lam and Birch 1991). *G. vaginalis* produces a pore-forming cytolysin, called vaginolysin, which is specific to mammalian epithelial cells and has been hypothesized to be involved in the pathogenesis of BV (Gelber, Aguilar et al. 2008).

The most successful media for isolation of *G. vaginalis* possesses both differential and selective features. The addition of human blood allows identification of beta-hemolytic *G. vaginalis* from nonhemolytic colonies, semiselective medium containing

colistin or nalidixic acid, to which *G. vaginalis* is resistant, allows for identification of resistant vs. sensitive bacteria, and addition of 1% corn starch allows for identification of *G. vaginalis* by the hydrolytic clearing of the opaque starch (Goldberg and Washington II. 1976; Mickelsen, McCarthy et al. 1977). We have employed a combination of selective and differential media, containing nalidixic acid (30µg/ml), starch, and the pH indicator bromocresol purple. The plates contain starch and when fermented by *G. vaginalis*, the pH indicator allows for selection of yellow colonies on a purple background. Incubation of *G. vaginalis* at 37ºC for 24-48 hrs in an anaerobic box with 10% CO_2 produces adequate growth of this fastidious organism.

Electron microscopy revealed a fibrillar exterior layer (**Figure 2**) and ruthenium red staining indicates it has a polysaccharide component; however, this component is not capsular (Criswell, Stenback et al. 1972; Greenwood 1983). Electron microscopy has also revealed pili radiating from the surface of *G. vaginalis* (Johnson and Davies 1984; Boustouller, Johnson et al. 1987). The authors noted that clinical isolates displayed more heavily piliated cells and that laboratory strains quickly lost pili upon frequent subculture (Johnson and Davies 1984; Boustouller, Johnson et al. 1987). Both the exopolysaccharide component and pili could be involved in *G. vaginalis* extraordinary ability to adhere to vaginal epithelial cells. It has been shown that *G. vaginalis* cells adhere better to vaginal epithelial cells scraped from the vaginal wall than do *L. acidophilus* cells (Mardh, Holst et al. 1984). Increasing acidity of the test medium also allowed for high adherence of *G. vaginalis* to vaginal epithelial cells (Sobel, Schneider et al. 1981). Pretreatment of *G. vaginalis* with subinhibitory concentrations of

Figure 2. Electron micrograph of *G. vaginalis*. Overnight *G. vaginalis* cultures grown in sBHI with 8% human serum were pelleted and washed with PBS, then negatively stained with phosphotungstic acid. Electron microscopy revealed a fibrillar exterior layer indicated by the arrow. (Electronmicrograph courtesy of Dr. Michael Harwich, VCU)


200 nm

metronidazole was able to significantly reduce the adherence of some of the strains tested (Peeters and Piot 1985). Not only can *G. vaginalis* adhere to epithelial cells, it can hemagglutinate red blood cells; although, it seems that different adhesins are responsible for adherence to the two cell types (Scott and Smyth 1987). Electron microscopy verified that adhesion to red blood cells occurred by means of pili, while adhesion to the epithelial cell line, McCoy, involved the outer fibrillar layer (Scott, Curran et al. 1989).

G. vaginalis is sensitive to ampicillin, which was originally used to treat BV; although, it is now treated with metronidazole or clindamycin, which have shown a higher degree of efficiency. *G. vaginalis* is resistant to low levels of metronidazole; however, it is highly susceptible to the hydroxyl-metabolite of metronidazole (Easmon, Ison et al. 1982). It is highly resistant to colistin, nalidixic acid, and amphotericin and can be resistant to tetracycline if the genome contains the TetM determinant, which is most often associated with a conjugative transposon located in streptococcal species (Huang, Gascoyne-Binzi et al. 1997).

Many animal models have been tested to analyze the role of *G. vaginalis* in BV; however a practical model is still lacking. Intravenous inoculation of mice with viable *G. vaginalis* produced no effects (Boustouller, Johnson et al. 1986). Different primate species were studied in an attempt to develop an animal model of BV infection; pigtailed macaques were colonized but no clue cells were found, tamarins and chimpanzees were not colonized, and grivet monkeys were able to be colonized with *G. vaginalis*, but only when a mixed culture of *G. vaginalis* and *Mobiluncus* were used to infection the monkey (Johnson, Ison et al. 1984; Mardh, Holst et al. 1984). There has also been work to

develop an equine model; *Gardnerella* strains have been detected in the genital flora of mares, the first observation of natural infection in animals (Salmon, Walker et al. 1991).

The significance of *G. vaginalis* in BV is disputed. However, approximately 95% of women suffering from BV are heavily colonized by *G. vaginalis* (Gardner and Dukes 1955; Holst, Svensson et al. 1984; Borchardt, Adly et al. 1989). The finding of a negative *G. vaginalis* culture had a 97% predictive value of exclusion of bacterial vaginosis (Amsel, Totten et al. 1983; Cristiano, Coffetti et al. 1989). *G. vaginalis* has also been detected as the sole infecting species in other infection sites, including the bloodstream, urogenital tract of both men and women, the kidneys, and bone tissue; this points to the pathogenic potential of *G.vaginalis* (Piot, Van Dyck et al. 1984; Reimer and Reller 1984; Reimer 1991; Amaya, Al-Dossary et al. 2002; Graham, Howes et al. 2009).

In the laboratory, *G. vaginalis* is sensitive to phagocytosis and killing by neutrophils in the presence of human serum, in a process mediated by complement activated by the alternative pathway (Easmon, Clark et al. 1985). However, it has been shown by Swidsinski et al. that *G. vaginalis* is able to form an adherent biofilm on the vaginal epithelium of women with BV (Swidsinski, Mendling et al. 2005). The formation of this biofilm may interfere with the immune response to *G. vaginalis*. A follow-up paper observed the resurgence of a dense and active bacterial biofilm on the vaginal mucosa, consisting primarily of *G. vaginalis*, after treatment of BV with metronidazole (Swidsinski, Mendling et al. 2008). They hypothesized that development of an adherent biofilm by *G. vaginalis* contributes to the pathogenesis and recurrence of BV (Swidsinski, Mendling et al. 2005). Little is known about the *G. vaginalis* biofilms;

therefore, we sought to characterize the biofilm, and identify genes involved in formation of the biofilm.

Biofilms

It has recently been recognized that planktonic cell growth does not accurately reflect bacterial growth in nature or in infectious diseases, where most bacteria grow as biofilms (O'Toole, Kaplan et al. 2000; Lindsay and von Holy 2006). Biofilms are communities of microorganisms that are surrounded by an extracellular polymeric matrix composed of polysaccharides, proteins and/or nucleic acids, which promote intercellular adhesion and adhesion to surfaces. Examples of microorganisms that utilize primarily polysaccharide components in their biofilm matrix include *S. aureus* which utilizes poly-N-acetylglucosamine or PNAG (O'Gara 2007) and *P. aeruginosa* which utilizes alginate (Ryder, Byrd et al. 2007). Examples of microorganisms that utilize primarily protein components in their biofilm matrix include *Acinetobacter baumannii* which utilizes a biofilm-associated protein or BAP (Loehfelm, Luke et al. 2008) and *Bacillus subtilis* which utilizes a polysaccharide and the protein TasA (Chai, Chu et al. 2008). The final type of biofilm matrix is a nucleic acid based matrix, normally composed of extracellular DNA (eDNA). Examples of microorganisms that utilize eDNA in their biofilm matrix include *Listeria monocytogenes* (Harmsen, Lappann et al. 2010) and *Bacillus cereus* (Vilain, Pretorius et al. 2009). These matrix components are not mutually exclusive; organisms able to utilize a combination of matrix components are quite common. *Salmonella* sp. are able to use curli, cellulose, and the biofilm-associated protein BapA to

form a biofilm (Jonas, Tomenius et al. 2007). *Enterococcus faecalis*, which produces a biofilm-associated protein Esp, also has been found to promote autolysis of its own cells to release eDNA which plays a role in biofilm formation by the organism (Thomas, Thurlow et al. 2008). Not only is there considerable variation in biofilm structure between different bacterial species, but a single species may also produce biofilms that vary considerably in matrix composition depending on environmental conditions. An example of this is *Staphylococcus epidermidis*, in which Hennig et al. described a spontaneous switch from biofilm formation mediated by polysaccharides to biofilm formation mediated by proteins in an *icaC* insertion mutant (Hennig, Nyunt Wai et al. 2007).

The biofilm mode of growth imparts on its constituent bacteria resistance to antimicrobial agents (Passerini de Rossi, Garcia et al. 2009), disinfectants (Kim, Ryu et al. 2007), drying (Vanderlinde, Harrison et al. 2009), ultraviolet light (Bak, Ladefoged et al. 2009), acid exposure (Kubota, Senda et al. 2009), phagocytosis (Cerca, Jefferson et al. 2006) and sheer stress (Giao, Azevedo et al. 2008). This resistant phenotype of biofilms is due to many factors including: restricted penetration of antibiotics/disinfectants, production of antimicrobial destroying enzymes, altered growth rate of some cells, and overexpression of stress response genes (del Pozo and Patel 2007).

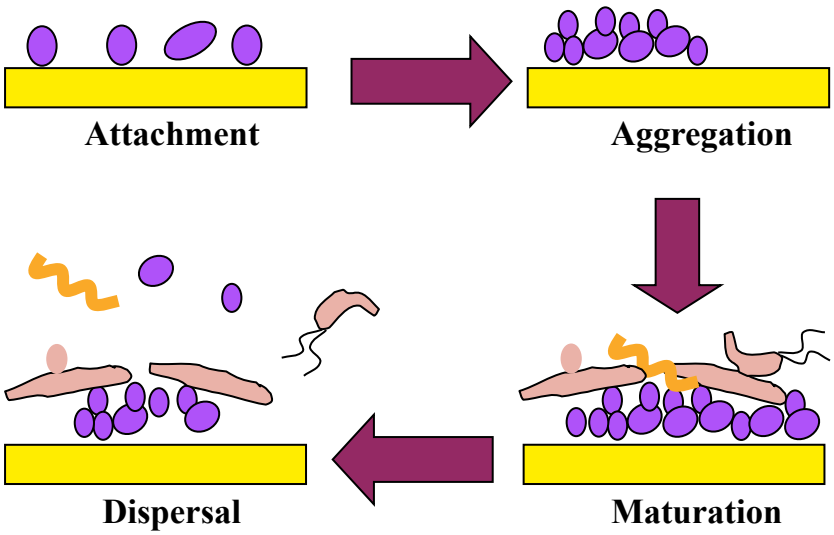
Biofilm development requires a series of complex but distinct and well-regulated steps. The exact molecular mechanisms differ from organism to organism, but the stages are similar across a wide range of microorganisms. The steps of biofilm development are: (1) microbial attachment to a surface, (2) intercellular attachment and aggregation of

bacterial cells, (3) biofilm maturation, and (4) dispersal of bacterial cells for production of new biofilms (**Figure 3**). Many environmental cues can stimulate or repress biofilm formation. For example, changes in salt concentration affect biofilm formation by the water pathogen *Vibrio cholera*, leading to an increase in bacterial mobility and increased infection rate during dry or flood conditions (Shikuma and Yildiz 2009). Examples of other environmental cues that can affect biofilm formation are low temperature, which increases expression of biofilms in *E. coli* and *Y. pestis* (Perry, Bobrov et al. 2004; White-Ziegler, Um et al. 2008), and growth in non-optimal growth conditions, which increases biofilm formation in environmental isolates of *E. coli* (Castonguay, van der Schaaf et al. 2006).

A well characterized biofilm whose development has been extensively researched, is the formation of a biofilm on the surface of a subvenous catheter. When inserting the catheter into the bloodstream, bacteria become attached to the tip and length of the catheter surface. Rapidly, blood plasma proteins, platelets, fibrin and leukocytes bind to the surface of the catheter; these host products serve as scaffolding for the developing biofilm and receptors for newly arriving bacteria (Cappelli, Tetta et al. 2005). *S. aureus* adheres to proteins like fibrinogen and fibronectin found on the catheter in the first step of biofilm development (Arciola, Campoccia et al. 2005; O'Neill, Humphreys et al. 2009).

Once bacteria are attached to the surface of the catheter, phenotypic changes in the bacteria alters protein expression to further produce adhesins that anchor the bacterial cells to each other and to the catheter surface (Vandecasteele, Peetermans et al. 2003). *S.*

Figure 3. Steps of biofilm development. (1) Attachment: Cells affix to substratum using surface proteins like MSCRAMMs and surface induced gene expression results in a protein profile significantly different from planktonic bacteria, (2) Aggregation: Cells orient themselves using surface proteins like Biofilm-associated proteins (BAPs), allowing for development of clusters and motility is lost, (3) Maturation: Cell communication (potentially through quorum sensing) leads to cell clusters reaching maximum thickness via production of exopolysaccharide, (4) Dispersal: Production of enzymes to disperse bacteria or in some bacteria, the protein profile begins to resume that of planktonic bacteria allowing for the dispersal of bacteria from the biofilm.



Attachment	**Aggregation**	**Maturation**	**Dispersal**
Use of MSCRAMMs, surface proteins to attachment to surface	Use of surface proteins like Bap and AAP to aggregate	Production of EPS to mature the biofilm	Use of enzymes like alginate lyase to disperse the biofilm, shearing

aureus bovine mastitis isolates produce a biofilm-associated protein called Bap that acts as an intercellular adhesion, anchoring bacteria to each other and causing maturation of the biofilm (Cucarella, Solano et al. 2001). As the bacterial cells multiply, they can produce exopolysaccharides or "slime" which leads to differentiation into true biofilms: exopolysaccharide-encased bacterial communities resistant to many negative environmental stimuli (Costerton, Stewart et al. 1999). Furthermore, just as there are environmental cues for events leading to biofilm development, there are cues which lead to maturation of the biofilm. Many bacteria utilize a communication system that employs "messengers" produced by individual bacterial cells; when these signals accumulate to a certain density they cause the expression of specific sets of genes, including genes involved in biofilm formation. An example of this communication system is quorum sensing in gram negative bacteria which utilize acylhomoserine lactone signals to talk between bacteria (Fuqua, Winans et al. 1994).

Finally, in order to colonize new surfaces the bacteria within the biofilm must detach and disseminate. Dispersal is accomplished by shearing, where pieces of the biofilm can break off in the flow of blood or urine and colonize new surfaces, or by enzymatic dispersal. An example of enzymatic dispersal is seen in *P. aeruginosa*, which produces an enzyme called alginate lyase that is able to digest the polysaccharide component of its biofilm, releasing bacteria into the environment (Boyd and Chakrabarty 1994).

The medical community has gradually recognized biofilms as important aspects of disease, especially in patients with indwelling medical devices or who are

immunocompromised (Lindsay and von Holy 2006). The biofilm phenotype is associated with chronic, indolent, and relapsing infections. Examples of widely studied and well-recognized biofilms include those found in dental plaque, catheter infections, pulmonary infections, and endocarditis. Most biofilm diseases are treated by antibiotic therapy and removal of the infected device (i.e. catheter, heart valve, artificial joint). Extended administration of antibiotics may be required and even prolonged therapy may not be effective. Device removal is not cost-effective and may extend the patient's recovery period; therefore, more effective therapies, like biofilm-dispersing enzymes are being developed. An important step in understanding the pathogenesis of BV would be characterization of biofilm formation in *G. vaginalis*. Identification and characterization of the gene responsible for biofilm formation in *Gardnerella* could lead to novel treatment modalities and an improved understanding of this inadequately described condition.

Chapter 2

Materials and Methods

BV-associated strains and culture conditions. All media components were from Becton Dickinson unless otherwise noted (BD, Franklin-Lakes, NJ). BV-associated bacterial species used in this study, references to their association with BV, and the source of the strains are listed in **Table 1**. The bacterial strains used in this study were purchased from ATCC or isolated from confirmed cases of BV (confirmed by Amsel criteria and Nugent score) at Brigham and Women's Hospital, Boston, MA (Onderdonk, Lee et al. 2003), or isolated as part of this study from Virginia Commonwealth University Women's Health Clinic. Strains from the VCU Women's Health Clinic were isolated from vaginal swab specimens from women diagnosed with BV by the Amsel criteria (Holmes, Spiegel et al. 1981). Individual colonies were isolated from Brucella Blood agar (Oxyrase, Mansfield, OH) and cultured in supplemented Brain Heart Infusion broth (sBHI); Brain Heart Infusion broth (Difco) containing 2% (w/v) gelatin, 0.5% yeast extract, 0.1% starch, and 0.1% glucose. All cultures were grown under anaerobic conditions, in approximately 10% CO_2 with the use of the AnaeroPack system (Mitsubishi Gas Chemical Co, Tokyo, Japan). For some experiments, cultures were grown in sBHI supplemented with 1% glucose (sBHIG), sBHI supplemented with 8% heat-inactivated human serum (sBHIs), or in standard BHI or BHIG.

Table 1. BV-associated bacterial species used in this study.

Organism	Strain and source	Reference to involvement in BV
Gardnerella vaginalis	5-1*, 465*, 101-1ǂ, 49145•	Menard et al. (2008), Oakley et al. (2008), Fredricks et al. (2005), Marrazzo et al. (2008)
Atopobium vaginae	FAǂ	Menard et al. (2008), Oakley et al. (2008), Fredricks et al. (2005), Marrazzo et al. (2008) Zhou et al. (2004)
Mobiluncus mulieris	BV 26-9ǂ	Menard et al. (2008), Oakley et al. (2008), Fredricks et al. (2005), Marrazzo et al. (2008)
Prevotella bivia	29303•, 715BVDǂ	Oakely et al. (2008), Fredricks et al. (2005)
Veillonella sp.	715BVAǂ	Oakley et al. (2008), Lefevre et al. (1985), Biagi et al. (2009)
Peptostreptococcus sp.	715BVCǂ	Oakley et al. (2008), Marrazzo et al. (2008)
*Peptoniphilus s*p.	718BVAǂ	Fredricks et al. (2005), Marrazzo et al. (2008)
Fusobacterium nucleatum	718BVCǂ	Oakley et al. (2008)

* Brigham and Women's Hospital.

ǂ VCU Women's Health Clinic.

• American Type Culture Collection.

16s Polymerase Chain Reaction (PCR) and identification of BV-associated anaerobes.

Genomic DNA was isolated from cultured BV-associated anaerobes from patients from the VCU Women's Health Clinic using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and amplified by PCR using universal primers for the 16S rRNA gene found in **Table 2**. PCR reactions were performed in a TC-312 thermocycler (Techne, Staffordshire, UK). PCR reaction mixtures consisted of 25 µl HiFi PCR Mix (Invitrogen, Carlsbad, CA) and 4 mM primers.

Conditions for PCR were as follows, one initial cycle at 94ºC for 2 minutes followed by 35 cycles of: 94ºC 30 seconds to denature, 50ºC 30 seconds to hybridize, and 71ºC 30 seconds for extension. A final extension at 72ºC for 5 min after the final cycle and the reactions were chilled to 4ºC. PCR products were sequenced at the VCU Nucleic Acids Core Facility under the supervision of Dr. Gregory Buck and species were identified by a 98% or greater identity to sequences in the NCBI database using BLASTn.

Non-BV related strains, culture conditions and plasmids used. *E. coli* was grown aerobically in Luria-Bertani broth (LB) and on LB agar with appropriate antibiotics at the following concentrations: ampicillin 100 µg/ml and chloramphenicol 35 µg/ml. *S. aureus* cultures were grown aerobically in TSB broth at 37ºC with appropriate antibiotics at the following concentrations: chloramphenicol 10 µg/ml and erythromycin 10 µg/ml. *L. lactis* cultures were grown aerobically in GM-17 broth at 30ºC (LB, TSB and GM-17 media from Sigma-Alderich, St. Louis, MS). Plasmids used are seen in **Table 3**.

Table 2. Primers used in this study.

Primer Label	**Sequence**
UnivFWD	5’-AGAGTTTGATCCTGGCTCAG-3’
UnivREV	5’-GGACTACCAGGGTATCTAAT-3’
AP1	5’-GTAATACGACTCACTATAGGGC-3’
AP2	5’-ACTATAGGGCACGCGTGGT-3’
GSP1	5’-TCTGACGAATTGGTTATATTCGCATC-3’
GSP2	5’-CAACAAGTTCACGACACCACTTATTG-3’
BapEpiIntein3	5’-GTGTGGTTGCTCTTCCGCAACCTACA-3’
BapEpiRev3	5’-GTGTGGCATATGGATATTGCAAATAA-3’
BapSbfFWD	5’GGTTGGCCTGCAGGCGTGATTAATTCATA TACGAATAAATTAC-3’
BapSbfREV	5’GGTTGGcctgcaggTTAGTCGCGCTTCTTCC TGCG-3’

Table 3. Non-BV organisms and plasmids used in this study.

Non-BV Organisms Used	Strain
Escherichia coli	CH3-Blue chemically competent bacteria (Bioline)
	BL21-CodonPlus (DE3)-RIL Competent bacteria (Stratagene)
Lactococcus lactis	ATCC 11454
Staphylococcus aureus	RN4220
	RN4220Δica (eyrthromycin 10 μg/ml)

Plasmids Used	Antibiotic Resistance
pCR2.1®Topo	*E. coli*, Ampicillin 100 μg/ml
pCR3.2®Topo-bapL	*E. coli*, Ampicillin 100 μg/ml
pKYB1	*E. coli*, Kanamycin 50 μg/ml
pKYB1-bapL	*E. coli*, Kanamycin 50 μg/ml
pRB473	*E. coli*, Chloramphenicol 35 μg/ml *S. aureus*, Chloramphenicol 10 μg/ml
pRB473-bapL	*E. coli*, Chloramphenicol 35 μg/ml *S. aureus*, Chloramphenicol 10 μg/ml

Agarose gels. Agarose gels were prepared by dissolving agarose in 1X Tris-Acetate-EDTA (TAE) buffer (Fisher Scientific, Pittsburgh, PA). Ethidium bromide was added to 0.15 µg/ml prior to pouring the gel. Generally, PCR products were run on a 1.5% agarose gels and 10X BlueJuice gel loading buffer (Invitrogen) was added to PCR products to reach a 1X dye concentration. Gels were run at 120 volts until the dye front had migrated roughly ¾ of the length of the gel and then visualized under UV light. DNA size was measured against a λ DNA ladder, which was made of 1/10 volume of HindIII digested λ DNA (New England Biolabs, Ipswich, MA), 1/5 volume of 10X Blue Juice gel loading buffer and 7/10 volume of deionized, distilled sterile water.

Gardnerella biofilm growth assay. *Gardnerella vaginalis* strain ATCC 49145 (American Type Culture Collection, Manassas, VA) and strains 5-1 and 465-5 (kindly provided by Dr. Robin Ross, Boston, MA) were grown under anaerobic conditions, in ~10% CO_2 using the AnaeroPack system (MGC, Tokyo, Japan). 96-well tissue culture plate assays were performed essentially as previously described to determine optimal growth conditions for biofilm formation (Christensen, Simpson et al. 1985). *G. vaginalis* was grown in Corning Cell-Bind Plates (Corning, Corning, NY) in various media without or with 1% supplemental glucose (G) including Luria broth (LB, LBG), Tryptic Soy Broth (TSB, TSBG), Mueller-Hinton (MH, MHG), Brain Heart Infusion (BHI, BHIG), De Man Rogosa Sharpe (MRS, MRSG), and a chemically defined medium resembling vaginal secretions (CDM, CDMG) (Onderdonk, Lee et al. 2003).

Biofilm assay of BV-associated organisms. Biofilms were grown using 96 well Corning Cell Bind Plates (Corning). Overnight cultures of *G. vaginalis* or BV-associated anaerobes were diluted 1:10 with fresh sBHI and individual wells of a sterile, polystyrene 96 well tissue culture plate were inoculated with 0.2 ml of dilute culture. The tissue culture plates were incubated for 24-72 hrs at 37 °C, under anaerobic conditions. Growth was analyzed using a 96-well plate spectrophotometer with a 595nm filter (BioTek 800 Plate reader, Winooski, VT). Spent medium was removed, wells were washed once with 200 µl phosphate buffered saline (PBS) to remove nonadherent bacteria, and the plates were air dried for 1 hr. Biofilms were stained with 200 µl of safranin for one min, wells were washed gently to remove the safranin, and the plates were air dried. After the biofilms were visually analyzed and imaged using a flatbed scanner, the safranin was solubilized with 200 µl of 33% (v/v) acetic acid per well. For quantitative results, the solubilized safranin was analyzed at optical density of 562nm using the 96-well plate reader (BioTek).

Cell line. ME-180 cervical epithelial cells (ATCC) were cultured at 37ºC in 5% CO_2 in McCoy's 5A medium (Quality Biologic, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 1IU ml^{-1} penicillin/streptomycin (MediaTech, Manassass, VA). ME-180 cervical epithelial cells were used in place of vaginal epithelial cells due to availability and cost effectiveness.

Confocal microscopic adherence assay. ME-180 epithelial cells were seeded into 6-well tissue culture plates (Greiner Bio One, Monroe, NC) at a density of 1.5 X 10^5 cells/well

and incubated for 72 hrs or until a monolayer of cells had formed. The growth medium was removed, the cell monolayer was washed twice with sterile PBS and stained with 2.5µl/ml per well of Vybrant DIL membrane stain (Invitrogen) for 30 min at 37 °C, 5% CO_2.

Following incubation, the monolayers were washed twice with sterile PBS to remove excess stain. Overnight cultures of the various bacterial species were standardized to an optical density of 600 nm (OD_{600nm}) of 0.150 using a NanoDrop 1000 (Thermo Scientific, Waltham, MA), and 2 ml of each culture was centrifuged to collect the cells. Bacteria were washed twice with 1 ml of sterile PBS and stained with 5µl of BacLight green bacterial stain in 1ml PBS (Invitrogen).The bacterial cells were incubated in the dark for 30 min at 37 °C, washed twice with 1 ml of sterile PBS to remove excess stain. 1 X 10^7 stained bacteria in 0.5 ml PBS were added per well containing ME-180 cells. The plates were centrifuged at 700 rpm (18 g) for 2 min to maximize contact between the bacteria and cells.

Time course assays indicated that adherence of bacteria to ME-180 cells did not increase after 15 minutes of contact (data not shown); therefore, the plates were incubated covered for 15 min at 37°C, 5% CO_2. After 15 minutes of exposure to *G. vaginalis* at 37°C the ME-180 cells began to exhibit cytopathogenic changes. *G. vaginalis* was therefore incubated at 10°C to prevent cytotoxicity; these conditions did not affect binding of any of the other species. Finally, the wells were washed vigorously twice with sterile PBS to remove nonadherent bacteria and the wells were analyzed by confocal microscopy using a muliphoton confocal scanning laser microscope (Zeiss LSM 510; Microscopy Core Facility, Neuroscience Department, VCU, Richmond, VA).

Adherence assays were repeated 3 times on separate days, with three fields of view assessed. Adherence scores were based on a qualitative scoring system ranging from (-) to (+++) where (-) denotes that no adherent bacteria were observed in any field of view on all days tested; (+/-) indicates that less than 25% of ME180 cells had bacteria attached in any field of view on 1 or 2 days, but it was not repeatable for all 3 experiments; (+) denotes that less than 25% of ME180 cells had bacteria attached in all fields of view on all days tested; (++) denotes that greater than or equal to 25% to less than 75% of ME180 cells had bacteria attached in all fields of view on all days tested; and (+++) denotes that greater than or equal to 75% to 100% of ME180 cells had bacteria attached in all fields of view on all days tested.

Adherence of *G. vaginalis* was also tested when bacteria had been pretreated with proteinase K. Overnight *G. vaginalis* cultures were centrifuged and resuspsended in PBS, and subjected to 20 µl/ml of a 20 mg/ml stock of proteinase K (Qiagen), rocking at room temperature for 1 hour. After an one hour incubation, the bacteria was collected and washed three times with 1 ml of sterile PBS to remove residual proteinase K. The bacteria were then used for confocal adherence assays as described above.

G. vaginalis bacteria vs. supernatant cytotoxicity assay. Overnight cultures of *G. vaginalis* strain 5-1 were centrifuged to remove bacteria and supernatant removed and dialyzed against 0.1X PBS overnight. ME-180 epithelial cells were seeded into a 6-well tissue culture plate at a density of 3.0 X 10^4 cells per well. After 72 hours, or when a monolayer of cells had formed, the media was removed, and approximately 1 X 10^6

bacteria in PBS or dialyzed supernatant were added to individual wells. The monolayers were analyzed for cytotoxicity by light microscopy every hour for 4 hours.

Cytopathogenicity was scored from 0 to 5 where 0 denotes no difference between the experimental well and control; a 1 indicates that less than 25% of cells were rounded; a 2 indicates that 25-50% of cells were rounded; a 3 denotes that greater than 50% of cells were rounded; a 4 indicates that greater than 50% of cells were rounded with partial disruption of the monolayer; and a 5 indicates complete disruption/absence of the monolayer. After the monolayers were analyzed for 4 hours and given a cytotoxicity score, the bacteria were removed by pipetting off the liquid from each well and microscopic images of each well were taken with an Olympus CK2 microscope (Olympus, Center Valley, PA).

Trans-well cytotoxicity assay. Overnight cultures of *G. vaginalis* strain 5-1 were collected and resuspended in 1X PBS. ME-180 epithelial cells were seeded into a 6-well tissue culture plate at a density of 3.0×10^4 cells per well. To assess the contact dependence of *G. vaginalis*-associated cytotoxicity, the media from the confluent 6-cell tissue culture plate was replaced with 2ml of 1X PBS, a sterile transwell with pores of either 0.45 μM or 8 μM was inserted into a well, and 1.5 ml of the bacterial suspension was added to the top portion of the transwell. The monolayers were monitored every hour by light microscopy for cytopathogenic changes, such as cell rounding, loss of adhesion, and disruption of the monolayer. Cytopathogenicity was scored using the scoring system described above and

microscopic images of each well were taken with an Olympus CK2 microscope at magnifications of 100X and 400X (Olympus).

BV-associated anaerobe cytotoxicity assay. ME-180 epithelial cells were seeded into a 96-well tissue culture plate at a density of 3.0 X 10^4 cells per well. After 72 hours, or when a monolayer of cells had formed, the media was removed, and approximately 1 X 10^6 bacteria in PBS were added to individual wells. The monolayers were analyzed for cytotoxicity by light microscopy every hour for 4 hours. Cytopathogenicity was scored using the scoring system described above and microscopic images of each well were taken with an Olympus CK2 microscope (Olympus).

Biochemical characterization of G. vaginalis biofilm matrix. For biofilm cultures, *G. vaginalis* strain ATCC 49145 was grown anaerobically in BHI in T75 tissue culture flasks (Corning). Planktonic cultures were grown in BHI with shaking. Media was removed from biofilms; planktonic bacteria were collected by centrifugation and were washed once with double-distilled water. Loosely associated surface material was extracted by the vigorous resuspension of bacteria in distilled, deionized water, for a final cell density of 10^9 CFU/mL, as estimated by optical density at 650 nm (OD_{650}). Protein concentrations in the extracts were determined with the use of Coomassie Protein Assay Reagent and the Bradford assay (Pierce Biotechnology, Rockford, IL). Nucleic acid content was estimated by measuring the optical density at 260 nm (OD_{260}). Hexosamine contents were estimated by standard phenol-sulfuric acid assay with the use of glucose dilutions as standards

(Dubois, Gilles et al. 1951) . Three separate experiments were performed, and statistical significance was determined with the Student *t* test.

Bradford assay for protein concentration. Protein concentrations were determined by performing a Bradford assay on known concentrations of bovine gamma globulin (Pierce Biotechnology) to generate a standard curve. This standard curve was applied to measurements taken from dilutions of sample protein. 20 µl of protein sample was added to 200 µl of Coomassie Protein Reagent (Pierce Biotechnology) and vortexed for 20 seconds. The absorbance at 595 nm was then measured on a NanoDrop 1000 spectrophotometer (Thermo Scientific) three times for each sample as well as each standard. The average was then used to generate the standard curve and determine protein concentration.

Lectin blots of G. vaginalis matrix material. For lectin blots, 100 µL of the cellular extracts of *G. vaginalis* were diluted serially 5-fold in PBS and dilutions were blotted onto nitrocellulose with a vacuum manifold and probed with peroxidase-conjugated lectins: wheat germ agglutinin (WGA), concanavalin A, and Glycine Max (Sigma), as described previously (Jefferson and Cerca 2006). The blots were developed with the use of the ECL-Plus Western blotting detection system (GE Healthcare, Piscataway, NJ).

Confocal microscopy of G. vaginalis biofilms. Strain 49145 was grown in BHIG in 35-mm collagen-coated glass-bottom dishes (MatTek, Ashland, MA) at 37°C anaerobically for 48

hours. Biofilms were stained with 3 µL SYTO 9 and 3 µL Alexa Fluor 633-conjugated WGA (Invitrogen) in 3 mL phosphate-buffered saline solution for 15 minutes with rocking. Biofilms were washed twice with PBS solution, and confocal imaging was performed with a multiphoton confocal scanning laser microscope (Zeiss LSM 510; Microscopy Core Facility, Neuroscience Department, VCU, Richmond, VA). The objective was an Acroplan 63X/0.9W (Carl Zeiss Microimaging, Inc, Thornwood, NJ). The excitation wavelengths that were used were 488 nm and 633 nm; bandpass filters for emitted light were 500 nm to 530 nm and 650 nm.

Biofilm degradation assays. To analyze the dissolution of established biofilms, 24 hour-old biofilms were subjected to 5-fold dilutions of various compounds in fresh BHIG (or in 50 mM/L NaOAc; pH 4.2 for sodium metaperiodate). Stock solutions were 20 mg proteinase K/mL (Qiagen), 20 mg trypsin/mL, 5U chitinase/mL, 30% H_2O_2, 10 M/L lactic acid, 20% sodium dodecyl sulfate (SDS), 4 mg sodium metaperiodate/mL in 50 mM/L sodium acetate pH 4.2, and 25 U deoxyribonuclease (DNasel)/mL (Ambion, Austin, TX). Biofilms were incubated for 20 hours, the media was removed, and the biofilms were washed with PBS solution, dried, and stained with safranin. Digital images of the biofilms were taken by an HP color LaserJet 2820 printer with scanning capabilities (Hewlett-Packard Company, Palo Alto, CA).

Luciferase assay for bacterial viability. The BacTiter luciferase assay for adenosine triphosphate (Promega Corp, Madison, WI) was used to determine bacterial viability. To

correlate viability with relative light units, planktonic cultures and biofilm cultures that had been resuspended by pipetting up and down were diluted serially 10-fold in BHI. 10 µL of each dilution was plated on BHI agar. The plates were incubated anaerobically for 72 hours, and colony forming units (CFUs) were determined, and 200 µL of each serial dilution was placed in Cell Bind–coated 96-well clear bottom black plates (Corning). Cells were collected by centrifuging the plates, and the media were replaced with 50 µL BacTiter reagent (Promega). After 10 minutes at 21°C, luminescence was analyzed with a Tropix luminometer (GE Healthcare, Piscataway, NJ) for 1 second.

Planktonic and biofilm culture sensitivity assays. For sensitivity assays, 200 mM hydrogen peroxide and 2 M lactic acid (Sigma) were diluted 2-fold in BHI or CDM and added to *G. vaginalis* biofilm and planktonic cultures. Plates were incubated anaerobically for 20 hours at 37°C; cells were collected by centrifugation, resuspended in 50 µL BacTiter reagent (Promega), and transferred to black 96-well plates (BD Falcon). Viability was assessed as described earlier for the luciferase asay. The concentration of H_2O_2 or lactic acid that was required to bring about a 90% decrease in relative light units was recorded as the MBC_{90} for each chemical.

Proteinase K-treated biofilm sensitivity assays. The effect of treating biofilms with 100 µg proteinase K/mL for 24 hours on viability was determined with the BacTiter assay, as described earlier. For proteinase K–treated biofilm sensitivity assays, the media in the wells was replaced with BHIG that contained 100 µg of proteinase K/mL. The biofilms

were preincubated with the protease for 1 hour; sensitivity to H_2O_2 and lactic acid was determined as described earlier. For a Proteinase K time course, biofilms were stained using Live/Dead BacLight stain, 100 μg of proteinase K/mL was added to a *G. vaginalis* biofilm, and confocal images of the same field of view were collected every 20 minutes for 280 minutes. Red color indicates live bacteria, while green color indicates dead bacteria.

Degradation assay with natural proteases. To analyze the dissolution of established biofilms by natural proteases, 24 hour-old biofilms were subjected to 5-fold dilutions of various enzymes in fresh PBS at pH 4.5 or 7.4. Stock solutions were 10 U Bromelain, 10 U Papain, 5.78 U Ficin, and 345 U Pepsin. Biofilms were incubated for 20 hours, the media was removed, and the biofilms were washed with PBS, dried, and stained with safranin. Digital images of the biofilms were scanned using an HP color LaserJet 2820 printer (Hewlett Packard Company). The safranin stained biofilms were solublized with 200 μl of 33% (v/v) acetic acid per well. For quantitative results, the solubilized safranin was analyzed at optical density of 562nm using a 96-well plate reader (BioTek). Technical replicates and biologic replicates were performed on each strain and the absorbance readings were averaged. These averaged absorbance readings were compared to control to give a percent reduction in biofilm mass.

Antibiotic sensitivity assay using natural proteases. 24 hour biofilms were grown in 96-well plates in BHIG anaerobically at 37°C. Planktonic cultures were grown in 50 ml conical tubes in BHIG with gentle shaking anaerobically at 37°C. Media was removed

from biofilm wells and replaced with either 200 µl BHIG as control, 200 µl of 1.8375 µg/ml clindamycin (3X MIC) in BHIG, 200 µl of 10 U papain in BHIG or 200 ul of 10 U papain containing 1.8375 µg/ml of clindamycin in BHIG. These cultures were incubated anaerobically for 16 hours at 37°C. Media was removed from another biofilm well and pretreated with 200 µl of 10 U papain in BHIG for 2 hours anaerobically at 37°C, media was removed and wells washed with 200 µl of sterile PBS and 200 µl of 1.8375 µg/ml clindamycin was added to the wells and incubated for 16 hours anaerobically at 37°C. After the 16 hour incubation, all bacteria were harvested by 3 pulses of a sonicator, 10-fold diluted in 200 µl of BHIG and drip plated for CFU.

Identification of Biofilm-Associated Protein (BAP) in G. vaginalis. A partial sequence of the *G. vaginalis* genome is available from the *Gardnerella vaginalis* Genome Project, Stanford Genome Technology Center, which is funded by the Ellison Medical Foundation. The amino acid sequence of the staphylococcal Bap protein was compared to the partial genome of *G. vaginalis* using blastP software. Structural similarities between the two proteins were analyzed by the RADAR software (EMBEL-EBI).

DNA extraction. Genomic DNA was isolated from *G. vaginalis* strain 5-1 using a phenol-chloroform extraction. Briefly, 100 ml of culture was centrifuged at 4000 rpm (4ºC) for 15 min to pellet the bacteria. The pellet was resuspended with 10 ml of lysis buffer containing lysozyme (10 mM Tris HCl pH 8.0, 10 mM NaCl, 10 mM EDTA, 1% Triton X100 and 10 mg Lysozyme) and allowed to incubate at 37ºC for 1 hr. 100 µl of Proteinase K (20 ug/ml stock) was added to the mixture and allowed to incubate at 65ºC for 1 hr

(Qiagen). 10 ml of a 1:1 mixture of phenol:chloroform was added, mixed gently as not to shear the DNA, and centrifuged at 12000 rpm (4ºC) for 5 min. The upper aqueous solution was removed to a clean tube and two chloroform washes were performed. The DNA was precipitated by addition of one tenth the volume of 3M sodium acetate and 2 volumes of ice-cold 100% ethanol. This was allowed to rest at -20C for at least 1 hr. The DNA was collected by centrifugation at 12000 rpm (4ºC) for 1 hr. The DNA was washed once with 70% ethanol and allowed to air dry for 15 minutes before being resuspended with an appropriate volume of nuclease-free water.

Genome walking. The 5' upstream region of the *bapL* locus of *G. vaginalis* was amplified using the genome walking technique. Briefly, a Universal Genome Walker Kit (Clontech, Palo Alto, CA) was used to construct a number of adaptor ligated *G. vaginalis* DNA fragment libraries (Sung, Chantler et al. 2006). These libraries were used as a template for PCR amplification using a gene specific primer (GSP1) and an adaptor specific primer provided by the genome walking kit (AP1) (see **Table 2** for primer list). These PCR products were then used as a template for a second nested PCR using an internal gene specific primer (GSP2) and an internal adaptor specific primer (AP2). Both PCRs were performed using a DNA polymerase suitable for long distance PCR (Clontech). A single band was excised from the gel, purified, and then sequenced using the nested adaptor specific primer to initiate sequencing.

Full G. vaginalis genome sequencing. Sequencing was performed in the VCU Nucleic Acids Core Facility using Roche 454 technology (Roche, Branford, CT). One full run of GS FLX and one half run GS FLX XLR were done for strain 5-1 (coverage of ~175 X). Five micrograms of DNA was sequenced as per the standard Roche sequencing protocol.

Production of pKYB1-bapL for expression of peptide fragment of BapL. Forward and reverse primers were used to amplify an antigenic region in the BapL protein, which was chosen by *in silico* analysis using the EMBOSS:antigenic program (EMBOSS explorer). The primers also introduced Sap1 and Nde1 restriction sites at the ends of the 0.5 kb PCR product; this allowed for the production of an intein-chitin binding domain (CBD) tagged fusion peptide (Zhang, Gonzalez et al. 2001). The forward primer used was BapEpiIntein3 and the reverse primer used was BapEpiRev3 (**Table 2**). The 0.5 kb PCR product was then digested with Sap1 and Nde1 using specific buffers and BSA per the manufacturer's instructions (New England Biolabs). After digestion, the PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen) as described by the manufacturer. The digested 0.5 kb PCR product was ligated into the linearized *E. coli* vector pKYB1 (kan^{50}) using Ready-to-Go T4 DNA Ligase tubes (Amersham Biosciences, Piscataway, NJ) with an hour incubation at 16ºC.

CH3 chemically competent E.coli transformation. 2 µl of purified plasmid DNA was added to 50 µl of CH3 blue chemically competent *E. coli* cells (Bioline, Taunton, MA). This mixture was incubated on ice for 10 minutes. The tubes were then transferred to a

water bath at 42ºC for 30 seconds and immediately after the incubation, 250 µl of SOC media (2% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, 85.5 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20mM glucose) was added to the tube and incubated for one hour at 37ºC with shaking. Following this recovery period, the cells were plated on LB plates with appropriate antibiotic selection.

Plasmid DNA purification and screening. Plasmid mini-preps were prepared from individual *E. coli* transformants that were grown overnight in 5 ml of LB broth with appropriate antibiotic, using a QIAprep Spin Miniprep Kit (Qiagen), as described by the manufacturer. Plasmids were screened for correct insert by digestion with Nde1 and Kpn1 (Bioline). Plasmids with correct insert sizes were sequenced to confirm insertion of the accurate antigenic region of BapL before being used to transform BL21-CodonPlus®(DE3)-RIL *E. coli* cells (Stratagene, La Jolla, CA).

Induction of recombinant BapL peptide. The pKYB1-based construct expressing the antigenic peptide from BapL was introduced into BL21 CodonPlus®(DE3)-RIL *E.coli* cells by transformation (Stratagene). Strains harboring this plasmid were grown in 6 liters of LB supplemented with kanamycin 50 µg/ml to an OD_{600} of 0.8 and then IPTG (Fisher) was added to 1mM to induce expression of the recombinant peptide.

Purification of recombinant peptide. After induction, cells were grown for 6.5 hours and harvested by centrifugation at 6,000 rpm at 4ºC for 15 minutes. Cell pellets were

resuspended in Impact buffer (20 mM Tris, 0.4 M NaCl, 0.1% Triton X100 and Complete™ protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)). Bacteria were lysed sequentially with a Thermo IEC French pressure cell press (Thermo Scientific) with 750 p.s.i. 3 times per 25 ml of resuspended bacteria, followed by sonication with a 60 Sonic Dismembrator at 15 watts (RMS) for 20 seconds 6 times on ice with 20 seconds rest between each repetition (Fisher). Cell lysates were centrifuged at 20,000 rpm at 4ºC for 30 minutes and supernatants were collected, filtered through a 0.45 µM filter, and run through a chitin column. The lysate was loaded onto a column made of chitin beads (New England Biolabs); the flow-thru was collected and passed back over the column to ensure protein binding to the column. The column was washed with 5 column volumes of cold, sterile Impact buffer, followed by 2 washes with 15 ml of Impact buffer and 1:1000 dilution of Triton X100. Finally, the column was washed with 15 ml of Impact buffer containing 750 µl DTT, about 1 inch of liquid was left in the column and the column was sealed with parafilm and left overnight at 4ºC. After the overnight incubation, the BapL peptide was eluted with the addition of 45 ml of Impact buffer. The BapL peptide was concentrated, analyzed by SDS-PAGE using 4-12% Tris-glycine gels (Invitrogen) and protein concentrations were estimated by the Bradford method (Bradford 1976). Approximately 10 mg of purified recombinant peptide was submitted to Invitrogen for production of polyclonal rabbit antiserum by their standard protocols.

Polyacrylamide Gel Electrophoresis. Protein samples were analyzed using the NuPAGE protein system (Invitrogen) and were separated on pre-cast NuPAGE Novex Bis-Tris 4-

12% gradient gels (Invitrogen). 4X NuPAGE LDS loading buffer was added to samples to achieve a 1X concentration and these samples were then boiled for 10 minutes prior to gel loading. Gels were run in 1X MES SDS Running Buffer (Invitrogen), rinsed in deionized water three times for 5 minutes each and then stained in Imperial Protein Stain (Thermo Scientific) for 1-2 hours, followed by destaining in water overnight.

Western blot analysis of BapL expression. Samples for western blot analysis were prepared as follows. Biofilm samples were grown for 48 hours in BHI or BHIG with media change after 24 hours. Planktonic samples were grown for 24 hours in BHI with gentle shaking. One BHIG biofilm was treated for 1 hr with 6 µl/ml of proteinase K (20 µg/ml stock, Qiagen) to disrupt the biofilm and bacteria collected by centrifugation of the media. Protein amount was standardized by pellet weight after centrifugation to collect the bacteria. Proteins were extracted from the samples by resuspending with 1 ml of lysis buffer containing lysozyme and incubating for 45 minutes at room temperature with rocking. Samples were centrifuged to collect the cell debris, supernatants collected, NuPAGE loading buffer added and samples boiled for 10 minutes. Proteins were separated on NuPAGE Bis-Tris 4-12% gels as described. Following electrophoresis, the polyacrylamide gel was rinsed in deionized water. PVDF membrane was wetted in 100% methanol then incubated in transfer buffer. Sponges were wetted in transfer buffer and used to surround the gel and membrane to ensure the system remained saturated with transfer buffer at all times. The transfer was performed at 35 volts for 1 hour and 20 minutes and later the membrane was incubated overnight in 1X PBS, 5% skim milk and

5% BSA (Difco, Franklin Lakes, NJ) at 4ºC. The membrane was then incubated for 2 hours at room temperature with shaking in a 1:1000 dilution of anti-BapL antibody in 1X PBS, 0.15% skim milk and 0.15% BSA. The membrane was then washed 4 times for 10 minutes each in 1X PBS, 0.05% Tween-20 (Fisher Scientific) followed by an incubation in a 1:10,000 dilution of goat anti-rabbit-HRP conjugate secondary antibody (Invitrogen) in 1X PBS, 0.15% skim milk and 0.15% BSA for 1 hour at room temperature. The membrane was washed four times for 10 minutes each in 1X PBS, 0.05% Tween-20. The blot was incubated in ECL™ Plus chemiluminesce developing reagent (GE Healthcare). The blot was then wrapped in saran wrap, exposed to X-ray film, and developed in an X-O-Mat.

Reverse-transcriptase PCR. The mRNA levels of *G. vaginalis* grown as a biofilm in BHI and BHIG and as a planktonic culture were analyzed for expression of bapL. Overnight cultures of *G. vaginalis* strain 5-1 were diluted 1:25 in fresh BHI or BHIG in T75 flasks. The flasks were grown anaerobically for 48 hours with fresh media change after 24 hours. Biofilms were washed with deionized, distilled H20 to remove non-adherent cells and bacteria were collected by addition of 1ml ddH20 and scraping of cells from the surface. Planktonic cultures of *G. vaginalis* strain 5-1 were grown in 250 ml flasks of BHI anaerobically for 48 hours with gentle stirring to keep bacteria in the planktonic state. All cultures were centrifuged to pellet the cells and RNA from both planktonic and biofilm cultures were extracted using a Fastpro RNA kit (MP Biomedicals, Solon. OH). Isolated RNA was DNase treated to ensure the absence of genomic DNA contamination. RNA was

converted to cDNA using *bapL* and *G. vaginalis 16S* reverse primers along with Accuscript reverse transcriptase (Stratagene, La Jolla, CA). Syber green PCR reagent containing MgCl2, dNTPs and Taq polymerase was combined with cDNA and either *bapL* or *16S* specific primers (Quantace, Norwood, MA). Amplification was carried out using a thermocycle profile that included: stage 1, 95ºC for 3 minutes; stage 2 consisting of 40 cycles of 95ºC for 15 seconds, 61.5ºC for 15 seconds, 72ºC for 15 seconds; and stage 3 consisting of 81 cycles of 55ºC for 15 seconds.

The absolute Ct values from 3 qPCR assays were averaged to determine the cycle threshold value from each sample. Expression of *bapL* was normalized to *16S* rRNA as determined by cycle threshold values using the following equation: E=2^(16S Ct – bapL Ct).

Immuno-fluorescence microscopy. Cultures of *G. vaginalis* and *L. lactis* were grown overnight as previously stated, and diluted to an equal OD_{600} to ensure equal numbers of bacteria. 1 ml of each diluted culture was centrifuged to pellet the bacteria and washed two times by resuspending with 1 ml of sterile 1X PBS. After washing, the pellets were resuspended with 1 ml of sterile 1X PBS, sonicated with 1 second pulses eight times to ensure the bacteria were not clumped, then a 1:10 dilution of BapL specific polyclonal rabbit serum was added to each tube and incubated at 37ºC for 30 minutes. After the incubation, both bacteria were washed three times with sterile 1X PBS and then a 1:100 dilution of Alexa-488 labeled goat-anti rabbit antibody was added to the tubes and incubated at room temperature in the dark for 30 min. The bacteria were washed an

additional three times, resuspended with 1 ml of PBS, sonicated with 1 second pulses eight times, and a small volume was placed on a microscope slide and observed with an Olympus BX51 fluorescence microscope using a FITC filterset. Both differential interference contrast (DIC) and fluorescence images were taken to localize the fluorescence staining to individual bacteria.

Purification of anti –bapL IgG antibodies. BapL-specific IgG antibodies were purified from harvested rabbit serum by a ProPur Protein G Antibody Purification kit (NUNC™ Thermo Fisher Scienctific, Rochester, NY). Briefly, 12 ml of filtered, harvested rabbit serum containing polyclonal antibodies against BapL were diluted 1:1 with binding buffer A, or 0.1 M sodium phosphate, 0.15 NaCl, pH 7.4. The sample was then pipetted onto an equilibrated Protein G spin column and centrifuged at 150 g for 30 minutes. The spin column was washed twice with 10 ml of binding buffer A to remove unbound contaminants. Bound IgG was eluted with 10ml elution buffer B2, or 0.2M Glycine/HCl pH 2.5 directly into a fresh centrifuge tube containing 1.3 ml neutralization buffer C, or 1 M Tris/HCl ph 9.0, to bring the pH of the sample to approximately 7.5. The tube was swirled to ensure thorough mixing of the final eluate with neutralization buffer C. The elution step was repeated, the samples pooled, and the antibody preparation was de-salted and concentrated using a 10 kDa MWCO ultrafiltration spinner that was supplied. Aliquots of 50 µl of purified BapL antibody were stored at -20ºC until further use.

Antibody inhibition assay of biofilm formation. Overnight cultures of *G. vaginalis* were diluted 1:100 in fresh BHIG and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. 200 µl of each culture was added to a 96-well plate and incubated anaerobically at 37ºC for 24 hours. The wells were washed twice with sterile PBS to remove nonadherent bacteria and stained with safranin to assess biofilm formation. Photographic images of the biofilm were taken by an HP color LaserJet 2820 printer with scanning capabilities (Hewlett-Packard Company).

Antibody inhibition assay of initial adherence. Overnight cultures of *G. vaginalis* were diluted 1:50 in fresh BHIG and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. 200 µl of each culture was added to a 96-well plate and incubated anaerobically at 37ºC for 2 hours. The wells were washed twice with sterile PBS to remove nonadherent bacteria and stained with safranin to assess biofilm formation. Photographic images of the biofilm were taken by an HP color LaserJet 2820 printer with scanning capabilities (Hewlett-Packard Company).

Antibody inhibition assay of aggregation. Overnight cultures of *G. vaginalis* were standardized to ensure equal numbers of bacteria and either treated with PBS as a control, 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. The OD_{600} of the

cultures was measured every 15 minutes for 120 minutes at room temperature and the measurements were plotted and slopes analyzed.

Antibody inhibition assay of adherence to epithelial cells. Overnight cultures of *G. vaginalis* were incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody, standardized via OD_{600} measurements to ensure equal numbers of bacteria then added to 80% confluent monolayers of ME180 cervical epithelial cells. The plates were centrifuged at 600 rpm for 2 minutes to maximize interaction between the bacteria and epithelial cells and incubated at 37ºC for 15 minutes in 5% CO_2. After the incubation, the wells were washed twice with PBS to remove nonadherent bacteria then bacteria were harvested by addition of 5% saponin for 5 minutes. The wells were diluted 10-fold in PBS and 10 µl of each dilution was plated on BPS agar plates and incubated for 48 hours. Colony-forming units were counted and percentage of adherence measured using the equation:

% adherence = (Experimental CFU/ Control CFU) x 100

Extracellular matrix (ECM) protein adherence assay. Overnight cultures of *G. vaginalis* were diluted 1:10 in PBS and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. Immulon plates (Thermo Fisher Scientific) were pre-coated with the ECM proteins fibrinogen, fibronectin, hyaluronic acid, elastin, and collagen IV by diluting the ECM proteins in 100 mM carbonate buffer to a final concentration of 20 µg/ml and adding 200 µl of each per

well and allowing the plate to incubate for 16 hours at 4ºC. 200 µl of each bacterial culture was added to the Immulon plate that had been previously immobilized with ECM proteins. The plates were centrifuged at 600 rpm for 2 minutes to maximize the interaction between the bacteria and ECM proteins then incubated at 37ºC for 10 minutes. After the 10 minute incubation, the wells were washed twice with 200 µl of sterile PBS to remove nonadherent bacteria, plates dried and the wells were stained with 200 µl of safranin. Photographic images of the biofilm were taken by an HP color LaserJet 2820 printer with scanning capabilities (Hewlett-Packard Company).

Mucin adherence assay. Overnight cultures of *G. vaginalis* were diluted 1:10 in PBS and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. Immulon plates (Thermo Fisher Scientific) were pre-coated with porcine mucin (stock 5%) in 100 mM carbonate buffer to a final concentration of 20 µg/ml and 200 µl of the diluted mucin was added to each well and incubated for 16 hours at 4ºC. 200 µl of each bacterial culture was added to the Immulon plate that had been previously immobilized with porcine mucin. The plate was centrifuged at 600 rpm for 2 minutes to maximize the interaction between the bacteria and porcine mucin then incubated at 37ºC for 10 minutes. After the 10 minute incubation, the wells were washed twice with 200 µl of sterile PBS to remove nonadherent bacteria, plates dried and the wells were stained with 200 µl of safranin. Photographic images of the biofilm were taken by an HP color LaserJet 2820 printer with scanning capabilities (Hewlett-Packard Company).

Mucin Dot Blot. A 5% and 1% porcine mucin solution in 1X PBS was dotted onto strips of nitrocellulose membrane. The membranes were allowed to dry at room temperature for 1 hour then incubated for 1 hour at room temperature in 1X PBS with 5% skim milk and 5% BSA. *G. vaginalis* surface proteins were extracted by pelleting an overnight culture of *G. vaginalis*, rinsing the cell pellet in PBS, then sonciating the pellet for 30 seconds three times on ice. Supernatant was collected and protein amount analyzed by OD_{280} measurements. 400 µg of G. vaginalis proteins were allowed to interact with the blocked membranes for 1 hour in 1X PBS, 0.05% Tween-20 (Fisher Scientific). After incubation with the *G. vaginalis* protein, the membrane was then washed 4 times for 10 minutes each in 1X PBS, 0.05% Tween-20, followed by an incubation with 10 µg/ml of purified anti-BapL antibody in 1XPBS, 0.05% Tween-20, 0.15% skim milk and 0.15% BSA for 1 hour. After the primary antibody, the membrane was washed as described above, and incubated with a 1:10,000 dilution of goat anti-rabbit-HRP conjugate secondary antibody (Invitrogen) in 1X PBS, 0.15% skim milk and 0.15% BSA for 45 minutes at room temperature. The membrane was then washed four times for 10 minutes each in 1X PBS, 0.05% Tween-20. The blot was incubated in ECL™ Plus chemiluminesce developing reagent (GE Healthcare). The blot was then wrapped in saran wrap, exposed to X-ray film, and developed in an X-O-Mat.

Opsonophagocytosis assay. White blood cells (WBC) were prepared from fresh human blood collected from a healthy adult volunteer. 25 ml was mixed with an equal volume of

dextran-heparin buffer and incubated at 37°C for 1 hr. The upper layer containing the leukocytes was collected, the cells were pelleted by centrifugation, and hypotonic lysis of the remaining erythrocytes was accomplished by resuspsion of the cell pellet in ddH_20. WBC were then washed three times and resuspended in RPMI buffer (Gibco). A hemocytometer was used to give a final WBC count of 1.5 X 10^6 WBC per 100 µl. The *G. vaginalis* bacterial strains to be evaluated for phagocyte-dependent killing activity of the anti-BapL antibody were grown anaerobically overnight in BHIG on 0.1 mm glass beads to stimulate biofilm formation. Biofilms on the glass beads were dispersed by sonication on ice for 10 seconds. The complement source was 50 µl of baby rabbit serum diluted in RPMI buffer.

The opsonophagocytic assay was performed with 100 µl of leukocytes, 50 µl of either dispersed bacteria or biofilm beads, 50 µl of complement solution, and 50 µl of 1:20 diluted anti-BapL antibody or preimmune antibody. The reaction mixture was incubated on a rotor rack at room temperature for 90 min. The tubes were sonicated for 10 sec and 10-fold diluted in RPMI buffer and plated on BPS agar plates. The percentage of killing was calculated by determining the ratio of CFU surviving in tubes with bacteria, leukocytes, complement and antibody to CFU surviving in control tubes. The percent killing was compared between biofilm samples and dispersed biofilm samples.

Sandwich western of ME180 proteins probed with G. vaginalis proteins. Monolayers of ME180 cervical epithelial cells were grown in T75 flasks until 100% confluent. Cells were harvested by trypsin digestion, centrifuged at 1000 rpm for 12 minutes to remove the

trypsin then resuspended in 1 ml of sterile PBS. Proteins were extracted by the addition of 200 µl of NuPAGE loading buffer and incubation for 10 minutes at room temperature while rocking. ME180 cervcial epithelial cell proteins were separated on NuPAGE Bis-Tris 4-12% gels as described earlier. Following electrophoresis, the polyacrylamide gel was rinsed in deionized water. PVDF membrane was wetted in 100% methanol then incubated in 1X transfer buffer (20X NuPAGE transfer buffer and 10% methanol). Sponges were wetted in transfer buffer and used to surround the gel and membrane to ensure the system remained saturated with transfer buffer at all times. The transfer was performed at 35 volts for 1 hour and 20 minutes following which the membrane was incubated for 1 hour in 1X PBS, 5% skim milk and 5% BSA (Difco, Franklin Lakes, NJ) at 4ºC. The membrane was then incubated for 1 hour with 200 µg of *G. vaginalis* surface proteins. *G. vaginalis* surface proteins were extracted by pelleting an overnight culture of *G. vaginalis*, rinsing the cell pellet in PBS, then sonciating the pellet for 30 seconds three times on ice. Supernatant was collected and protein amount analyzed by OD_{280} measurements. After incubation with the *G. vaginalis* protein, the membrane was then washed 4 times for 10 minutes each in 1X PBS, 0.05% Tween-20 (Fisher Scientific) followed by an incubation with 10 µg/ml of purified anti-BapL antibody in 1XPBS, 0.05% Tween-20, 0.15% skim milk and 0.15% BSA for 1 hour. After the primary antibody, the membrane was washed as described above, and incubated with a 1:10,000 dilution of goat anti-rabbit-HRP conjugate secondary antibody (Invitrogen) in 1X PBS, 0.15% skim milk and 0.15% BSA for 45 minutes at room temperature. The membrane was then washed four times for 10 minutes each in 1X PBS, 0.05% Tween-20. The blot was incubated in

ECL™ Plus chemiluminesce developing reagent (GE Healthcare). The blot was then wrapped in saran wrap, exposed to X-ray film, and developed in an X-O-Mat.

Immunoprecipitation of ME180 proteins that interact with BapL. The buffer of purified anti-BapL antibody was exchanged and desalted using Econo-Pac 10DG columns from the Affi-Gel® Hz Immunoaffinity Kit (BioRad, Hercules, CA). The desalted purified anti-BapL antibody was then coupled to Affi-Gel Hz Hydrazide Gel using the Affi-Gel® Hz Immunoaffinity Kit (BioRad). Briefly, that purified anti-BapL antibody was oxidized by adding one-tenth the volume of sodium periodate to the purified IgG sample. This mixture was rotated end-over-end for 1 hour at room temperature. The oxidized antibody was then desalted again as described previously. The oxidized, desalted anti-BapL antibody was incubated end-over-end for 16 hours at room temperature with washed Affi-Gel Hz Hydrazide Gel. After the coupling reaction was complete, the gel was washed with PBS 0.5 M NaCl, pH 7.4. 10 mg of *G. vaginalis* protein and 10 mg of ME180 cervical epithelial cell protein was allowed to interact for 30 minutes at room temperature while rotating. This mixture was added to the washed Affi-Gel Hz Hydrazide Gel and allowed to rotate for 2 hours at 4ºC. The Affi-Gel Hz Hydrazide Gel was then placed into a column, washed 2 times with PBS 0.5 M NaCl pH 7.4 and 2 times with PBS 50 mM NaCl pH 7.4. Bound protein was eluted by addition of 10 ml of 0.2M Glycine/HCl pH 2.5 into a tube containing 1.3 ml 1M Tris/HCl pH 9.0 to neutralize the protein solution. The protein was concentrated using a 3 kDa MWCO filtration spinner. The protein was separated by SDS-

PAGE and the gel was stained overnight with Imperial Protein stain (Thermo Scientific), following by destaining in water.

Ethyl methanesulfonate (EMS) mutagenesis. A kill curve using liquid EMS (Sigma, molecular weight 124.16) amounts of 10 µl, 50 µl, and 250 µl for 5 minutes, 15 minutes, 30 minutes and 1 hr showed that 50 µl of EMS for 30 minutes produced approximately 90% killing of *G. vaginalis* bacteria. 3 mls of an overnight culture of *G. vaginalis* was centrifuged to collect the cells. The bacteria were washed once with sterile PBS and resuspended in 500 µl of sterile PBS. 50 µl of stock EMS compound was added to the bacteria and allowed to rock at 37°C for 30 minutes. After the 30 minute incubation, the bacteria were rinsed four times with sterile BHIG. The final resuspension was in 1 ml of BHIG; this was allowed to incubate for 24 hours anaerobically at 37°C. An additional 5 ml of sterile BHIG was added to the mutagenized bacteria after the 24 hour incubation; this was allowed to incubate for an additional 24 hours before being used for FACS analysis. EMS is highly toxic and appropriate precautions were used including a lab coat and eye protection. EMS usage was in a chemical safety hood and all equipment that came into contact with EMS was rinsed in 1M NaOH for at least 24 hours before being disposed of appropriately.

Fluorescence-activated cell sorting (FACS) staining and analysis. A 48 hour EMS mutagenized culture of *G. vaginalis* was centrifuged to collect the cells. The pellet was washed two times with sterile PBS and sonicated for 8 one second pulses to ensure the

bacteria were not clumped. The bacteria were stained with a 1:10 dilution of anti-BapL antibody for 30 minutes at 37°C. After this incubation, the bacteria were collected by centrifugation and washed two times with sterile PBS. The bacteria were then stained with a 1:100 dilution of Alexa-488 labeled goat anti-rabbit secondary antibody in the dark at room temperature for 30 minutes. After this incubation, the bacteria were collected by centrifugation and washed two times with sterile PBS, before being sonicated for 3 one second pulses. These stained bacteria were then analyzed by FACS, gating on fluorescence intensity. Individual bacterial cells with low fluorescence intensity, signifying a potential *bapL* mutant, were collected in wells of a 96-well plate. After the FACS run, the 96-well plate harboring low fluorescent bacteria was incubated anaerobically at 37°C for 48 hours. Wells with growth after the 48 hour incubation were collected and added to snap-cap tubes containing 5 ml of BHIG. These tubes were allowed to incubate anaerobically at 37°C for 24 hours before the culture was analyzed for biofilm formation using the 96-well assay described previously.

Long-Distance PCR of the bapL gene. Long-distance PCR reactions were performed in a TC-312 Techne thermocycler (Techne). Primers used were BapSbfFWD and BapSbfREV which incorporated Sbf1 restriction sites onto each end of the PCR product (**Table 2**). PCR reaction mixtures consisted of 2.5 mM dNTPs, 5X Phire Buffer, 100 pM forward and reverse primers, F-120S Phire Hot start polymerase (Finnzymes, Espoo, Finland), water and genomic DNA.

Conditions for PCR were as follows, one initial cycle at 94ºC for 2 minutes followed by 10 cycles of: 94ºC 30 seconds to denature, 52ºC 30 seconds to hybridize, and 70ºC 10 minutes for extension. Immediately following, there was a second step with 30 cycles of: 94ºC 30 seconds to denature, 56ºC 30 seconds to hybridize, and 72ºC 10 minutes for extension. A final extension at 72ºC for 15 minutes after the final cycle and the reactions were chilled to 4ºC. A two cycle protocol was used; cycle one utilized a lower annealing temperature to increased the number of *bapL* gene products that could be used for amplification, while cycle two utilized a higher annealing temperature that produced highly specific amplification of the *bapL* gene.

Production of pRB473-bapL for heterologous expression. The 9.1 kb *bapL* PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen) as described by the manufacturer. The resulting PCR product was subcloned into the plasmid pCR2.1®-Topo (Invitrogen) to construct the recombinant plasmid pCR2.1-Topo-*bapL*. This plasmid was used to transform CH3 blue chemically competent *E.coli* cells (Bioline) and plated on LB ampicillin 100µg/ml plates. The recombinant plasmid was purified from pooled colonies of *E. coli* and subjected to Sfb1 restriction digest using its specific buffer and BSA per the manufacturer's instructions (New England Biolabs) to drop out the *bapL* PCR product. The digested *bapL* PCR product was ligated into the Pst1-linearized *E. coli*/*S. aureus* shuttle vector pRB473 (amp^{100}/cm^{10}) using Ready-to-Go T4 DNA Ligase tubes (Amersham Biosciences) with an hour incubation at 16ºC. 2 µl of purified plasmid DNA

was added to 50 µl of CH3 blue chemically competent *E. coli* cells and transformed as described above.

Plasmid mini-preps were prepared from individual *E. coli* transformants that were grown overnight in 5 ml of LB broth with ampicillin concentration of 100 µg/ml, using a QIAprep Spin Miniprep Kit (Qiagen), as described by the manufacturer. Plasmids were screened for correct insert by digestion with Pst1 (New England Biolabs). Plasmids with correct insert sizes were used to transform *S. aureus* RN4220.

Production of electrocompetent S. aureus RN4220. Electrocompetent *S. aureus* cells were prepared from cultures grown in TSB at 37ºC with shaking. Cultures were grown to exponential phase (OD_{600}= 0.5-0.8) and then incubated on ice for 10 minutes. The cultures were collected by centrifugation for 15 minutes at 4,000 rpm at 4ºC. Care was taken to ensure all subsequent steps were performed on ice. The pellet was then washed three times with one tenth the volume of ice-cold sterile water. The pellet was then washed one time with one fifth the volume of cold sterile 10% glycerol. The pellet was then resuspended in one tenth the volume of ice-cold sterile 10% glycerol and incubated at room temperature for 15 minutes. The culture was collected by centrifugation for 5 minutes at 4,000 rpm at 4ºC, resuspended in 500 µl of ice-cold 10% glycerol, and aliquots (100 µl) were then stored at -80ºC until further use.

Transformation of electrocompetent S. aureus. Plasmid DNA isolated as described above was used to transform *S. aureus* RN220. Plasmid DNA (5 µl) was added to 75 µl of

electrocompetent *S. aureus* and incubated on ice for 10 minutes. The mixture was then transferred to an electroporation cuvette with a gap length of 0.1 cm and pulsed one time using the MicroPulser (BioRad) pre-set *S. aureus* setting Sta (1.8 kV, 2.5 msec, 25 µF). Following electroporation, 250 µl of LB broth was added immediately to the cuvette and this mixture was then transferred to an eppendorff tube. The antibiotic to be used for selection was added at a subinhibitory concentration (chloramphenicol = 0.1 µg/ml) and the cells were incubated for 1 hour with shaking at 37ºC. Aliquots (75 µl and 225 µl) were then spread on TSA supplemented with appropriate antibiotic selection and incubated at 30ºC for 48 hours.

S. aureus biofilm assay. Biofilms were grown using 96 well Corning Cell Bind Plates (Corning). Overnight cultures of *S. aureus* RN4220 with empty vector or pRB473-*bapL* or *S. aureus* RN4220Δ*ica* with empty vector or pRB473-*bapL* were diluted 1:10 in fresh media and individual wells of a sterile, polystyrene 96 well tissue culture plate were inoculated with 0.2 ml of dilute culture. The tissue culture plates were incubated for 24-72 hrs at 37 °C, under aerobic conditions. Growth was analyzed using a 96-well plate spectrophotometer with a 595nm filter (BioTek 800 Plate reader, Winooski, VT). Spent medium was removed, wells were washed with 200 µl once with phosphate buffered saline (PBS) to remove nonadherent bacteria, and the plates were air dried for 1 hr. Biofilms were stained with 200 µl of safranin for one min, wells were washed gently to remove the safranin, and the plates were imaged using a HP color LaserJet 2820 printer with scanning capabilities (Hewlett-Packard Company).

Poly-N-acetyl glucosamine (PNAG) blot. Standardized amounts of overnight bacterial cultures were centrifuged to pellet the cells and resuspended in 250 µl of 0.5 M EDTA. The samples were boiled for 5 minutes then centrifuged to pellet cell debris. The supernatant was transferred to a new tube, 20 µl of proteinase K was added then the sample was heated at 65ºC for 1 hour. After the 1 hour incubation, the samples were boiled for 5 minutes to inactivate the proteinase K. The samples were sonicated for 3-5 seconds and dilutions made in 1X PBS. A piece of nitrocellulose blotting membrane (BioTrace NT, Pall Corporation) was cut to fit inside the blot apparatus (7.5 cm wide by 11.5 cm long). The cover paper was removed from the nitrocellulose and the membrane was soaked in 50 ml of 5% methanol for 1-2 minutes. The nitrocellulose membrane was rinsed in 50 ml of 1X PBS; the nitrocellulose membrane was placed within the blotting apparatus and care was used to ensure that the instrument gasket had produced a tight seal. 200 µl of stock or diluted PNAG samples were loaded into individual wells in the blotting apparatus and the machine was allowed to run for 10 minutes or until all sample have been soaked up by the membrane. The membrane was removed carefully and allowed to dry overnight between pieces of kimwipe.

The dried nitrocellulose membrane was soaked in 25 ml of 5% bovine serum albumin (BSA) solution for 1 hour at room temperature on a rocker. The membrane was probed with 15 ml of primary antibody solution containing 3 µl of goat anti-PNAG antibody (1:5000 dilution), 1 ml 5% BSA solution, and 14 ml of 1X PBS for 3 hours at room temperature on a rocker. The membrane was washed 3X with 15 ml of 1X PBS for 4

minutes each. After the wash steps, the membrane was probed with l5 ml of secondary antibody solution containing 1.5 µl of rabbit anti-goat-HRP labeled antibody (1:10,000 dilution) and 15 ml of 1X PBS for 1 hour at room temperature on a rocker. The membrane was again washed 3X with 15 ml of 1X PBS for 4 minutes each. The blot was incubated in ECL™ Plus chemiluminesce developing reagent (GE Healthcare). The blot was then wrapped in saran wrap, exposed to X-ray film, and developed in an X-O-Mat.

Phage Transduction. The generalized transducing phage 80φ was used to create the allelic exchange mutation of *ica* in *S. aureus* RN4220. A freezer stock of Sa113 Δ*ica* was inoculated onto TSA supplemented with erythromycin, and grown overnight at 37ºC. A single colony was subcultured onto a new plate and allowed to grow overnight at 37ºC. All of the culture was removed from the plate and resupended in 0.5 ml of TSB in a small tube. 200 µl of this culture was added to 0.4% soft agar in a new tube, along with 5 µl of 2M $CaCl_2$ and either 75 µl of phage 80φ or no phage. This mixture was plated on TSA plates and monitored for lysis.

Following lysis, the soft agar was collected, centrifuged, and the supernatant was filter sterilized through a 0.45 µm filter. Aliquots of this lysate, either 75 or 150 µl, were then mixed with 150 µl of recipient bacteria, or *S. aureus* RN4220, and 5 µl of 2M $CaCl_2$. The mixture was incubated with shaking at 37ºC for exactly 20 minutes, 5 ml of 20mM sodium citrate was added, the culture was centrifuged to pellet the bacteria, the pellet washed twice with 20 mM sodium citrate, then incubated for 1 hour and 45 minutes in 500

µl of 20 mM sodium citrate. Finally, the mixture was plated on TSA supplemented with erythromycin and sodium citrate and these plates were incubated for 48 hours at 37ºC.

CHAPTER 3

Analysis of Virulence Potential of *Gardnerella vaginalis*

Introduction

Gardnerella vaginalis is present in up to 95% of cases of BV (Catlin 1992; Marrazzo, Thomas et al. 2008); however, with the advancement of molecular tools, it has been shown that the number and diversity of anaerobes associated with BV is high (Oakley, Fiedler et al. 2008). One study found that pure cultures of *G. vaginalis* do not always cause BV and the organism can occur, albeit in low numbers, in healthy women (Gardner and Dukes 1955; Fredricks, Fiedler et al. 2007). Together, these findings cast doubt on the role of *G. vaginalis* in the pathogenesis of BV. Recently, the term functional equivalent pathogen/pathogroup (FEP) has been proposed for polymicrobial infections in which no one species alone is capable of causing disease, but rather the synergistic effects of the consortium give rise to the pathophysiology (Dowd, Wolcott et al. 2008). Inarguably, the vagina is colonized by numerous anaerobic bacteria during BV, but whether the symptoms and the syndrome are a consequence of the multi-species consortium or whether there is a key species in each case that precipitates the disorder has been a matter of debate for decades. A follow-up study to the report that *G. vaginalis* in pure culture did not reliably cause BV found that fresh cultures initiated the infection more frequently (Criswell, Ladwig et al. 1969). This study may suggest that under conditions

that foster expression of virulence determinants, *G. vaginalis* may have greater pathogenic potential. Furthermore, recent studies indicate that *G. vaginalis* is equipped with a number of virulence properties and consequently, the idea that it is the etiologic agent of BV is being revisited (Swidsinski, Mendling et al. 2005; Gelber, Aguilar et al. 2008).

One such virulence determinant is the ability of *G. vaginalis* to form a biofilm. Swidsinski et al. showed that *G. vaginalis* was able to form an thick, tenacious biofilm on the vaginal epithelium of women with BV (Swidsinski, Mendling et al. 2005). This biofilm incorporated other bacterial groups into its layers, suggesting that the biofilm may enable other anaerobes to colonize the vagina. Not only could formation of a biofilm allow other anaerobes to colonize the vagina, but the biofilm phenotype allows its constituent bacteria to be resistant to many negative environmental stimuli. This biofilm phenotype may explain the high rates of relapse seen with BV.

G. vaginalis also produces the toxin vaginolysin, which is a member of the cholesterol-dependent family of pore-forming toxins that lyse human red blood cells and vaginal epithelial cells (Gelber, Aguilar et al. 2008). Vaginolysin has been hypothesized to be involved in the pathogenesis of BV. *G. vaginalis* can also adhere well to vaginal epithelial cells and attaches equally well to exfoliated vaginal epithelial cells and to tissue culture cells (Sobel, Schneider et al. 1981; Scott and Smyth 1987). Finally, not only does *G. vaginalis* have numerous virulence factors that could contribute to the pathogenesis of BV, it has also been implicated as the sole etiologic agent of infections outside of the reproductive tract. There have been reports of *G. vaginalis* as the causative agent of vertebral osteomyelitis and discitis, retinal vasculitis, acute hip arthritis, and bacteremia

(Lagace-Wiens, Ng et al. 2008; Graham, Howes et al. 2009; Neri, Salvolini et al. 2009; Sivadon-Tardy, Roux et al. 2009).

Many of the bacterial species associated with BV have evolved to exist only in humans and consequently a reliable animal model for BV does not exist. This has stymied efforts to tease out the pathogenesis of BV, hence studies of the etiology of BV are lacking. Reports of the frequency with which different species are isolated exist but point only to association and not cause. For example, *Atopobium vaginae* is frequently isolated from BV and one study indicates its potential to induce an inflammatory response, but there has not been a demonstration that it is equipped with specific virulence factors (Libby, Pascal et al. 2008). In an effort to begin to unravel this etiologic mystery, we designed a simple model to test adherence to vaginal epithelial cells and used established models to analyze biofilm forming capacity and cytotoxicity of *G. vaginalis* and a number of additional BV isolates including *A. vaginae*, *Prevotella bivia*, *Mobiluncus mulieris*, a *Veillonella* sp., a *Peptostreptococcus* sp., a *Peptoniphilus* sp., and *Fusobacterium nucleatum* (Verstraelen, Verhelst et al. 2004; Nikolaitchouk, Andersch et al. 2008; Oakley, Fiedler et al. 2008).

Results

Adherence of BV-associated anaerobes to cervical epithelial cells

We analyzed the ability of *G. vaginalis* strains 5-1, 465, and 101-1 to bind to ME-180 cervical epithelial cells. Overnight cultures were standardized to ensure equal numbers of bacteria. The bacteria were stained with a green fluorophore, and allowed to

interact with the monolayer of red fluorescent-labeled ME-180 cervical epithelial cells for 15 minutes before washing to remove nonadherent bacteria. **Figure 4** shows representative confocal microscopic images of the *G. vaginalis* adherence assay. All *G. vaginalis* strains tested exhibited considerable adherence. We next analyzed the ability of BV isolates: *A. vaginae*, *M. mulieris*, *P. bivia, Veillonella*, *Peptostreptococcus*, *Peptoniphilus*, and *F. nucleatum* (**Table 1**) to bind to ME-180 cervical epithelial cells. **Figure 5** shows representative confocal microscopic images of red ME-180 cells and green BV-associated bacteria; *G. vaginalis* is shown as a positive control. Both *G. vaginalis* and *Peptoniphilus* exhibited considerable adherence. *Peptoniphilus* adhered more homogenously to the ME-180 cells whereas *G. vaginalis*, which is very aggregative, adhered as large clusters. As shown in **Figure 5**, *F. nucleatum* and *A. vaginae* exhibited some adherence to the epithelial cells, although to a significantly lesser degree than *G. vaginalis* or *Peptoniphilus*. *M. mulieris*, *P. bivia* strain 715BVD, *P. bivia* strain 29303 (data not shown), *Veillonella*, and *Peptostreptococcus* were unable to adhere to the cervical epithelial cells under these conditions. **Table 4** lists the adherence scores for each species after the 15 minute incubation with the monolayer; the scores correlate with the confocal microscopy images. Each experiment was repeated three times with similar results

Figure 4. Adherence of *G. vaginalis* to cervical epithelial cells. Bacteria were grown in sBHIG anaerobically at 37 °C for 24 hrs. Bacteria (green) were standardized to ensure equal numbers, added to vaginal epithelial cells (red) and incubated for 15 minutes before washing to remove nonadherent bacteria. Qualitative estimates of adherence were ascertained by confocal microscopy.

GV adherence
Control-GV only
Control-ME180 only

Figure 5. Adherence of *G. vaginalis* and BV-associated anaerobes to cervical epithelial cells. Bacteria were grown in sBHIG anaerobically at 37ºC for 24 hours. Bacteria (green) were standardized to ensure equal numbers, added to cervical epithelial cells (red) and incubated for 15 minutes before washing to remove nonadherent bacteria. Qualitative estimates of adherence were ascertained by confocal microscopy. *P. bivia* strain 29303 is not shown but did not exhibit adherence.

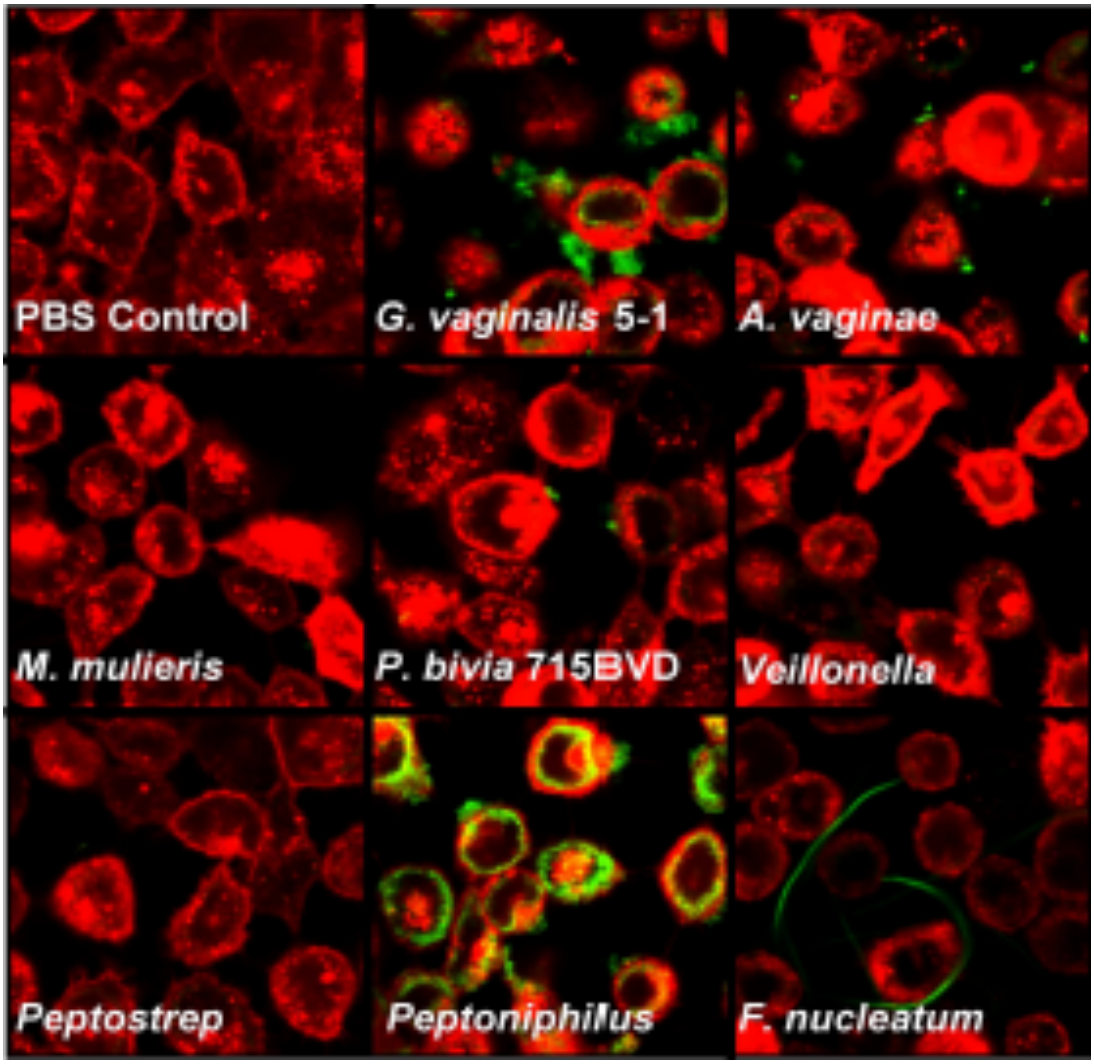

PBS Control
G. vaginalis 5-1
A. vaginae
M. mulieris
P. bivia 715BVD
Veillonella
Peptostrep
Peptoniphilus
F. nucleatum

Table 4. Adherence and Cytotoxicity scores for the BV-associated anaerobes.

Strain	**Cytotoxicity score***	**Adherence score†**
PBS control	0	-
G. vaginalis 5-1	5	+++
G. vaginalis 101-1	5	+++
G. vaginalis 465	5	+++
A. vaginae FA	0	++
M. mulieris BV26-9	0	±
P. bivia 29303	0	±
P. bivia 715BVD	0	±
Veillonella sp. 715BVA	0	-
Peptostreptococcus sp. 715BVC	0	-
Peptoniphilus sp.	0	+++
F. nucleatum 718BVC	0	+

Adherence and cytotoxicity scores of the bacterial strains. Adherence scores ranged from (-), indicating no bacteria atdhered to the ME180 cells, to (+++), indicating many bacteria adhered to the ME180 cells. Cytotoxicity scores ranged from 0, indicating no change, to 5, indicating complete lysis of the ME180 monolayer. Experiments were repeated three times and produced similar results each time.

Cytotoxicity of *G. vaginalis*

G. vaginalis strains 5-1, 465, and 101-1 were tested for their ability to cause cell rounding and lysis of ME-180 cervical epithelial cells. Equal numbers of each bacterial species were added to a confluent monolayer of ME-180 cervical epithelial cells. Cytotoxicity scores (**Table 4**) and microscopic images (**Figure 6**) of the monolayer were recorded after 4 hours. All *G. vaginalis* strains tested were a 5 on the cytotoxicity scale, causing complete lysis of the monolayer within 4 hours. Supernatant from a *G. vaginalis* culture was also tested for cytotoxicity; however, supernatant had no cytopathic effect on the cervical epithelial cells.

Trans-well assay to elucidate contact dependence of *G. vaginalis* cytotoxicity

Other cholesterol-dependent cytolysins depend upon adherence to exert their activity (Madden, Ruiz et al. 2001). To investigate whether *G. vaginalis* requires direct contact with cervical epithelial cells to exert its cytotoxic effects, we performed cytotoxicity assays as described above; however, the bacteria were separated from the cervical epithelial cell monolayers with trans-well filters with a pore size of either a 0.45 µm or 8.0 µm. The trans-well filter with a pore size of 0.45 µm prevents direct contact of the bacteria and monolayer but does allow proteins produced by *G. vaginalis* to interact with the monolayer; whereas, the trans-well filter with a pore size of 8.0 µm allows direct contact of the bacteria with the monolayer. Cytotoxicity was observed when the 8.0 µm transwell was used but not when the 0.45 µm transwell separated the bacteria from the

Figure 6. Cytotoxic changes of cervical epithelial cell monolayers challenged with *G. vaginalis* strains. Bacteria were grown in sBHIG anaerobically at 37ºC for 24 hours, standardized to ensure equal numbers, added to cervical epithelial cells and incubated for 4 hours. Light microscopy images were taken after the 4 hour incubation. (magnification 100x)

100X
Control - PBS
100X
Gardnerella Bacteria
100X
Gardnerella supernatant

epithelial cells (**Figure 7**) suggesting that direct contact between the bacteria and monolayer is required for cytotoxicity.

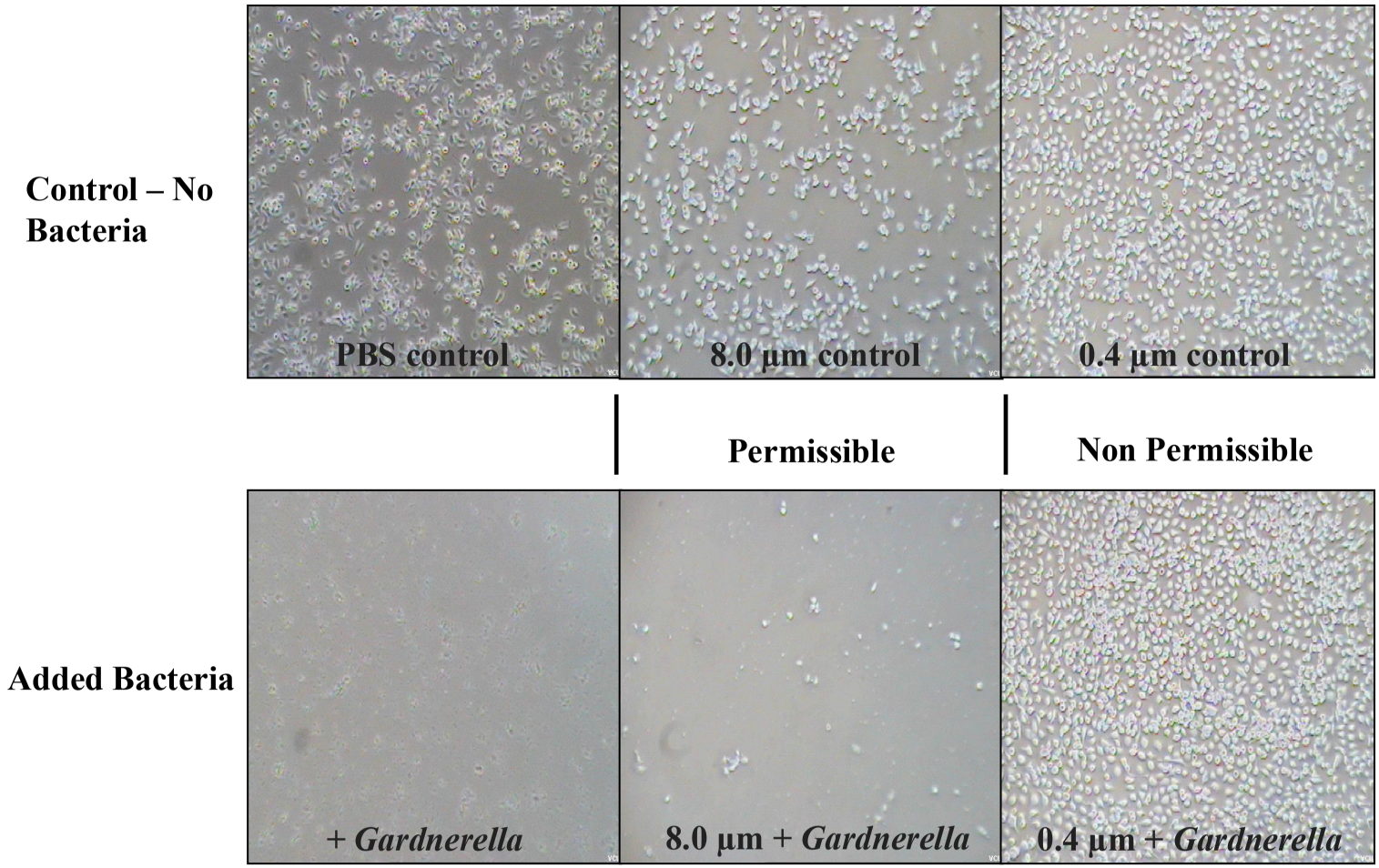
Cytotoxicity of the BV-associated anaerobes

The BV-associated anaerobes were tested for their ability to cause cell rounding and lysis of ME-180 cervical epithelial cells. Equal numbers of each bacterial species were added to a confluent monolayer of ME-180 cervical epithelial cells. Cytotoxicity scores (**Table 4**) and microscopic images (**Figure 8**) of the monolayer were recorded after 4 hours. As shown in **Figure 8**, only the *G. vaginalis* strains induced rounding and cell death of the ME-180 cells. All of the *G. vaginalis* strains were given a score of 5 on the cytotoxicity scale, indicating complete lysis of the monolayer within 4 hours. All other anaerobes tested lacked detectable cytotoxic activity and remained a zero on the cytotoxicity scale. Each experiment was repeated three times with similar results.

Biofilm formation by BV-associated anaerobes

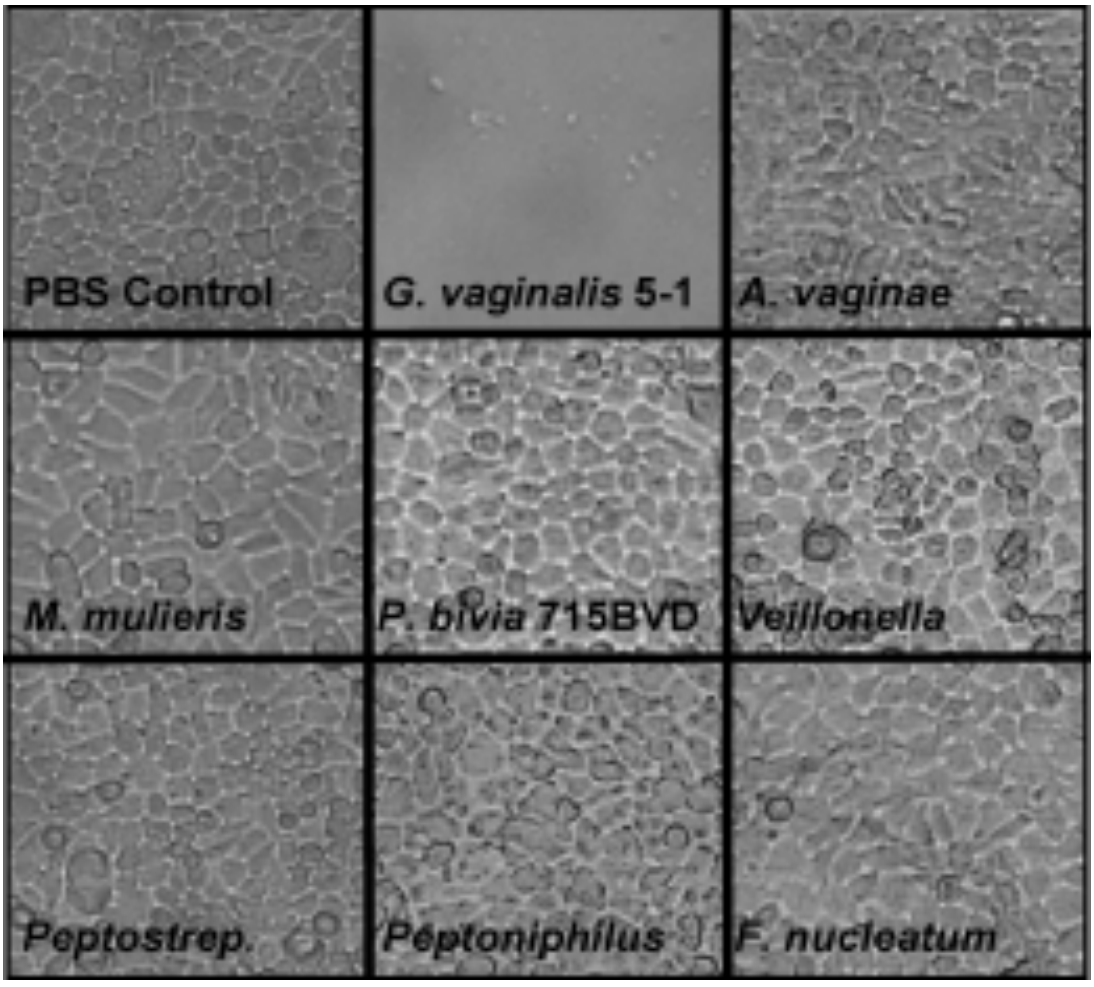
Biofilm assays were performed to analyze the biofilm-forming potential of *G. vaginalis* and other BV-associated anaerobes. When cultured overnight on tissue-culture-treated polystyrene, *G. vaginalis* stains 5-1, 465 and 101-1 produced a thick, tenacious biofilm (**Figure 9A**). *F. nucleatum* was able to produce a moderate, fluffy biofilm that was partially dislodged by washing. The ease with which *F. nucleatum* biofilms were dislodged by washing is reflected by the large standard deviation in **Figure 9B**. The other anaerobes tested: *A. vaginae, M. mulieris, P. bivia, Veillonella, Peptostreptococcus,* and

Figure 7. Cytotoxicity caused by *G. vaginalis* strains is contact-dependent. Bacteria were grown in sBHIG anaerobically at 37ºC for 24 hours. Equal numbers of bacteria were loaded into trans-well filters containing either 0.45 μm or 8 μm pores. The cells were monitored microscopically every hour for a 4 hour incubation and light microscopy images were taken after the 4 hour incubation. (magnification 100x)



(Magnification x 100)

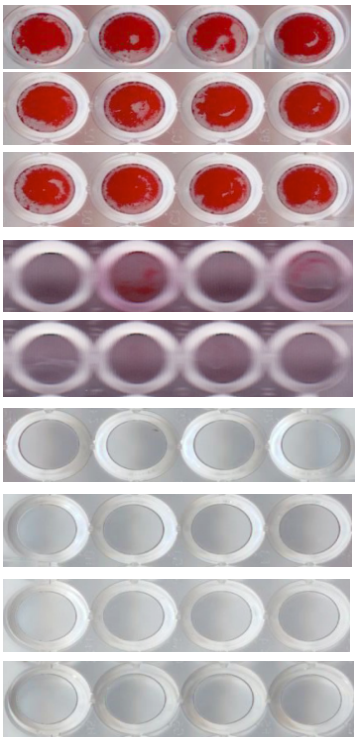
Figure 8. Cytotoxic changes of cervical epithelial cell monolayers challenged with various BV-associated anaerobes. Bacteria were grown in sBHIG anaerobically at 37ºC for 24 hours, standardized to ensure equal numbers, added cervical epithelial cells and incubated for 4 hours. Light microscopy images were taken after the 4 hour incubation. *P. bivia* strain BVD is not shown but was similar to *P. bivia* strain 29303, which did not exhibit cytotoxicity. (magnification 1000x)



(Magnification x 1000)

Figure 9. Biofilm formation of *G. vaginalis* strains and various BV-associated anaerobes. (A) Bacteria were grown in sBHIG anaerobically at 37ºC for 24 hours. Nonadherent cells were removed from the wells and the adherent bacteria were stained with safranin. OD_{595} readings were determined to ensure similar growth of each species. Experiments were repeated 3 times with similar results and a representative image is shown. (B) Quantitiative assessment of the biofilm-forming capacities were made by dissolving the safranin stain in 33% acetic acid and measuring OD_{562}. 8 technical replicates and 3 biologic replicates (N=24) were performed on each stain and the absorbance readings were averaged. Error bars represent standard deviations. *P. bivia* strain BVD is not shown but was similar to *P. bivia* strain 29303, which did not exhibit biofilm formation.

A.



G. vaginalis 5-1

G. vaginalis 101-1

G. vaginalis 465

Fusobacterium sp.

Peptostreptococcus sp.

Veillonella sp.

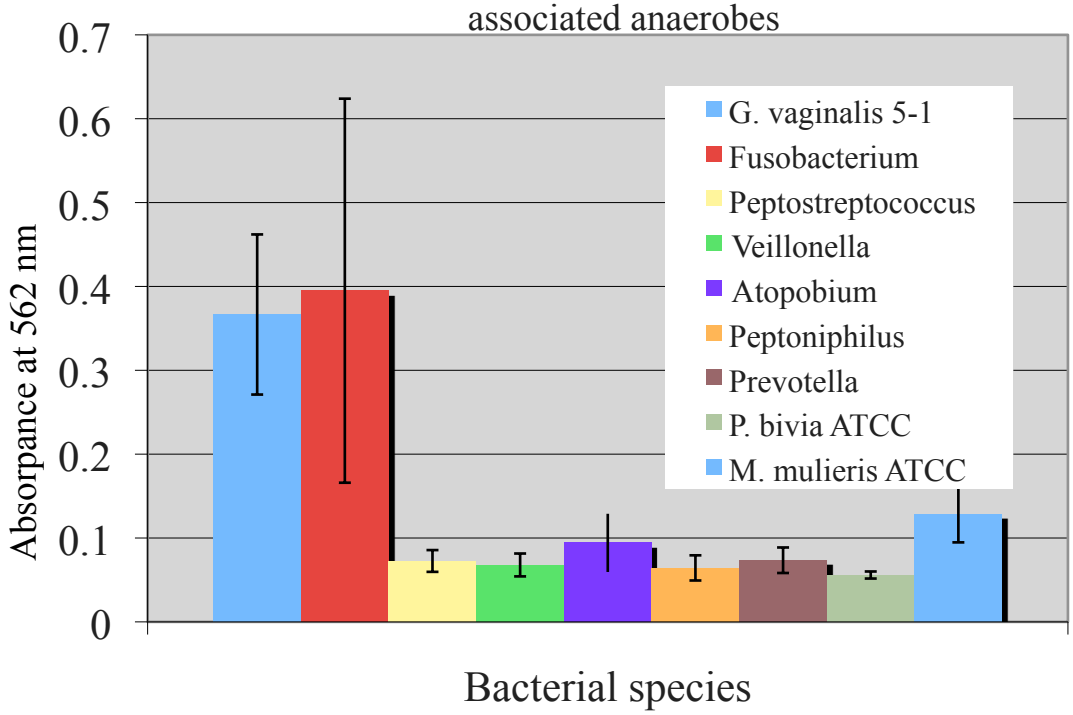
Atopobium sp.

Prevotella sp.

Peptoniphilus sp.

B.

Biofilm formation by *G. vaginalis* strains and other BV-associated anaerobes



Peptoniphilus, demonstrated significantly lower biofilm formation relative to *G. vaginalis* (student's t-test, $p<0.0001$).

Discussion

Despite the fact that *G. vaginalis* can be isolated in greater than 95% of BV cases, a study with healthy human volunteers indicating that pure cultures of the bacteria did not always cause BV, cast perpetual doubt on its pathogenic potential (Gardner and Dukes 1955; Criswell, Ladwig et al. 1969). However, the link between vaginolysin and BV along with studies showing *G. vaginalis* as the predominant species in the biofilm that forms on the vaginal epithelium during BV suggests that the role of this species as a pathogen should be readdressed.

As animal models for BV are lacking, we designed a series of in vitro experiments to compare the relative virulence capacities of *G. vaginalis* and other BV-associated anaerobes. Adherence is a key first step in pathogenesis, as a potential pathogen must first adhere to host tissues in order to avoid clearance by host defense mechanisms such as the mucociliary escalator, urine flow, and the flow of vaginal secretions. Furthermore, adherence is the first step in the formation of a biofilm and the capacity to form a biofilm on polystyrene is irrelevant to pathogenesis unless the bacteria can first adhere to the vaginal epithelium. We therefore analyzed the ability of BV-associated anaerobes to adhere to cervical epithelial cells in tissue culture. Only, *G. vaginalis* and *Peptoniphilus* adhered avidly to ME-180 cervical epithelial cells. *G. vaginalis* tended to form clumps of bacteria that adhered to epithelial cells, while *Peptoniphilus* was more evenly distributed

around the borders of the epithelial cells. Our data confirms previous studies showing that *G. vaginalis* adheres to McCoy cells and human red blood cells (Scott, Curran et al. 1989). *Peptoniphilus* has been only recently named and is associated with persistent cases of BV, with 36% of persistent cases of BV having the organism (Marrazzo, Thomas et al. 2008). This is the first study demonstrating its robust adherence to cervical epithelial cells, which is an interesting and potentially important observation. Perhaps *Peptoniphilus* associates with *G. vaginalis* during cases of BV and leads to the pathogenesis seen with the disorder; this hypothesis warrants more research into the role *Peptoniphilus* plays in the pathogenesis of BV. *A. vaginae* and *F. nucleatum* exhibited only moderate adherence, and adherent *M. mulieris*, *P. bivia* and *Peptostreptococcus* were not detected. The process of dental biofilm formation is initiated by bacterial species with affinity for the tooth pellicle such as the streptococci and actinomycetes. These species are referred to as initial colonizers. Other bacterial species adhere to the initial colonizers and this leads to the development of a complex biofilm community composed of a large variety of species, many of which are unable to adhere to teeth on their own. Specifically, *F. nucleatum* expresses receptors that adhere to a large variety of other bacterial species and acts as a bridge between early and late colonizers (Kolenbrander, Andersen et al. 2002; Periasamy and Kolenbrander 2009). Because *G. vaginalis* is able to adhere to and form a biofilm on cervical epithelia, it is plausible that it is an initial colonizer that paves the way for additional species with low innate pathogenic potential to become established in the vagina.

The production of a cytolysin by *G. vaginalis* was first reported in 1990, but has only recently been named vaginolysin and characterized as a pore-forming cytotoxin which utilizes the complement regulatory molecule CD59 to activate the epithelial p38 mitagen activated protein kinase pathway in human epithelial cells, leading to cell death (Rottini, Dobrina et al. 1990; Gelber, Aguilar et al. 2008). IgA antibodies against vaginolysin have been linked to the mucosal immune response during BV, further supporting the role of vaginolysin in BV pathogenesis (Cauci, Driussi et al. 2002). We analyzed the relative cytotoxic activity levels of the BV-associated anaerobes and found that only *G. vaginalis* was able to induce rounding and lysis of ME180 cervical epithelial cells. The other BV-associated species did not elicit detectable cytopathologic changes, even when the bacteria were incubated with the ME180 cells overnight (data not shown). It is important to note that the cytotoxic effect of *G. vaginalis* was contact dependent; this phenotype has been described in *Streptococcus pyogenes* which utilizes an injection pathway for streptolysin O called cytolysin-mediated translocation (Madden, Ruiz et al. 2001). The streptolysin O forms a pore in the eukaryotic cell through which an effector is translocated and the cytolysin and effector act synergistically to trigger cytoxicity (Madden, Ruiz et al. 2001). Future studies should analyze whether vaginolysin is transported by cytolysin-mediated translocation and if it helps move effector molecules into cervical epithelial cells.

Next, we analyzed biofilm formation. Biofilm formation is an important virulence factor because it confers heightened antibiotic tolerance and resistance to host immune defenses. Due to the fact that biofilm bacteria are not effectively cleared by the immune system or completely killed by antibiotics, biofilm-related infections tend to be chronic

and/or relapsing. BV tends to be a smoldering infection with a high rate of relapse or recurrence. Indeed, direct evidence from the microscopic analysis of vaginal biopsies of women with BV revealed the presence of a bacterial biofilm on the vaginal epithelium (Swidsinski, Mendling et al. 2005). A follow-up study revealed that metronidazole therapy failed to clear the biofilm and suggested that this was related to relapse (Swidsinski, Mendling et al. 2008). These studies revealed biofilm formation as an important virulence determinant in BV. In our in vitro assay, *G. vaginalis* formed a moderate or strong biofilm, depending on the strain and *F. nucleatum* formed a moderate biofilm that was easily dislodged. None of the other BV-associated anaerobes were able to from a significant biofilm.

Our study did not include all of the bacterial species found to be associated with cases of BV. BV is a polymicrobial disorder and the diversity of species associated with the disorder continues to increase as detection methods such as 16S rRNA gene pyrosequencing improve. Because of the large number of candidates and because many bacteria found to be associated with BV are unculturable, we focused instead on a relatively small number of commonly isolated species. The in vitro model of adherence used in this study is limited by the fact that cell monolayers of ME180s are not polarized as are cervical epithelial cells in vivo. The assay for biofilm formation is limited by the fact that the growth medium does not contain the same factors found in vivo and some in vivo cues may turn on expression of biofilm-related genes. These limitations aside however, in vitro models can be very informative and are key to furthering our understanding of BV as the lack of a tractable animal model for BV has thwarted efforts to understand the etiology

of this common and important women's health concern. While it is plausible that our in vitro study did not test for virulence properties that play an important role in BV pathogenesis or that the conditions used did not allow for full expression of virulence factors, our results are suggestive of a higher innate virulence potential of *G. vaginalis* relative to other BV-associated species. While a number of the BV-associated anaerobes had one of the three virulence properties tested, only *G. vaginalis* displayed robust cytotoxicity, strong adherence to vaginal epithelial cells and a propensity to form a dense biofilm, suggesting a key role for *G. vaginalis* in BV pathogenesis. Additional virulence factors produced by *G. vaginalis* include sialidase and prolidase, two hydrolytic enzymes that may have a role in degrading several key mucosal protective factors, such as mucins, as well as contributing to exfoliation and detachment of vaginal epithelial cells (Cauci, Culhane et al. 2008). In sum, there is strong evidence that *G. vaginalis* does possess innate pathogenic potential. Other BV-associated anaerobes may also have as yet un-described virulence factors, or may be more pathogenic in the presence of other species. It is also likely that the primary etiologic agent of BV, if there is one, varies on a case-by-case basis. However, this study suggests that due to its collection of virulence factors, it is likely that *G. vaginalis* has a pathogenic capacity and could be the key agent in certain cases of BV.

Although we have shown that *G. vaginalis* is able to form a thick, tenacious biofilm, not much is known about how *G. vaginalis* actually forms this biofilm. Many bacteria are able to produce biofilms and this biofilm mode of growth imparts tolerance to many negative environmental stimuli; therefore, in order to produce therapies that have

higher efficacy and lower rates of relapse, it is imperative to characterize the biofilm produced by *G. vaginalis*.

CHAPTER 4

Characterization of the *Gardnerella vaginalis* biofilm matrix

Introduction

Recent evidence suggests that, in many infections, bacteria assume a biofilm rather than a planktonic mode of growth. Biofilms are adherent communities of microorganisms that are held together by a polymeric matrix that is composed of polysaccharides, proteins, and/or nucleic acids. The distinct gene expression pattern and the physical structure of biofilms increase bacterial resistance to many negative stimuli. Bacterial biofilm infections are particularly problematic because the biofilm phenotype allows for increased tolerance to antibiotics, inhibition of normal immune responses, and an increase in difficulty of eradicating the infection. Biofilm cells are capable of persisting in the presence of antimicrobials at concentrations that are 1,000-fold higher than those necessary to eradicate planktonic bacteria (Hamilton 2002; Cerca, Martins et al. 2005). Biofilm infections can become chronic and recalcitrant; treatment often results in long periods of hospitalization, morbidity, severe functional impairment and increased mortality (Chaignon, Sadovskaya et al. 2007). Because of this, biofilm research has focused on

analyzing the architecture, antibiotic resistance profile and gene expression in biofilms in order to improve current antibiotic therapy and discovery of novel targets for therapy.

Although BV does not have a high rate of morbidity and mortality, it is associated with a high rate of relapse or recurrence. This is due to the fact that in most cases of BV, a thick, adherent biofilm, with *G. vaginalis* being the predominant species, forms on the vaginal epithelium (Swidsinski, Mendling et al. 2005). In addition, previous studies show that *G. vaginalis* biofilms can tolerate higher concentrations of certain antibiotics (Muli and Struthers 1998; Swidsinski, Mendling et al. 2008). We hypothesize that the biofilm phenotype confers a survival advantage on BV-associated anaerobes within the vagina and is an important component of the pathogenesis of BV.

In order to combat the high rate of recurrence seen with BV, new therapeutic modalities need to be developed. Some researchers speculate that introduction of probiotic strains of lactobacilli, or ones in which copious amounts of lactic acid and hydrogen peroxide are produced, could kill the biofilm bacteria and repopulate the vagina. We hypothesize that elaboration of a biofilm could enable *G. vaginalis* to survive, even in the presence of lactobacilli-derived H_2O_2 and lactic acid, and therefore probiotic therapy will prove ineffective. To test this we compared sensitivities of biofilm vs. planktonic cultures to H_2O_2 and lactic acid.

The most promising new therapies for BV are ones in which the biofilm matrix is targeted, leaving more susceptible planktonic bacteria in the place of biofim. It has been shown that some bacteria, like *Actinobacillus actinomycetemcomitans*, produce a biofilm dispersing enzyme that allows bacteria to be released from the biofilm matrix to populate

other locations (Kaplan, Ragunath et al. 2003). The enzyme is called Dispersin B (DspB) and it has been shown to disperse biofilms composed of β (1,6)-linked N-acetylglucosamine. Other enzymes with biofilm dispersing potential are deoxyribonucelase 1 (DNase1) which has been shown to degrade *P. aeruginosa* biofilms (Barken, Pamp et al. 2008), streptokinase (Varidase) which has been shown to degrade *S. aureus* biofilms (Nemoto, Hirota et al. 2000) and proteases from the Antarctic krill shrimp referred to as Krillase which have been shown to degrade oral biofilms (Berg, Kalfas et al. 2001).

Other agents that are not designed to specifically target biofilms also have biofilm-dispersing properties. For examples, sodium metaperiodate hydrolyzes carbon:carbon bonds and degrades certain polysaccharides, and can therefore disperse biofilms with susceptible polysaccharide matrices. Proteases like trypsin and proteinase K can degrade bacterial biofilms that require a proteinaceous component for intercellular adhesion (Chaignon, Sadovskaya et al. 2007; Badel, Laroche et al. 2008; Fredheim, Klingenberg et al. 2009).

The plant cysteine-proteinases bromelain, papain and ficin offer a wide spectrum of therapeutic efficacies; they demonstrate in vitro and in vivo, antiedemateous, anti-inflammatory, antithrombotic and fibrinolytic activities and may prove more specific and less harmful to human tissue (Maurer 2001; Tysnes, Maurer et al. 2001). Bromelain is a crude extract from the stems and immature fruits of pineapples, constituting an unusually complex mixture of different thiol-endopeptidases, phosphatases, glucosidases, peroxidases, cellulases, and glycoproteins (Taussig and Batkin 1988; Maurer 2001).

Papain is found in the latex of the papaya fruit and along with bromelain, has been used therapeutically in wound debridement (Kravitz, McGuire et al. 2008; Ramundo and Gray 2008). Ficin is derived from fig latex and has been shown to have a negative effect on the cuticle of nematodes and therefore has antihelmintic activity (de Amorin, Borba et al. 1999; Stepek, Buttle et al. 2005). Finally, Pepsin is an enzyme whose precursor is released by the chief cells in the stomach and upon contact with the hydrochloric acid is autocatalytically cleaved to activate itself. Pepsin degrades food proteins into peptides and functions best in acidic environments (Fruton 2002).

The architecture and matrix material of *G. vaginalis* biofilms has not yet been characterized. Therefore, in this study, we employed biochemical assays to determine the amounts of protein, carbohydrate and nucleic acid found in the biofilm matrix. We employed lectin blots to identify any polysaccharide component and used confocal microscopy to characterize the biofilm architecture. Finally, we treated the biofilms with a variety of compounds to determine which class of compounds was able to disperse the bacteria.

Results

Biofilm formation by *G. vaginalis* in vitro

To determine optimal conditions for in vitro biofilm formation, *G. vaginalis* was cultured anaerobically in 96-well plates in Luria Bertani broth (LB), Tryptic Soy broth (TSB), Brain Heart Infusion broth (BHI), Mueller Hinton broth (MH), Man, Rogosa and Sharpe broth (MRS), or a chemically defined medium resembling vaginal secretions

(CDM) (Geshnizgani and Onderdonk 1992). Because glucose increases biofilm formation in other bacterial species (Hall-Stoodley and Stoodley 2002), *G. vaginalis* was also cultured in the presence of 1% glucose (LBG, TSBG, BHIG, MHG, MRSG and CDMG). Biofilms were stained with safranin, which binds to DNA, polysaccharides and other biologic materials and imparts a red color to biofilms. As shown in **Figure 10**, biofilm formation was poor in LB, TSB, TSBG, MRS and MRSG. Optimal biofilm formation was observed in BHI with1% glucose.

Biochemical characterization of *G. vaginalis* biofilms

The crude biochemical composition of the extracellular matrix of *G. vaginalis* biofilms was characterized by extracting loosely-associated material from biofilm and planktonic bacteria. Protein concentrations in the extracts were determined with the use of Coomassie Protein Assay Reagent and the Bradford assay, nucleic acid content was estimated by measuring the optical density at 260 nm, and carbohydrate content was estimated by the standard phenol-sulfuric acid assay with the use of glucose dilutions as standards. Biofilm extracts contained more than 4 times as much total carbohydrate per 10^6 bacterial cells compared to planktonic bacteria (**Table 5**) but protein and nucleic acid concentrations were not significantly different ($p>0.05$ and $p>0.5$, respectively) suggesting that neither proteins nor nucleic acids are a major component of the extracellular biofilm matrix.

To further characterize the carbohydrate component of the biofilm matrix, matrix extracts were blotted onto nitrocellulose membranes and probed with various lectins,

Figure 10. Biofilm formation by *G. vaginalis* in vitro in different media. *G. vaginalis* strain ATCC 49145 was grown in various media with or without added glucose anaerobically at 37ºC for 24 hours. Non-adherent cells were removed from the wells and the adherent bacteria were stained with safranin. Strains 465 and 5-1 produced similar results.

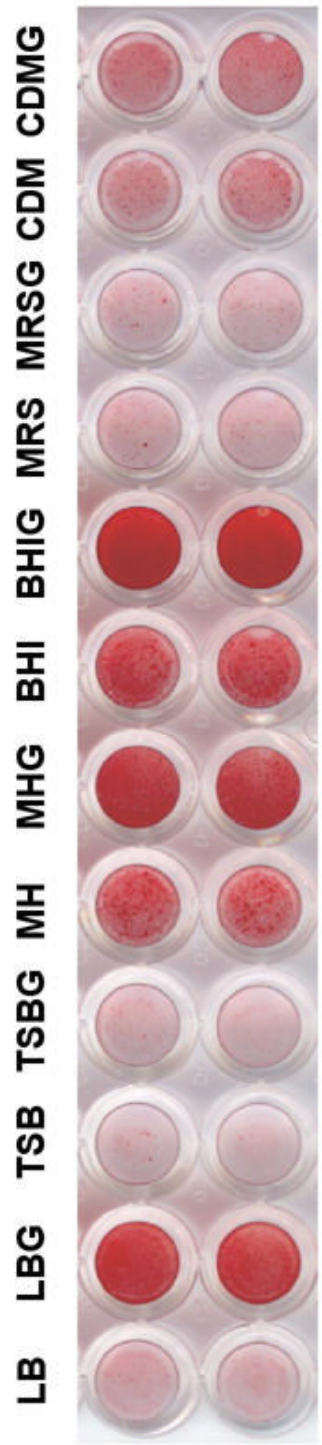

LB
LBG
TSB
TSBG
MH
MHG
BHI
BHIG
MRS
MRSG
CDM
CDMG

Table 5. Biochemical analysis of extracts from planktonic and biofilm bacteria.

	mg protein[#]	mg nucleic acid[*#]	mg carbohydrate[#]
Planktonic	0.44 ± 0.06	0.13 ± 0.01	0.28 ±0.09
Biofilm	0.21 ± 0.14	0.14 ± 0.02	1.23 ± 0.5[α]

[#] mg of protein, nucleic acid and carbohydrate are all per 10^9 CFU of bacteria

[*] Nucleic acid concentration in mg nucleic acid/10^9 CFU were calculated using the equation OD_{260}Xdilution factorX0.05

[α] $p<0.05$ by Student's t-test

which are sugar-binding proteins that are highly specific for their sugar moieties, which were conjugated to horseradish peroxidase (HRP). Glycine Max, which binds to N-acetylgalactosamine bound weakly to extracts from biofilms grown in CDM but not to planktonic extracts or extracts from biofilms grown in BHI (**Figure 11B**). Concanavalin A, which binds to α-mannose and α-glucose, did not bind to extracts from biofilms or planktonic bacteria (**Figure 11C**). Wheat germ agglutinin, which binds to N-acetylglucosamine, reacted with all extracts, but biofilm bacteria grown in BHI contained approximately 25-fold more of the material that reacted with the lectin in comparison to planktonic bacteria grown in BHI (**Figure 11A**) suggesting that N-acetlyglucosamine is a major component of the biofilm matrix.

Structural analysis of *G. vaginalis* biofilms

Confocal microscopy was used to further analyze the N-acetylglucosamine-containing polysaccharide component of *G. vaginalis* biofilms cultured in BHIG. Biofilm bacteria were stained with the green nucleic acid stain SYTO® 9 and Alexa Fluor® 633-conjugate of Wheat germ agglutinin, which binds to polysaccharides within the biofilm matrix. **Figure 12** shows that the biofilm was ~44 μm thick and exhibited a characteristic heterogeneous biofilm structure with peaks and "valleys" and microcolonies separated by channels. WGA adhered to material around the bacteria (red), but did not co-localize with the nucleic acid stain (green) suggesting that, as expected, the carbohydrate was extracellular and possibly only loosely associated with the cell surface.

Figure 11. Lectin blots of biofilm extracts react with Wheat germ agglutinin (WGA) but not with Concanavalin A (ConA) or Glycine max (Gly). Serial 5-fold dilutions of extracts from biofilms grown in BHI, BHIG, or CDM and planktonic cultures grown in BHI or CDM were blotted onto nitrocellulose and probed with peroxidase-conjugated lectins. Panel A was probed with WGA-HRP, panel B was probed with Gly-HRP and panel C was probed with ConA-HRP.

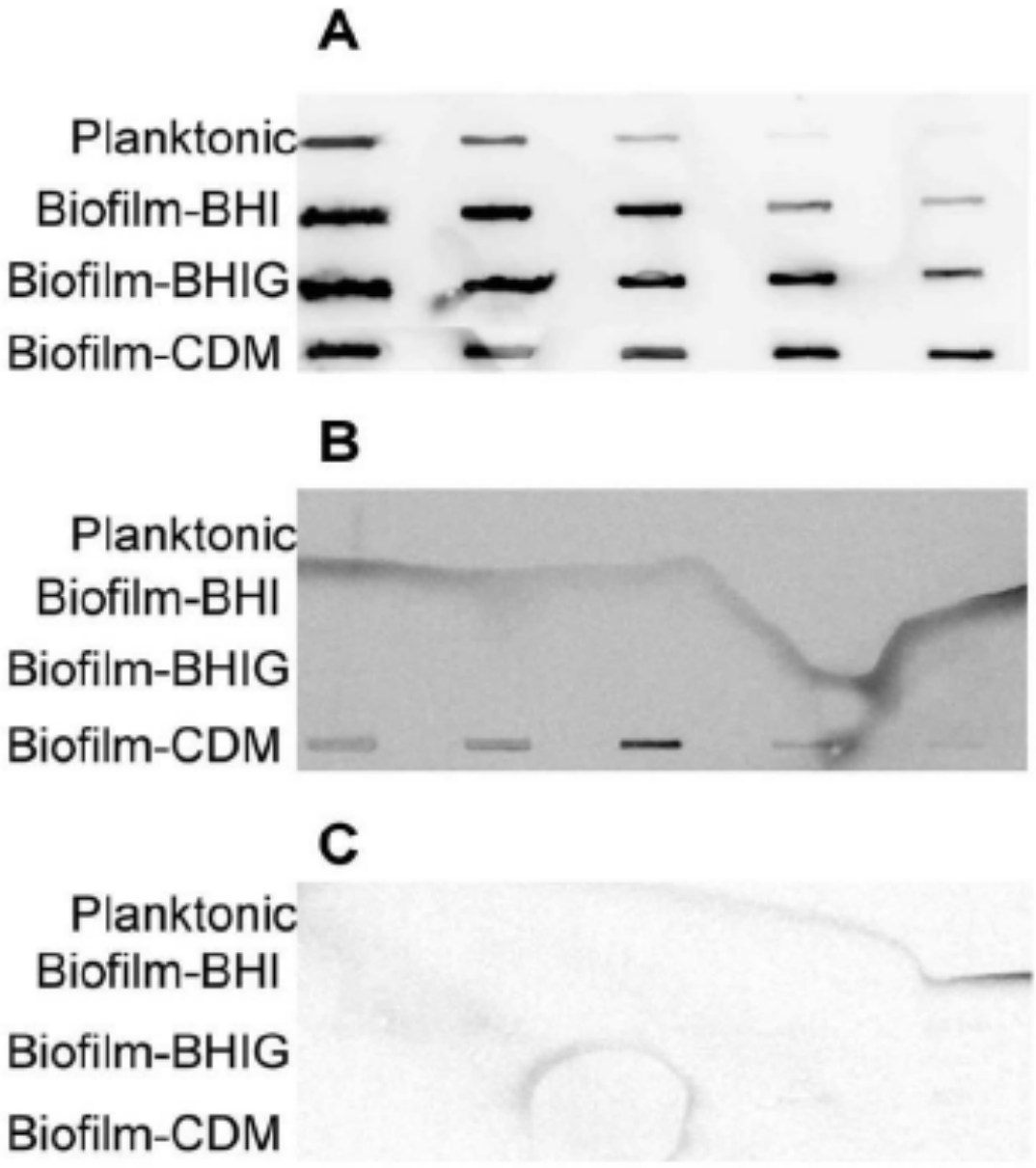
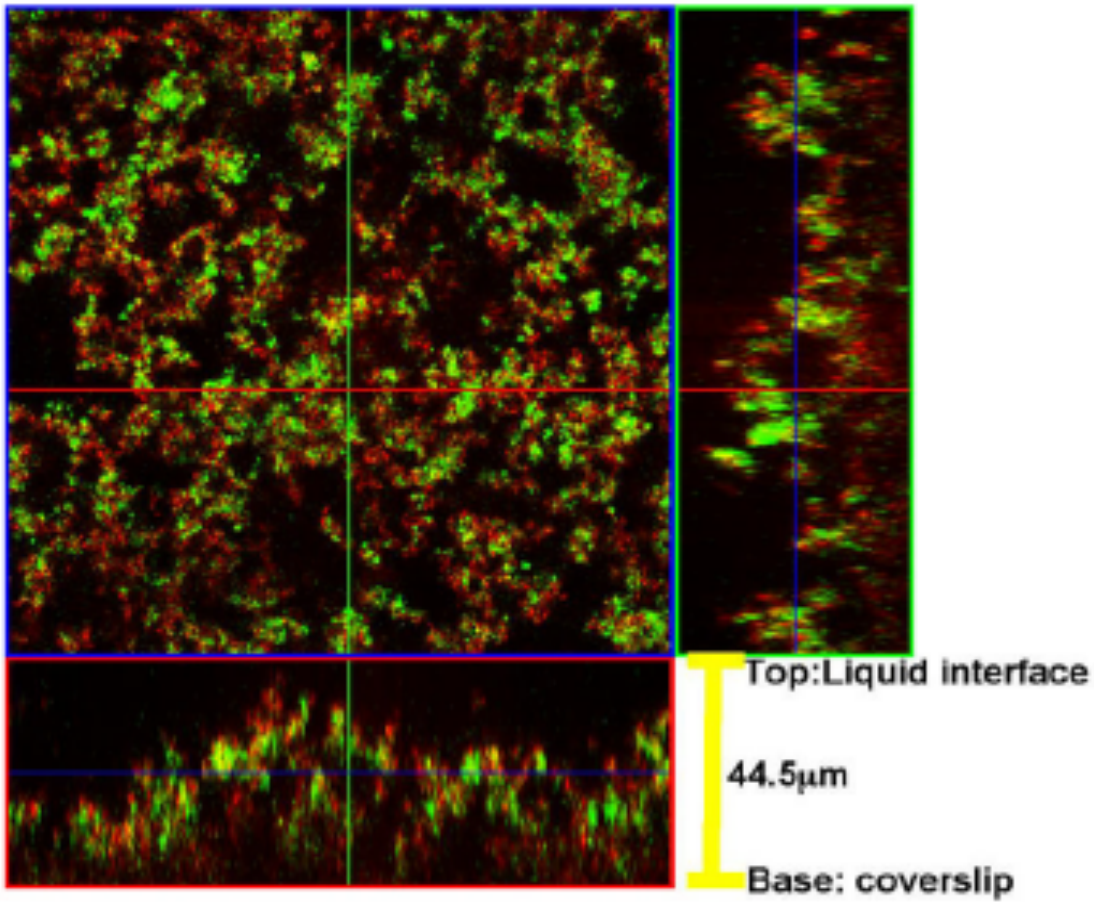

A
Planktonic
Biofilm-BHI
Biofilm-BHIG
Biofilm-CDM
B
Planktonic
Biofilm-BHI
Biofilm-BHIG
Biofilm-CDM
C
Planktonic
Biofilm-BHI
Biofilm-BHIG
Biofilm-CDM

Figure 12. Confocal microscopy of *G. vaginalis* biofilm structure. Biofilms were stained with SYTO® 9 and a fluorescent Alexa Fluor® 633-conjugate of WGA and analyzed by confocal scanning laser microscopy. The large panel is a "bird's-eye view of the biofilm. The right panel is a side view of an x-axis section and the lower panel is a side-view of the y-axis section. Green color indicates nucleic acid while red staining indicates presence of polysaccharide. The biofilm shown was formed by strain 465; biofilms formed by other strains yielded similar results.


Top:Liquid interface
44.5µm
Base: coverslip

Sensitivity of *G. vaginalis* biofilms versus planktonic cultures

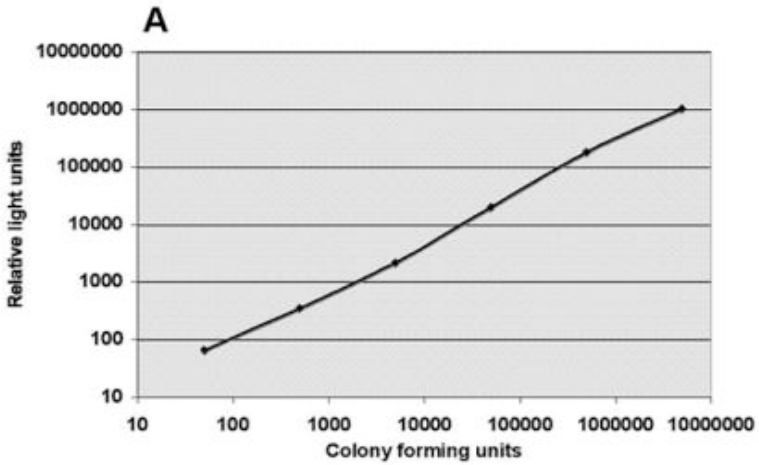
To determine if biofilm bacteria were more resistant than planktonic bacteria to H_2O_2 and lactic acid, we exposed biofilm and planktonic cultures to these agents and determined their viability by luciferase assay. The luciferase reagent is added directly to the bacterial cells; it supports bacterial cell lysis and generation of a luminescent signal. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of viable cells in culture. When reproducing these results with *G. vaginalis*, **Figure 13A** demonstrates that the relative light units (RLU) emitted from the luciferase assay directly related to the number of colony forming units of *G. vaginalis* present. **Figure 13B** and **13C** show the MBC_{90}'s (minimum concentration required to reduce viability by at least 90%) for H_2O_2 and lactic acid. Biofilms were more resistant and tolerated 4-fold higher concentrations of H_2O_2 and 8-fold higher concentrations of lactic acid than planktonic cultures.

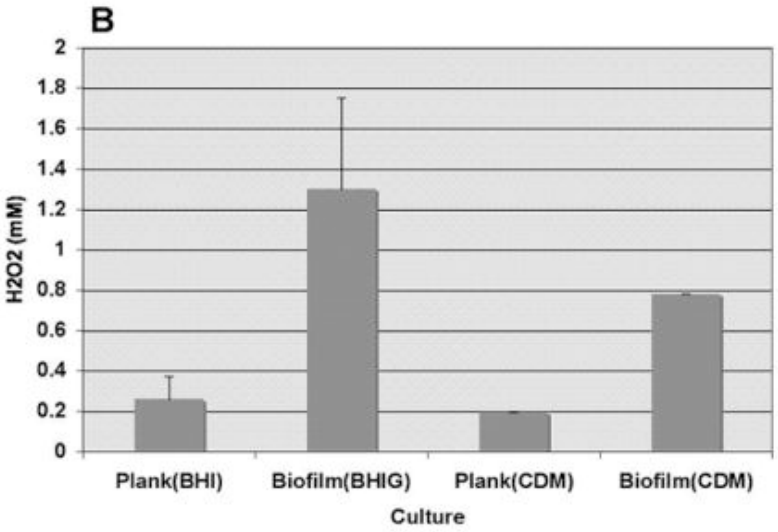
To better replicate the conditions that *G. vaginalis* would encounter in the vagina, we grew biofilms and planktonic cultures in CDM and tested the sensitivity of the cultures to H_2O_2 and lactic acid. As shown in **Figure 13**, biofilms grown in CDM were resistant to 4-fold higher concentrations of H_2O_2 and 4-fold higher concentrations of lactic acid.

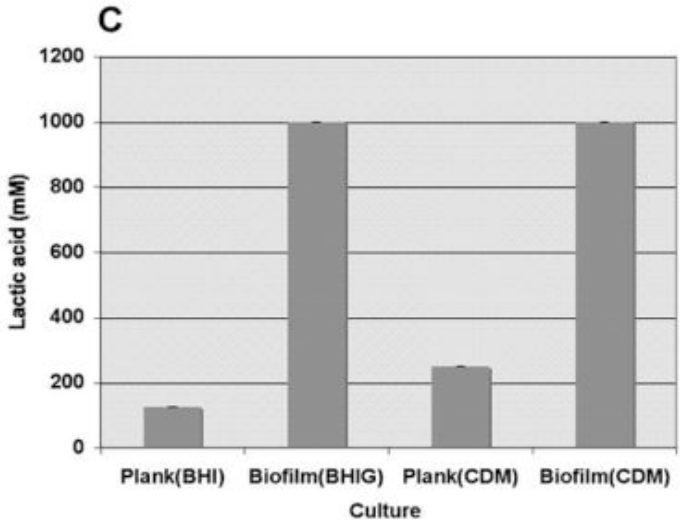
Dissolution of *G. vaginalis* biofilms

We hypothesized that degradation of the exopolysaccharide component within the biofilm matrix would lead to dissolution of the biofilms and would restore sensitivity to different agents. To test this, biofilms were treated with sodium metaperiodate, which

Figure 13. Sensitivity assays demonstrate that biofilms exhibit increased tolerance to H_2O_2 and lactic acid. (A) Illustrates the direct correlation between the number of colony forming units (x-axis) and relative light units (y-axis) measured in the viability assay. (B) Shows the MBC_{90} of H_2O_2, or the concentration required to decrease viability of biofilms and planktonic cultures grown in BHI or CDM by at least 90% is shown. (C) Shows the concentration of lactic acid required to decrease viability of biofilm and planktonic cultures grown in BHI or CDM by at least 90% is shown. Bars in Panels B and C represent the averages of three separate experiments and error bars represent the standard deviations.


A
10000000
1000000
100000
10000
1000
100
10
Relative light units
10
100
1000
10000
100000
1000000
10000000
Colony forming units


B
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H2O2 (mM)
Plank(BHI)
Biofilm(BHIG)
Plank(CDM)
Biofilm(CDM)
Culture


C
1200
1000
800
600
400
200
0
Lactic acid (mM)
Plank(BHI)
Biofilm(BHIG)
Plank(CDM)
Biofilm(CDM)
Culture

hydrolyzes polysaccharide, and chitinase, which degrades beta-1,4-linked polymers of N-acetylglucosamine. As shown in **Figure 14**, chitinase reduced the biofilm thickness only very slightly, even at the highest concentration tested and sodium metaperidoate had no effect.

We then tested other catabolic enzymes and compounds on mature biofilms to determine their biofilm-dispersing activities (**Figure 15**). Concentrations of proteinase K and trypsin as low as 32 µg/ml and 6.4 µg/ml (respectively) effectively disrupted the biofilms produced by all three strains. H_2O_2, lactic acid, SDS, and DNaseI, which have been shown to disrupt some *Pseudomonas aeruginosa* biofilms, did not have an effect (Whitchurch, Tolker-Nielsen et al. 2002).

Effect of Proteinase K on biofilm structure

Confocal microscopy was used to further analyze the structural changes associated with proteinase K treatment of *G. vaginalis* biofilms. For the proteinase K time course, stained biofilms were treated with 100 µg proteinase K/mL and confocal images of the same field of view were collected every 20 minutes for 280 minutes. **Figure 16** shows the proteinase K time course; at time zero microscopy revealed a thick, characteristically heterogeneous *G. vaginalis* biofilm with peaks and "valleys" and microcolonies separated by channels. By 40 minutes, the structure of the *G. vaginalis* biofilm was lost, with no microcolonies or channels remaining. By the end of the time course, no distinguishable biofilm remained. This shows that proteinase K is effectively cleaving a component of the biofilm that is very important in structure and architecture. Because proteinase K cleaves

Figure 14. Treatment of *G. vaginalis* biofilms with chitinase and sodium metaperiodate. *G. vaginalis* strain 465 was grown as a biofilm and treated for 20 hours at 37ºC with chitinase and sodium metaperiodate. The reagents were serially diluted 5-fold horizontally across the plate. The biofilm wells were washed and stained with safranin to shown biofilm dissolution as a decrease in red color. Biofilms formed by strains 5-1 and ATCC 49145 yielded similar results.

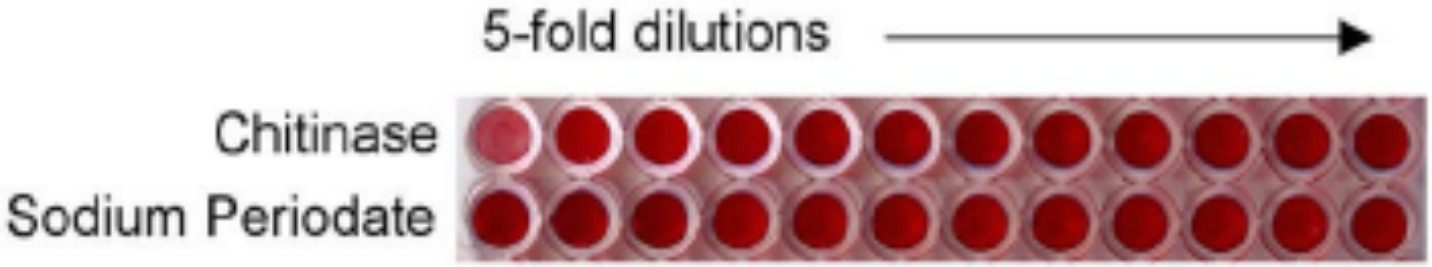

5-fold dilutions
Chitinase
Sodium Periodate

Figure 15. Treatment of *G. vaginalis* biofilms with proteases. *G. vaginalis* strain 465 was grown as a biofilm and treated for 20 hours at 37ºC with proteinase K, trypsin, H_2O_2, lactic acid, SDS or DNaseI. The reagents were serially diluted 5-fold horizontally across the plate. The biofilm wells were washed and stained with safranin to shown biofilm dissolution as a decrease in red color. Biofilms formed by strains 5-1 and ATCC 49145 yielded similar results.

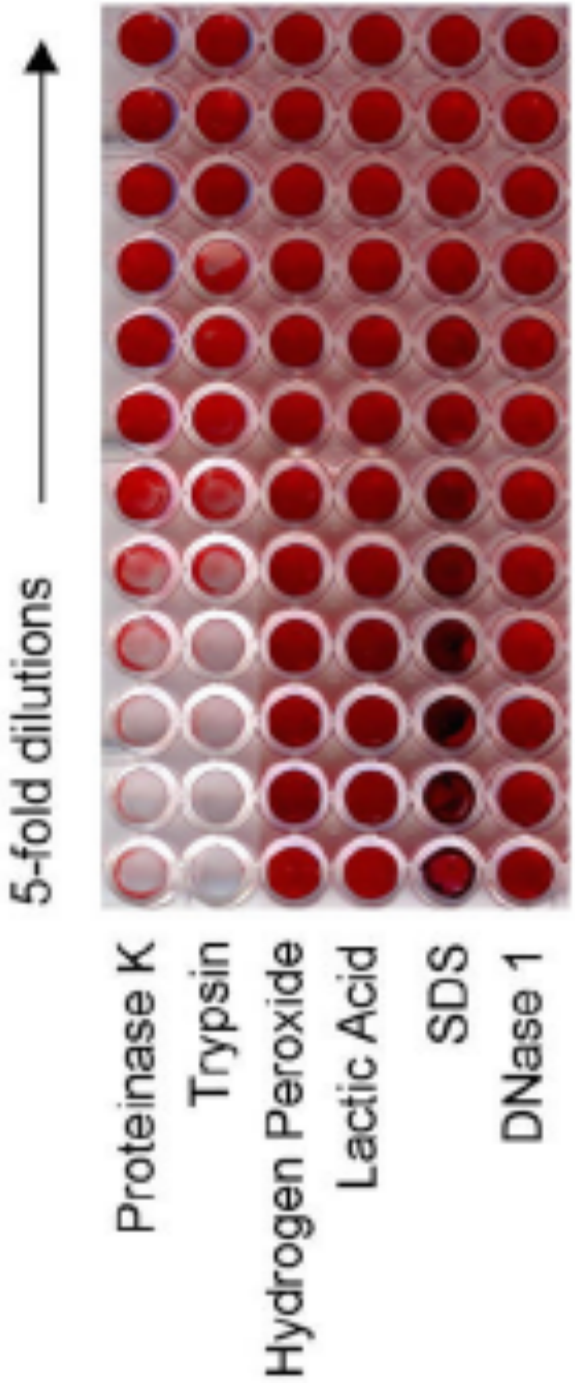
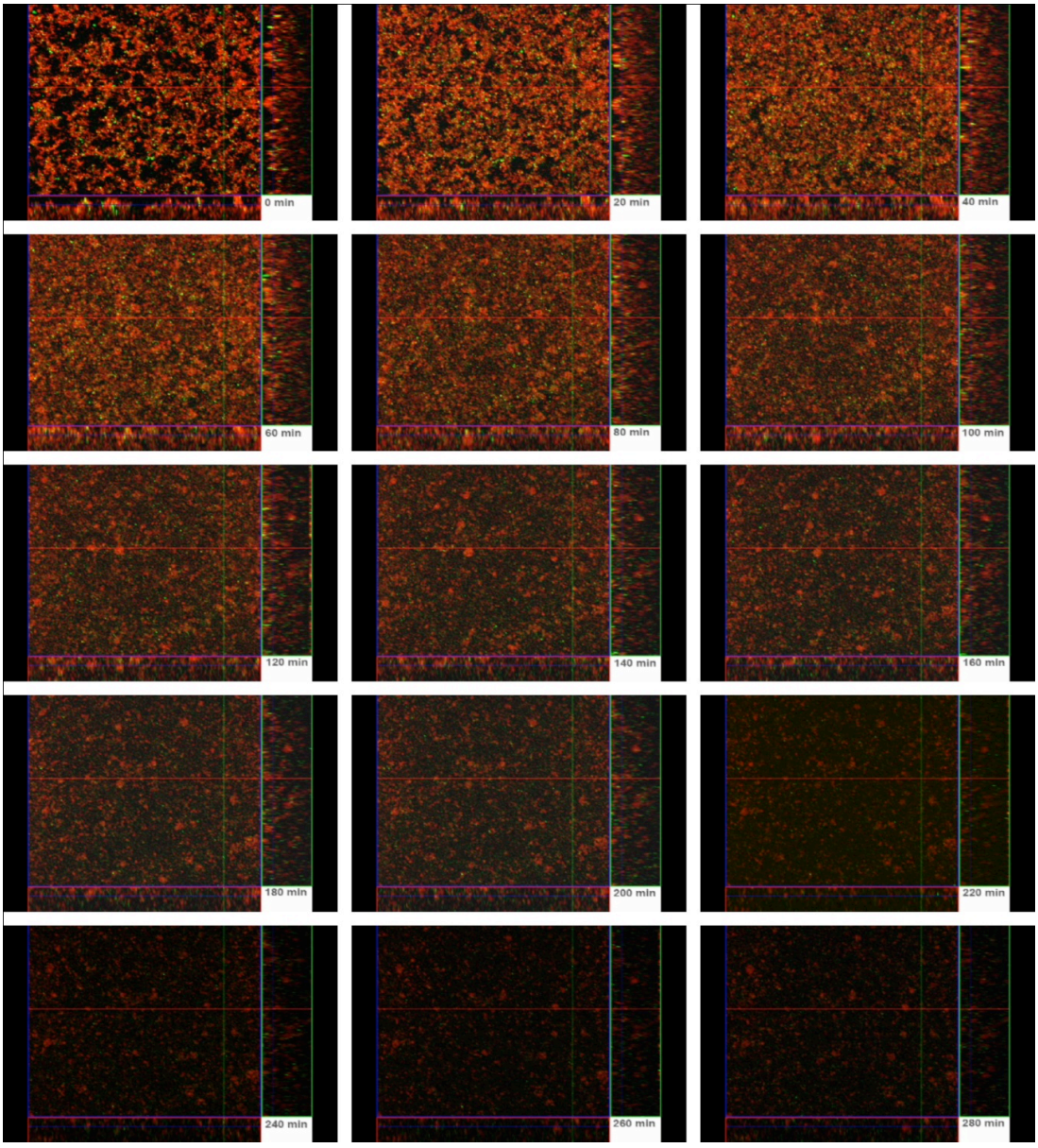

5-fold dilutions
Proteinase K
Trypsin
Hydrogen Peroxide
Lactic Acid
SDS
DNase 1

Figure 16. Effect of proteinase K treatment on biofilm structure over time. Biofilms were stained with Live/Dead BacLight staining and analyzed by confocal scanning laser microscopy. The large panels are a "bird's-eye view of the biofilm. The right panels are a side view of an x-axis section and the lower panels are a side-view of the y-axis section. Images were taken every 20 minutes for a total of 280 minutes. Red color indicates viable bacteria while green color indicates dead bacteria. The biofilm shown was formed by strain 465; biofilms formed by other strains yielded similar results.


0 min
20 min
40 min
60 min
80 min
100 min
120 min
140 min
160 min
180 min
200 min
220 min
240 min
260 min
280 min

proteins, this points to a proteinaceous adhesin playing an important role in *G. vaginalis* biofilm structure and architecture.

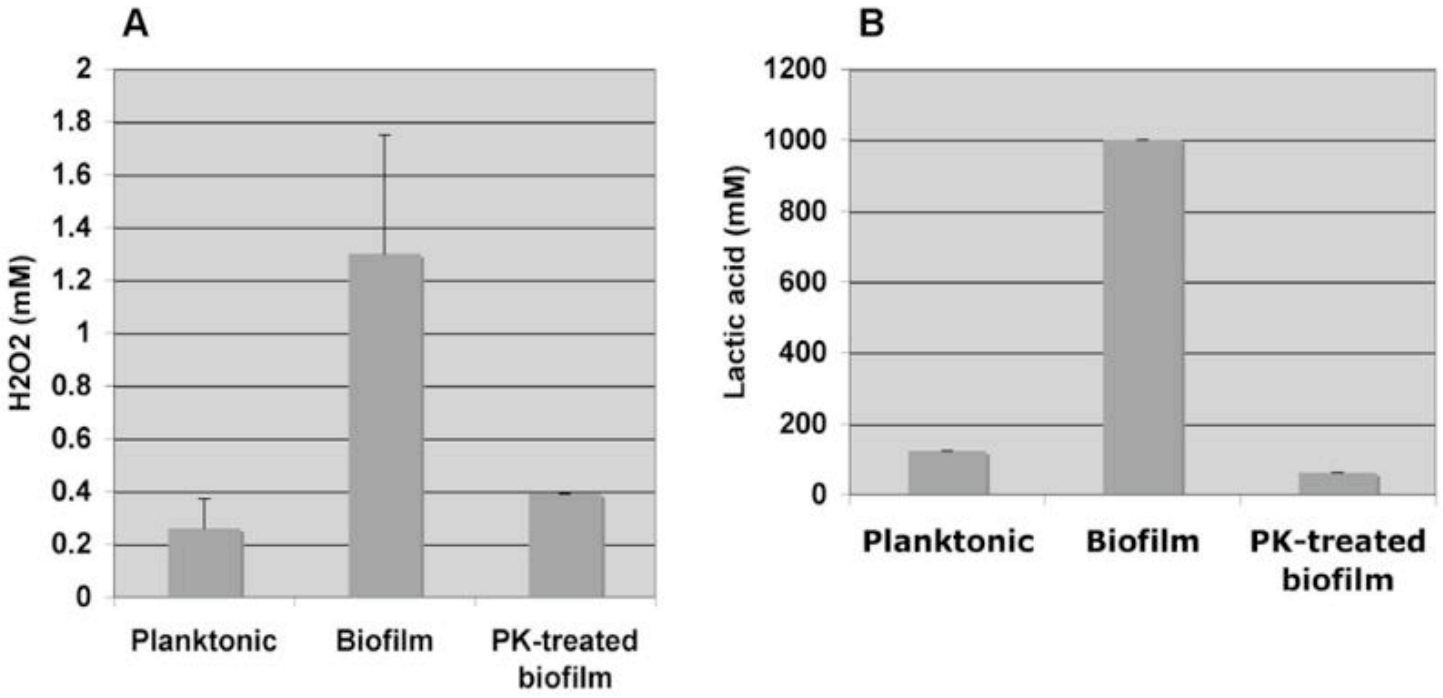
Effect of biofilm dissolution on sensitivity

To determine whether proteolytic dissolution of biofilms would restore the MBC_{90} to levels associated with planktonic bacteria, we pre-treated biofilms with proteinase K to degrade the biofilm structure before adding H_2O_2 and lactic acid. Proteinse K alone did not affect bacterial viability (data not shown); however, treatment of biofilms with the protease significantly increased the sensitivities to H_2O_2 and lactic acid back to planktonic levels (**Figure 17**).

Effect of natural proteases on *G. vaginalis* biofilm

To determine the ability of natural proteases to degrade the *G. vaginalis* biofilm, 24 hour biofilms were grown anaerobically, then subjected to 5-fold dilutions of ficin, bromelain, papain and pepsin in either PBS pH 7.4, which would represent how the enzyme could be potentially stored, and PBS pH 4.5, which would represent the average pH of the vagina during BV. Stock concentrations used were 10 U of bromelain and papain, 5.78 U of ficin and 345 U of pepsin. **Figure 18A** shows representative images of the degradation seen with each protease; both pH conditions gave similar results. The best degradation was seen with the enzyme ficin. Ficin was highly effective at degrading *G. vaginalis* biofilms at both pH 7.4 and pH 4.5. At the highest concentration of enzyme tested, 5.78 Units, ficin exhibited 93 and 92% biofilm reduction at pH 7.4 and 4.5,

Figure 17. Sensitivity of proteinase-K-treated biofilms to H_2O_2 and lactic acid. The concentrations of H_2O_2 (A) and lactic acid (B) required to decrease viability of planktonic cultures, biofilms and proteinase-K-treated biofilms by at least 90% are shown. Proteinase-K treated biofilm cultures exhibited sensitivities similar to that of planktonic cultures. Bars represent the averages of three separate experiments and error bars represent the standard deviations.


A
H2O2 (mM)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Planktonic
Biofilm
PK-treated biofilm
B
Lactic acid (mM)
1200
1000
800
600
400
200
0
Planktonic
Biofilm
PK-treated biofilm

respectively (**Figure 18B**). Bromelain was also an effective biofilm degrading agent regardless of pH, it was able to cause a 76% reduction in biofilm mass (**Figure 18B**). Similar efficient degradation was seen with papain; at the highest concentration of enzyme tested, papain exhibited 89 and 85% biofilm reduction at pH 7.4 and 4.5, respectively (**Figure 18B**). The enzyme pepsin was not effective at degrading *G. vaginalis* biofilms (**Figure 18A and 18B**). **Table 6** shows the amount of enzyme required to reduce *G. vaginalis* biofilm density by at least 75% at pH 7.4 and 4.5; this enzyme concentration could have therapeutic potential when used in conjunction with antibiotics. The percent biofilm reduction for each enzyme concentration tested is seen in **Appendix A**.

Effect of natural protease papain on antibiotic sensitivity of *G. vaginalis*

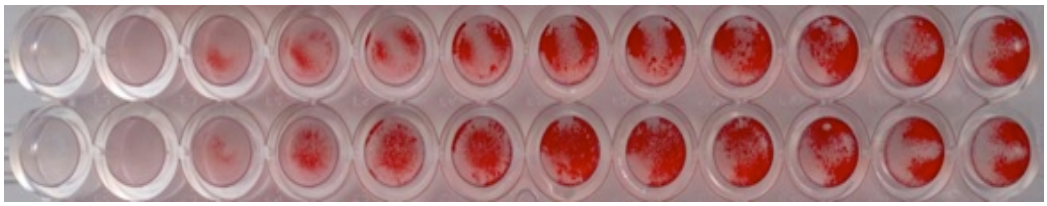
To determine the ability of the enzyme papain to increase the sensitivity of *G. vaginalis* to the antibiotic clindamycin, 24 hour biofilm and planktonic cultures were grown anaerobically at 37°C. Biofilms were either treated with BHIG as a control, with 10 U of papain as a control, or with 3 times the MIC of clindamycin (1.84 µg/ml) in BHIG, then incubated for 16 hours before bacteria were harvested, diluted and plated for CFU. Biofilms were either dispersed with 10 U of papain for 2 hours prior to treatment with clindamycin and nonadherent bacteria were removed by washing, or clindmycin was added directly to papain and allowed to incubate for 16 hours before bacteria were harvested, diluted and plated for CFU. Planktonic cultures were either treated with BHIG or clindamycin as a control for 16 hours before bacteria were harvested, diluted and plated for

Figure 18. Treatment of *G. vaginalis* biofilms with natural proteases. *G. vaginalis* strain 5-1 was grown as a biofilm and treated for 20 hours at 37ºC with ficin, bromelain, papain and pepsin. The reagents were serially diluted 5-fold horizontally across the plate. The biofilm wells were washed and stained with safranin to shown biofilm dissolution as a decrease in red color. (A) Images of stained biofilms. (B) Quantitative measures of biofilm degradation. Stained *G. vaginalis* biofilms were resolublized in 33% acetic acid and optical density of the solution was measured at 562 nm. The percentage of biofilm reduction was calculated using the equation described in the text. Biofilms fomed by strains 5-1 and ATCC 49145 yielded similar results.

A.

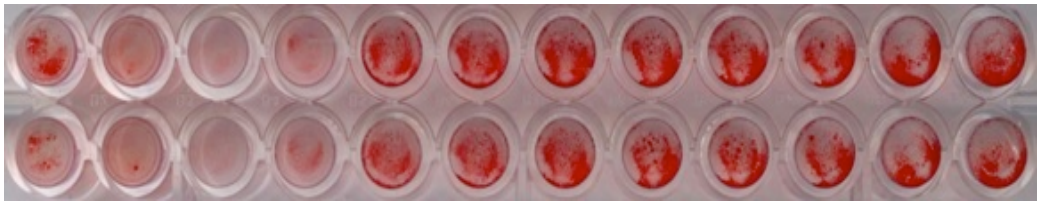
Ficin
5.78 U

5-fold dilutions → Control



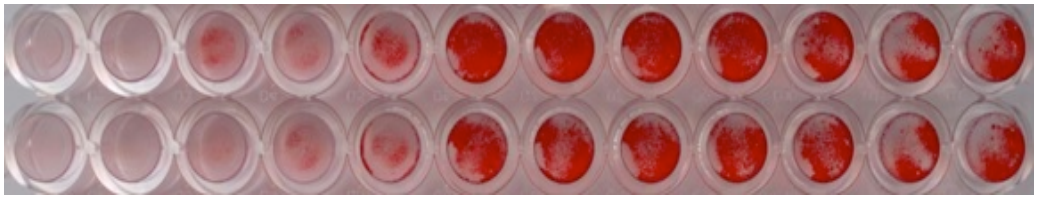
Bromelain
10 U

5-fold dilutions → Control



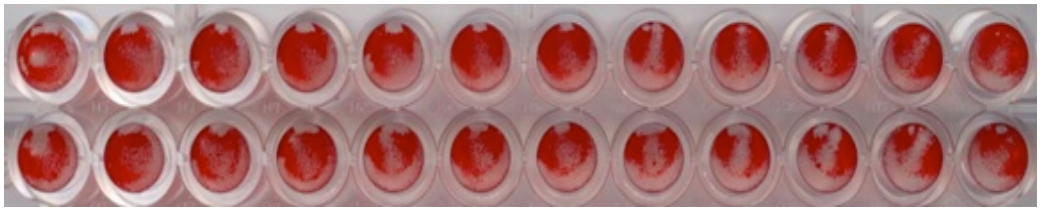
Papain
10 U

5-fold dilutions → Control

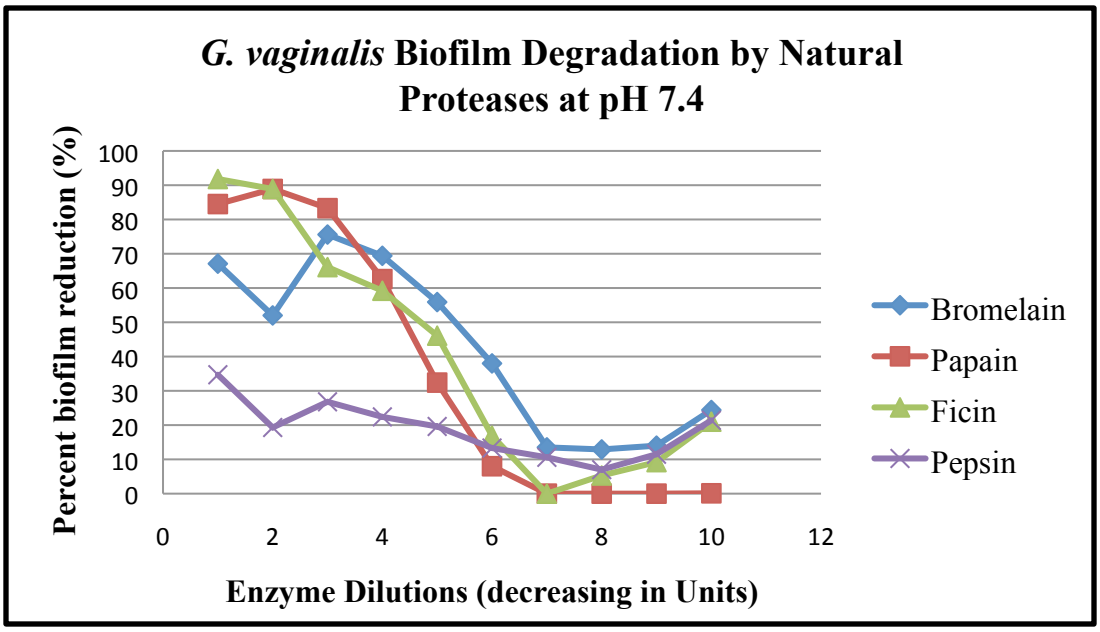


Pepsin
345 U

5-fold dilutions → Control



B.


G. vaginalis Biofilm Degradation by Natural Proteases at pH 7.4
Percent biofilm reduction (%)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Enzyme Dilutions (decreasing in Units)
Bromelain
Papain
Ficin
Pepsin

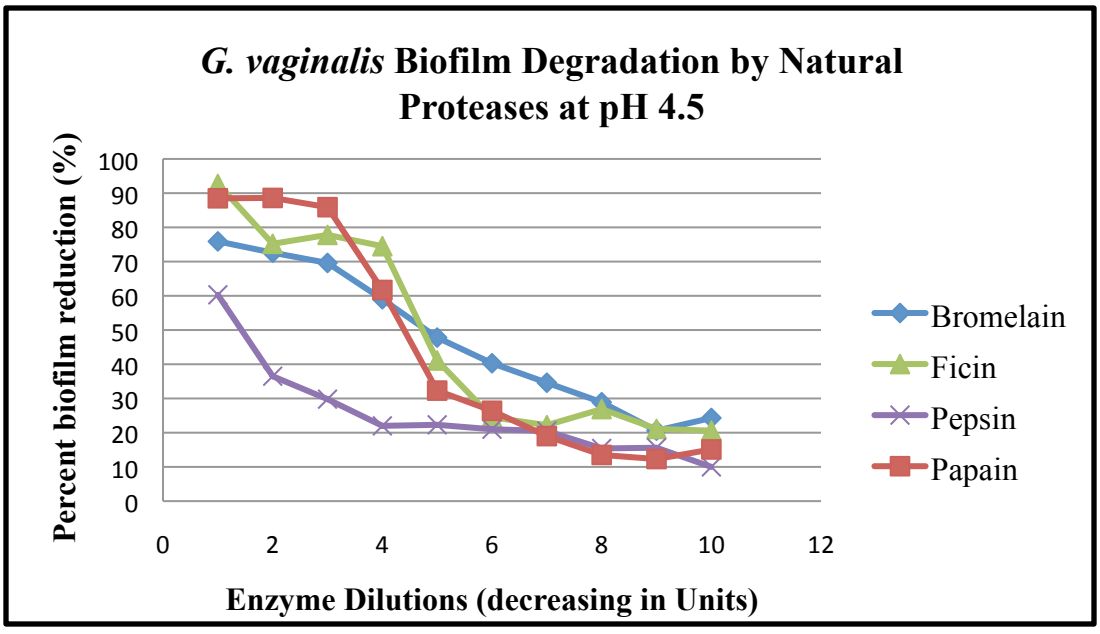

G. vaginalis Biofilm Degradation by Natural Proteases at pH 4.5
Percent biofilm reduction (%)
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Enzyme Dilutions (decreasing in Units)
Bromelain
Ficin
Pepsin
Papain

Table 6. Amount of enzyme required to reduce biofilm density by at least 75% at pH 4.5 and pH 7.4. (N/A indicates that pepsin was unable to reduce biofilm density by at least 75%, even at the highest enzyme concentration tested, 345U)

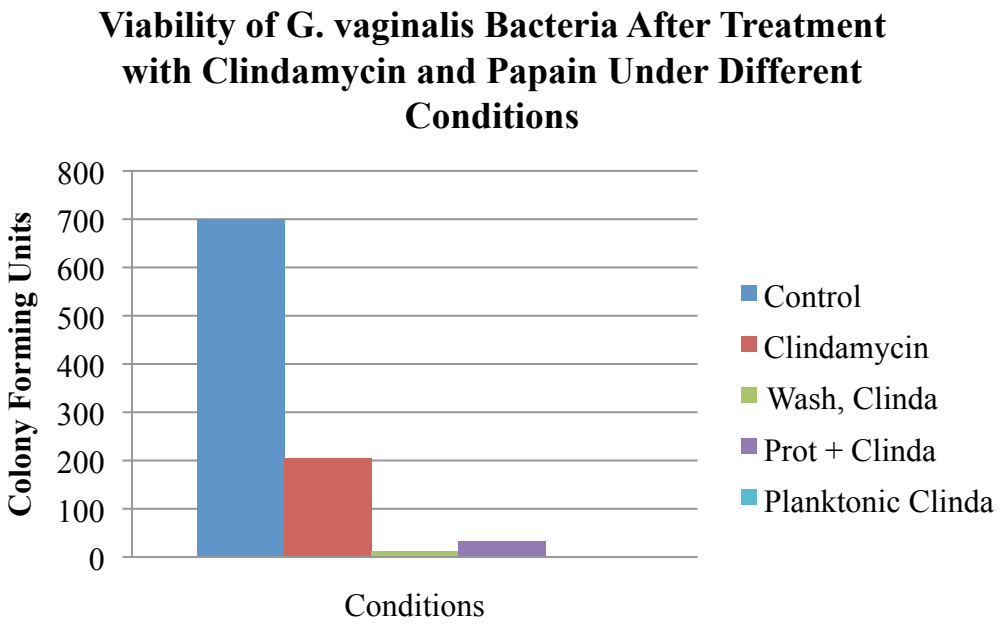
Proteases	pH 4.5	pH 7.4
Bromelain	10 U	0.4 U
Papain	0.4 U	0.4 U
Ficin	0.2312 U	1.156 U
Pepsin	N/A	N/A

CFU. **Figure 19** shows the CFU counts of bacteria treated under various conditions. Clindamycin was able to reduce the CFU count of biofilm bacteria by approximately 70%; however, no recoverable bacteria were seen when planktonic bacteria were treated with the same amount of antibiotic. This showcases the need to find a compound that can help reduce the resistance of biofilm bacteria to antibiotics. When the biofilm was first dispersed by papin and nonadherent cells removed by washing, this was able to reduce the CFU count of biofilm bacteria by approximately 98%. When clindamycin was added directly to papain and allowed to interact with the biofilm, it reduced the CFU count of biofilm bacteria by approximately 95%. Papain itself was able to reduce the CFU count of biofilm bacteria; therefore, more research is needed into whether papain acts in conjunction with the antibiotic, or if the enzyme is all that is needed to reduce the recoverable CFUs. This data suggests that papain treatment in combination with an antibiotic could be an effective new therapy for BV.

Discussion

The etiology and pathogenesis of BV have remained enigmatic ever since initial reports of the condition in 1954 (Gardner and Dukes 1954). Confounding issues include the polymicrobial nature of BV and the role of host factors, both genetic and behavioral. The recent demonstration that BV is associated with the formation of a biofilm in which the predominant species is *G. vaginalis* (Swidsinski, Mendling et al. 2005) is an important advance towards understanding its pathogenesis. We therefore sought to develop a simple

Figure 19. Treatment of *G. vaginalis* biofilm with papain increased the sensitivity to antibiotic challenge. 24 hour biofilm and planktonic cultures were grown anaerobically in BHIG at 37°C. Biofilms were treated with either BHIG as a control (Control), 10 U of papain in BHIG as a control, 1.8375 μg/ml of clindamycin in BHIG (Clindamycin), papain and clindamycin in BHIG (Prot + Clinda), or biofilms were dispersed with 10 U of papain for 2 hours prior to treatment with clindamycin (Wash, Clinda). Planktonic cultures were treated with BHIG as a control or clindamycin (Planktonic Clinda). The cultures were incubated for 16 hours anaerobically at 37°C and bacteria were harvested, 10-fold diluted and plated for CFU.


Viability of G. vaginalis Bacteria After Treatment with Clindamycin and Papain Under Different Conditions
Colony Forming Units
800
700
600
500
400
300
200
100
0
Conditions
Control
Clindamycin
Wash, Clinda
Prot + Clinda
Planktonic Clinda

model for studying *G. vaginalis* biofilms in vitro. We found that when cultured anaerobically in BHI + 1% glucose, the bacteria formed a thick, tenacious biofilm on polystyrene.

Growth of *G. vaginalis* is normally suppressed by healthy lactobacilli. This inhibition is attributed to the production of lactic acid and H_2O_2. We hypothesized that the formation of a biofilm would increase the resistance of the bacteria to H_2O_2 and lactic acid leading to colonization even in the presence of lactobacilli. Bacterial sensitivity is normally assessed by analyzing the minimum inhibitory concentration (MIC) required to inhibit the growth of suspension of bacteria at low cell density. This type of assay cannot be used to measure biofilm sensitivities because the bacteria within biofilms are at a high cell density. We therefore measured bacterial viability of biofilms and high cell density planktonic cultures using a luciferase reagent that quantitatively measures ATP. Using this method, MBC_{90}'s, or the concentrations required to reduce viability by at least 90%, for planktonic bacteria were ~0.2 mM H_2O_2 and ≥ 125-250 mM lactic acid. The concentrations of lactic acid required to kill *G. vaginalis* were considerably higher than the amounts reported to be secreted by lactobacilli (~30-60 mM) (McLean and McGroarty 1996), but since our assay measured bactericidal rather than bacteriostatic activity, it is possible that the concentration of lactic acid required to suppress growth is lower. Biofilm cultures were more resistant and tolerated 4-5 fold higher concentrations of H_2O_2 and 4-6 fold higher concentrations of lactic acid than planktonic cells. The finding that concentrations of H_2O_2 (0.8-1.3 mM) and lactic acid (1,000 mM) required to produce a 90% decrease in viability in biofilm bacteria were substantially greater than the MBC_{90}'s

for planktonic bacteria, could imply that *G. vaginalis* forms a biofilm in the vaginal environment in order to resist the detrimental effects of lactic acid- and H_2O_2-producing lactobacilli.

We hypothesized that enzymatic dissolution of biofilms would restore the sensitivity to H_2O_2 and lactic acid to planktonic-like levels. In order to find a compound to dissolve the biofilms, we preliminarily characterized the composition of the biofilm matrix. Biochemical analysis suggested that the extracellular matrix of the biofilms was predominantly composed of carbohydrate rather than protein and/or nucleic acid. Most previously characterized biofilms contain an exopolysaccharide intercellular adhesion so it is likely that the carbohydrate component we detected is a polysaccharide (Heilmann, Schweitzer et al. 1996; Kaplan, Ragunath et al. 2004; Wang, Preston et al. 2004; Overhage, Schemionek et al. 2005). Wheat germ agglutinin, a lectin from *Triticum vulgaris* bound to the matrix material, suggesting that the matrix contains N-acetylglucosamine. We therefore tested chitinase, an enzyme that cleaves the β-1-4 linkage between N-acetylglucosamine residues in chitin, for the ability to dissolve *G. vaginalis* biofilms. At the highest concentration tested, chitinase reduced the biofilm thickness only slightly. The extracellular polysaccharide adhesion involved in biofilm formation by a variety of unrelated bacterial species including *Staphylococcus aureus* and *S. epidermidis*, *E. coli* and *Actinobacillus actinomycetemcomitans* is a β-1-6-linked polymer of N-acetylglucosamine referred to as PGA or PNAG (McKenney, Hubner et al. 1998; Kaplan, Velliyagounder et al. 2004; Wang, Preston et al. 2004). The β-1-6-linkage of PNAG is resistant to chitinase and it is possible that the polysaccharide matrix of *G.*

vaginalis biofilms has a similar structure and is therefore not degraded by chitinase. We also tested sodium metaperiodate, which cleaves polysaccharides by oxidation and chemical cleavage of carbon-carbon bonds for biofilm dissolving activity. Surprisingly, sodium metaperiodate did not affect the *G. vaginalis* biofilms. It is possible that a component of the biofilms affected sodium metaperiodate activity or that other non-polysaccharide based adhesions are involved. Even though our biochemical analyses did not suggest a significant nucleic acid component to the biofilms, we tested DNaseI against *G. vaginalis* biofilms. It is known that *P. aeruginosa* biofilm matrix contains a significant amount of nucleic acid and that these biofilms are dispersed by DNaseI treatment (Whitchurch, Tolker-Nielsen et al. 2002). However, no degradation of *G. vaginalis* biofilms by DNaseI was observed. Treatment of the biofilms with the proteases proteinase K and trypsin did, however, effectively remove the biofilm from the polystyrene wells. Biofilm extracts did not contain significantly more protein than planktonic bacteria suggesting that the extracellular matrix is not largely proteinaceous. It is possible however, that surface proteins are required to anchor the polysaccharide matrix to the bacteria or to somehow link the polysaccharides together. Cleavage of the protein anchors could, in this theoretical model, release the bacteria from the polysaccharide matrix and hence from the biofilm.

Dissolution of biofilms with proteinase K increased the sensitivities to both H_2O_2 and lactic acid. The finding that biofilm dispersal increases sensitivity suggests that an enzyme that specifically targets the biofilm structure could be therapeutically useful. The use of probiotic therapy for BV, whereby healthy lactobacilli are re-introduced into the

vagina, has been proposed, and this study suggests that *G. vaginalis* would be more sensitive to probiotic therapy if it were planktonic (Reid 2001). In addition to increasing the sensitivity of the bacteria to H_2O_2 and lactic acid, biofilm dissolution could increase the rate of explusion of the bacteria from the vagina by the natural flow of mucous. Biofilm dispersal may also increase the susceptibility of the bacteria to the immune defenses. Proteinase K is a nonspecific protease that would likely damage the vaginal epithelium and would not be considered for clinical use.

We next tested the ability of four natural proteases to degrade the *G. vaginalis* biofilm. The four fruit proteases, ficin, bromelain and papain, were effective biofilm degraders. Papain produced a 75% reduction in biofilm mass at the lowest enzyme concentration used, 0.4 U, at both pH 7.4 and 4.5. Although ficin produced an overall higher percentage of biofilm reduction, with 93 and 93% biofilm reduction at pH 7.4 and 4.5, respectively, papain was used in the follow-up protease/antibiotic sensitivity assay due to its low cost and abundance in the laboratory. Pepsin was a poor biofilm degrader; however, this is not surprising due to its limited activity in non-acidic environments.

Sensitivity assays using papain show that while 1.84 µg/ml of clindamycin led to a 70% reduction in biofilm CFU, biofilms that had been pretreated with papain for 2 hours, then washed to remove nonadherent bacteria, had a 98% reduction in CFU counts. Also, when papain and clindamycin were added to biofilms and incubated overnight, CFUs were reduced by 95%. This suggests that treatment of patients with BV with both papain and an antibiotic, may increase the bacterial sensitivity to the antibiotic. Although biofilm bacteria are more tolerant to antibiotics, as seen by the viability of 30% of the biofilm

bacteria, biofilms that have been dispersed by proteases such as papain, led to an almost 100% eradication of biofilm bacteria; the few remaining planktonic bacteria may be more easily killed by the immune system. More research is needed into the in vivo effects of the fruit proteases on the vaginal epithelium; the identification of an animal model would increase the knowledge of whether fruit proteases would be a valuable, harmless addition to antibiotic therarpy

This data suggests that a proteinaceous adhesin has an important role in biofilm formation in *G. vaginalis*. Therapies aimed at disrupting this adhesin could prove extremely important in reducing the number of relapsing BV cases seen after antibiotic treatment. The idea that bacteria can produce a biofilm matrix whose most important component is a protein is not new; however, the idea of targeting this protein in order to produce more effective anti-biofilm therapies is a recent one. Lending precedence to the idea that BV may one day have an anti-biofilm therapy is the recently developed dispersal enzyme, Dispersin B, which is in the research phase at Walter Reed Army Institute of Research to analyze its efficacy in inhibiting or disrupting single- and mixed-species bacterial biofilms associated with combat trauma wound infections. Although we have shown that *G. vaginalis* biofilms are able to be degraded by proteases suggesting a role for a proteinaceous adhesin, it is imperative to identify and characterize the gene(s) involved in biofilm formation.

CHAPTER 5

Identification and Characterization of a *Gardnerella vaginalis* Biofilm-Associated Protein

Introduction

Biofilms are communities of microorganisms that are surrounded by an extracellular polymeric matrix composed of polysaccharides, proteins and/or nucleic acids, which promote intercellular adhesion and attachment to a surface. The biofilm mode of growth imparts on its constituent bacteria tolerance to antimicrobial agents, dessication, pH changes, ultraviolet light, acid exposure, phagocytosis and sheer stress (Lindsay and von Holy 2006; del Pozo and Patel 2007).

In most bacterial biofilms that have been characterized, the major intercellular adhesin holding the community together is an exopolysaccharide. In the staphylococcal species, the biofilm matrix is composed of an extracellular polysaccharide homopolymer of β-1,6-linked N-acetylglucosamine (PNAG); this polymer is essential for biofilm formation and causes hemagglutination and bacterial aggregation (Heilmann, Schweitzer et al. 1996; Mack, Riedewald et al. 1999; Vuong, Voyich et al. 2004). Many other bacteria can produce PNAG, including *Acinetobacter baumanni* and *E. coli* (Cerca and Jefferson 2008; Choi, Slamti et al. 2009). Although many bacteria produce a polysaccharide based

biofilm matrix, others are able to produce biofilms cemented by proteins (Cucarella, Solano et al. 2001; Latasa, Roux et al. 2005; Branda, Chu et al. 2006; Loehfelm, Luke et al. 2008). Henning et al. described a spontaneous switch from biofilm formation mediated by polysaccharides to biofilm formation mediated by proteins in a *Staphylococcus epidermidis icaC* insertion mutant (Hennig, Nyunt Wai et al. 2007). In *S. epidermidis*, several surface proteins involved in biofilm formation have been described, including SSP1, SSP2 and the AtlE autolysin (Veenstra, Cremers et al. 1996; Heilmann, Hussain et al. 1997). Other reports show that *Staphylococcus* sp. produce a protein adhesin named Bap, for biofilm-associated protein, which is involved in primary attachment and biofilm maturation (Cucarella, Solano et al. 2001). Bap is a large, cell surface-associated protein containing a series of repeats that is found in isolates of several coagulase negative staphylococcal species, as well as *S. aureus* mastitis isolates (Cucarella, Solano et al. 2001). This protein falls within a family of surface proteins involved in biofilm formation, called biofilm-associated proteins, which are found on the bacterial surface, have a high molecular weight, and contain a core domain of tandem repeats (Lasa and Penades 2006). Other proteins found within this family include the newly identified Bap protein of *Acinetobacter baumannii*, the Esp protein of *E. faecalis* and the BapA protein of *Salmonella* sp. (Latasa, Roux et al. 2005; Tendolkar, Baghdayan et al. 2005; Loehfelm, Luke et al. 2008).

We have demonstrated that compounds that degrade polysaccharide-based biofilms, such as sodium metaperiodate, have little effect on *Gardnerella* biofilms, while proteases are able to effectively degrade the biofilms (Patterson, Girerd et al. 2007). This

suggests that a proteinaceous adhesin, like Bap, may mediate biofilm formation in *G. vaginalis*. In this study, a partial genome sequence of *G. vaginalis* was analyzed for homology to the staphylococcal Bap protein. This search identified a large hypothetical protein, with a core of internal repeating units, which we call BapL for Bap-like. An antibody was produced against a small antigenic peptide of this protein and was used for inhibition of function assays. It has also been shown that certain biofilm-associated proteins, like the enterococcal SgrA protein, can act as initial adhesins, binding to extracellular matrix proteins (Hendrickx, van Luit-Asbroek et al. 2009); therefore, the BapL protein was analyzed for its ability to bind to ECM proteins by blockage with the anti-bapL antibody. Also, biofilm associated proteins has been found to have homology to mucus-binding protein; therefore, the BapL protein was also analyzed for its ability to bind to mucin.

It has been shown that *G. vaginalis* adheres robustly to ME180 cervical epithelial cells (Patterson, Stull-Lane et al. 2010). To help elucidate whether the BapL protein interacts with a component on the surface of ME180 epithelial cells, immunoprecipitation assays were performed. Random mutagenesis was performed using the mutagen ethyl methanesulfonate (EMS) to produce a *bapL* deficient mutant, and finally, the BapL protein was expressed in the heterologous host *S. aureus* RN4220.

Results

Identification of a biofilm-associated protein

In order to identify new factors involved in biofilm formation, we searched a partial genome sequence of *G. vaginalis*, provided by the *Gardnerella vaginalis* Genome Project, Stanford Genome Technology Center (funded by the Ellison Medical Foundation) for sequence homology to the *S. aureus* Bap protein using the tBLASTn program provided by NCBI. Our analysis revealed one incomplete open-reading frame encoding a hypothetical protein with significant similarity to Bap. The identified gene encodes a protein of large size, with 9 Rib (resistance to proteases) domains, and 35% homology to the staphylococcal Bap protein, therefore, we have designated the gene BapL for Bap-like. Rib domains are found in group B streptococci and confer protective immunity to the bacteria (Stalhammar-Carlemalm, Stenberg et al. 1993). The BapL protein also has homology to other known biofilm-associated proteins (**Table 7**). Using Softberry's FGENESB and BPROM programs, it was determined that the identified gene was missing the beginning of the open reading frame and promoter; therefore, we initiated genome walking experiments to identify the remainder of the gene. The additional sequencing data found by genome walking was confirmed by pyrosequencing, which was performed at the VCU Nucleic Acids Core Laboratory using Roche 454 technology. One full run of GS FLX and one half fun GS FLX XLR were done for *G. vaginalis* strain 5-1 (coverage of ~175X). The fully sequenced open reading frame is 9,144 base pairs and encodes a protein with a predicted molecular mass of 325 kDa. Analysis of the amino acid sequence reveals a repetitive structure consistent with the Bap family of proteins, as well as a cell wall-anchoring motif consistent with surface expression in Gram-positive organisms (**Figure 20**).

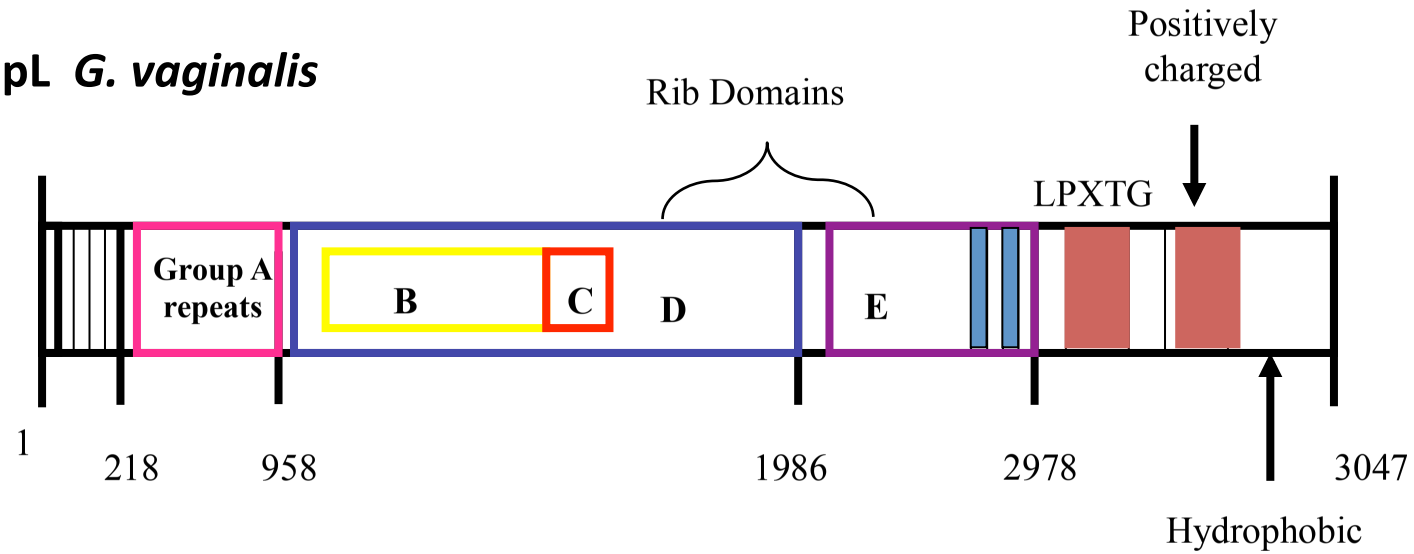
Table 7. Homologies between the Bap protein of *S. aureus* and the BapL protein of *G. vaginalis* to other known biofilm-associated proteins.

Organism	Protein	Similarity to Bap	Similarity to BapL
E. faecium	Esp	38%	39%
S. epidermidis	Aap	71%	34%
Salmonella sp.	BapA	29%	34%
A. baumanii	Bap	33%	34%

Figure 20. Structural schematic of the BapL protein from *G. vaginalis* compared to the Bap protein from *S. aureus*. Both proteins are large, cell wall anchored surface proteins with tandem repeating units. BapL is 325 kDa, contains 9 Rib (resistance to proteases) domains which are associated with protective immunity, and 35% homology to the staphylococcal Bap protein.

Bap *S. aureus*

BapL *G. vaginalis*



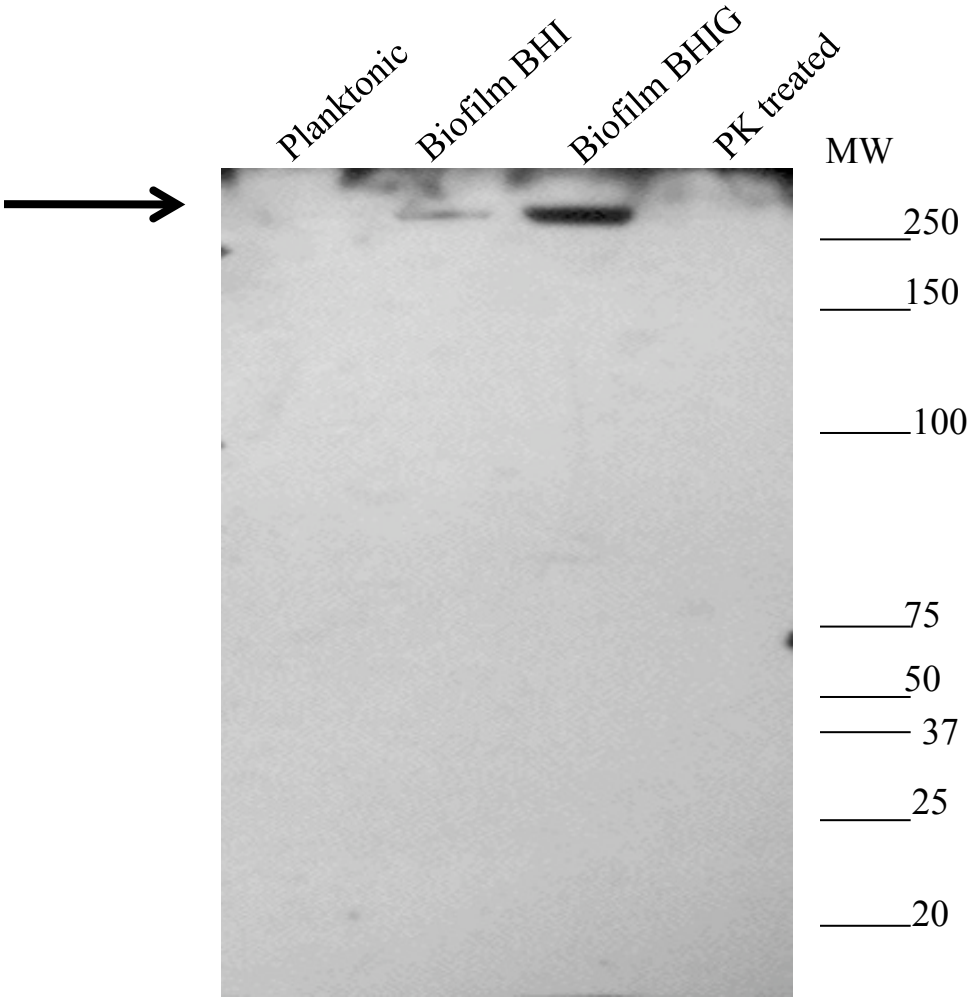
The BapL protein is highly expressed under biofilm conditions

In order to determine which conditions induce BapL expression, surface associated proteins were extracted from planktonic and biofilm cultures grown in BHI, as well as from biofilm cultures grown in BHIG. Bacterial pellets were standardized by weight then proteins extracted. The proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with purified polyclonal IgG rabbit antibodies raised against a small, antigenic peptide of BapL. As shown in **Figure 21**, a band of approximately 325 kDa is seen in the biofilm samples, while it is absent in the planktonic sample. This suggests that BapL is more highly expressed under conditions that promote biofilm formation. A biofilm grown in BHIG was treated with proteinase K to disrupt its biofilm structure and subjected to the same treatment to remove cell surface associated proteins. The absence of a BapL specific band in **Figure 21** indicates that the BapL protein was removed by this proteinase K treatment. The finding that proteinase K treatment disrupts biofilms and removes BapL from the surface suggests that BapL could possibly be involved in the biofilm structure.

The *bapL* gene is highly expressed under biofilm conditions

We next analyzed the expression of the *bapL* gene under different conditions. Overnight cultures of *G. vaginalis* strain 5-1 were diluted in fresh BHI or BHIG in T75 flasks and incubated anaerobically for 48 hours with fresh media change after 24 hours. Planktonic cultures of *G. vaginalis* were grown in 250 ml flasks of BHI anaerobically for 48 hours with gentle stirring to keep the bacteria in the planktonic state. Bacteria were

Figure 21. Western blot analysis of *G. vaginalis* proteins grown under different conditions. *G. vaginalis* cultures were grown overnight as biofilms in BHI or BHIG and as planktonic cultures in sBHIs and BHIG. Proteins were then extracted by sonication and loaded into each well. Proteins were separated by SDS-PAGE, transferred to PVDF membrane and probed with anti-bapL antibodies. Molecular mass standards are indicated in kilodaltons.


Planktonic
Biofilm BHI
Biofilm BHIG
PK treated
MW
250
150
100
75
50
37
25
20

harvested and mRNA extracted. Isolated mRNA was converted to cDNA using *bapL* and *16S* reverse primers and analyzed by real time reverse transcriptase PCR. Expression of the *bapL* gene was normalized to *16s* rRNA. **Figure 22** shows that expression of the *bapL* gene is highest in biofilms grown in BHI, whereas, expression is lowest in planktonic cultures. Biofilms grown in BHI had two times more expression of *bapL* than biofilms grown in BHIG. Planktonic expression of *bapL* was very low, with only a 0.0025 fold increase in *bapL* expression compared to expression of the 16S rRNA genes. This data shows that the *bapL* gene is significantly upregulated under biofilm conditions.

Anti-BapL antibody is specific and binds to the surface of *G. vaginalis*

Confocal microscopy was used to determine whether the anti-BapL antibody was specific and if the BapL protein was surface associated. Cultures of *G. vaginalis* and *Lactococcus lactis*, used as a negative control, were collected and washed with PBS then stained sequentially with purified anti-BapL antibody and labeled goat anti-rabbit secondary antibody. **Figure 23** shows that the anti-BapL antibody bound specifically only to *Gardnerella* bacteria, as demonstrated by the high fluorescence seen with addition of the anti-BapL antibody, while unrelated species, like *Lactococcus lactis*, showed no fluorescence with the addition of the anti-BapL antibody. Binding of the antibody to the surface of *G. vaginalis* confirms that the BapL protein is surface associated.

Anti-BapL antibody does not inhibit biofilm formation of *G. vaginalis*

Figure 22. The *bapL* gene is highly expressed in biofilm cultures. Overnight cultures of *G. vaginalis* were diluted in fresh BHI or BHIG in T75 flasks. Planktonic cultures were grown in 250 ml flasks of BHI. Each culture was grown anaerobically for 48 hours, either with media changer after 24 hours (biofilm only) or with gentle stirring (planktonic only). Cultures were harvested by centrifugation to pellet cells and RNA from all cultures was extracted using a Fastpro RNA kit. Isolated RNA was DNase treated to ensure the absence of genomic DNA contamination and converted to cDNA using *bapL* and *16S* reverse primers. The *bapL* gene expression was then analyzed by reverse transcriptase PCR. The absolute C_t values from 3 qPCR assays were averaged to determine the cycle threshold value from each sample. Expression of *bapL* was normalized to 16s rRNA as determined by cycle threshold values using the following equation: E=2^(16s C_t – bapL C_t).

Expression of *bapL* under planktonic and biofilm conditions

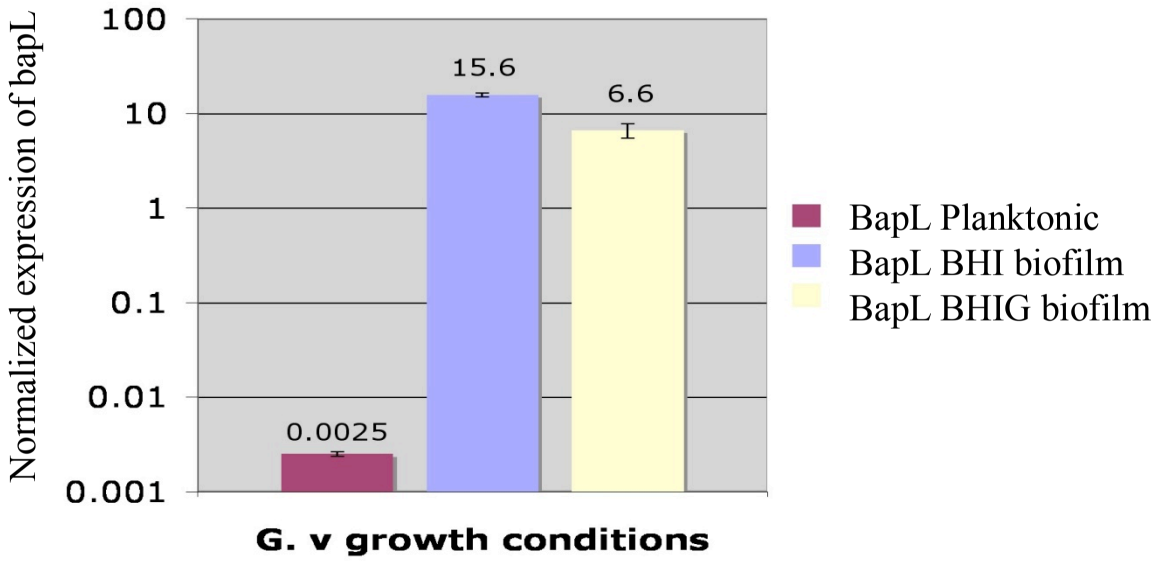
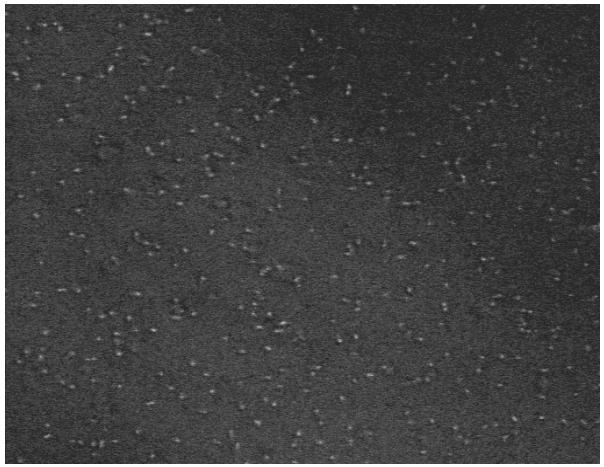


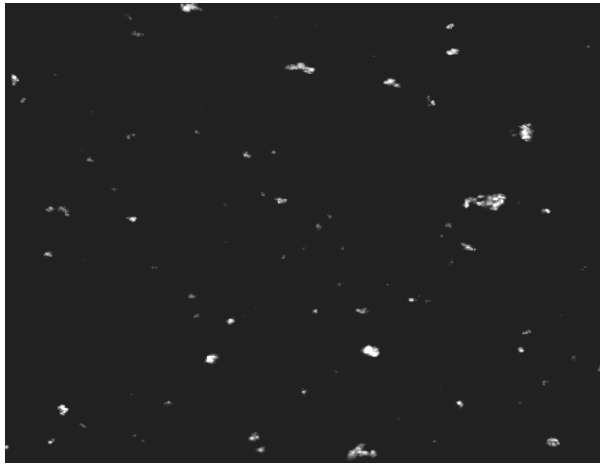
Figure 23. Confocal microscopy confirms surface expression of BapL. Cultures of overnight *G. vaginalis* and *Lactococcus lactis*, which was used as a negative control, were pelleted and washed with PBS, then stained sequentially with the anti-BapL Ab and Alexa 488 labeled goat ant-rabbit antibody and analyzed by confocal microscopy.

DIC *Lactococcus*

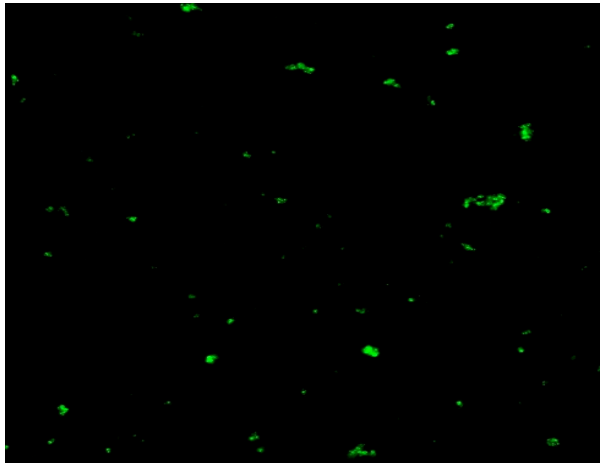


Alexa Fluor 488 *L.l*

DIC *Gardnerella*



Alexa Fluor 488 *G.v*



To determine if the BapL protein plays a role in biofilm formation by *G. vaginalis*, antibody inhibition assays were performed. Overnight cultures of *G. vaginalis* were diluted 1:100 in fresh BHIG and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. 200 µl of each culture was added to a 96-well plate and incubated anaerobically at 37ºC for 24 hours. The wells were washed with PBS to remove nonadherent bacteria and stained with safranin, which imparts a red color to biofilms. **Figure 24** shows that bacteria treated with anti-BapL antibody and preimmune antibody formed biofilms that were no different from wildtype bacteria. This shows that the addition of anti-BapL antibody was unable to inhibit biofilm of *G. vaginalis*.

Anti-BapL antibody does not inhibit initial adherence of *G. vaginalis*

Because it has been shown that the staphylococcal Bap protein has roles in both primary attachment and biofilm maturation, we next analyzed whether the BapL protein was playing a role in initial adherence to a surface (Cucarella, Solano et al. 2001). Overnight cultures of *G. vaginalis* were diluted 1:50 in fresh BHIG and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or preimmune antibody. 200 µl of each culture was added to a 96-well plate, centrifuged to maximize interaction with the surface of the 96-well plate, and incubated anaerobically at 37ºC for 2 hours. After the 2 hour incubation, the wells were washed with PBS to remove nonadherent bacteria and stained with safranin. **Figure 25** shows that bacteria treated with anti-BapL antibody and preimmune antibody produced no significant difference in initial

Figure 24. Biofilm formation by *G. vaginalis* is not inhibited by addition of anti-BapL antibody. An overnight culture of *G. vaginalis* was treated with either anti-BapL antibody or preimmune antibody for 30 minutes at room temperature on a rotator. The culture was then plated and incubated anaerobically at 37ºC for 24 hours. Nonadherent cells were removed by washing with PBS and adherent bacteria were stained with safranin, which imparts a red color to biofilms.

No antibody | Anti-bapL antibody | Preimmune antibody

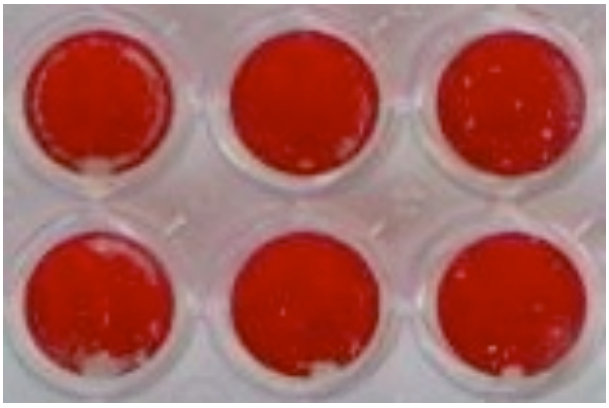
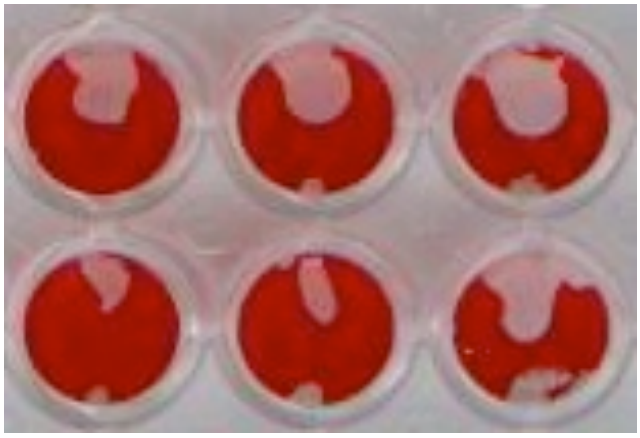


Figure 25. Initial adherence of *G. vaginalis* is not affected by the anti-BapL antibody. An overnight culture of *G. vaginalis* was treated with either anti-BapL antibody or preimmune antibody for 30 minutes at room temperature on a rotator. The culture was then plated and incubated anaerobically at 37ºC for 2 hours. Nonadherent cells were removed by washing with PBS and adherent bacteria was stained with safranin, which imparts a red color to biofilms

No antibody | Anti-BapL antibody | Preimmune antibody



adherence than wildtype bacteria. This shows that the addition of anti-BapL antibody was unable to inhibit initial adherence of *G. vaginalis*.

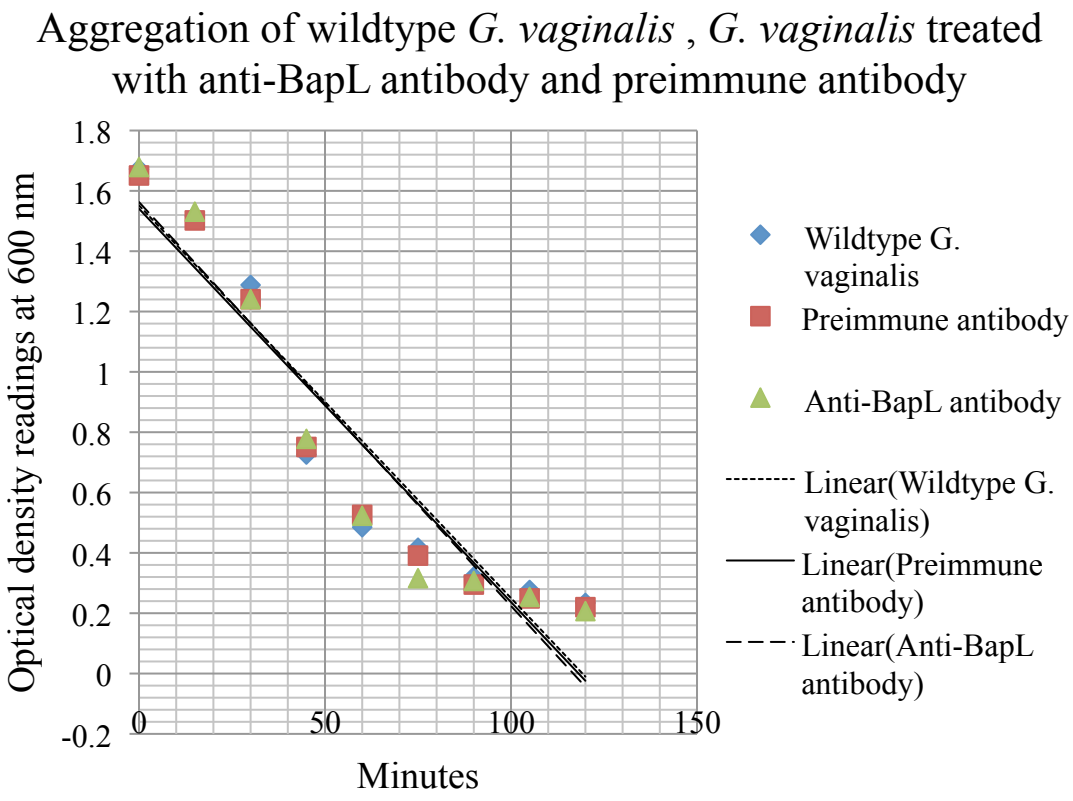
Anti-BapL antibody does not affect aggregation of *G. vaginalis* bacteria

Aggregation is an important ability of many bacteria and sometimes utilizes the same intercellular adhesins involved in biofilm formation (Schroeder, Jularic et al. 2009; Thompson, Abraham et al. 2010). To analyze whether BapL was playing a role in aggregation, we again employed antibody inhibition. Overnight cultures of *G. vaginalis* were standardized to ensure equal amounts of bacteria and either treated with PBS as a control, anti-BapL antibody or preimmune antibody. The OD_{600} of the cultures was measured every 15 minutes for 120 minutes, and the measurements were plotted and slopes analyzed. Slope values similar to the slope seen with wildtype bacteria would indicate the anti-BapL antibody had no effect on bacterial aggregation. **Figure 26** shows that the slope of the lines for wildtype bacterial aggregation, aggregation of bacteria treated with anti-BapL antibody and bacteria treated with preimmune antibody were similar, ranging from -0.0130 to -0.0134. This data suggests that the anti-BapL antibody does not affect aggregation.

Anti-BapL antibody does not affect *G. vaginalis* adherence to cervical epithelial cells

Because the anti-BapL antibody did not affect biofilm formation or initial adherence, we next tested whether the antibody could inhibit adherence of *G. vaginalis* to cervical epithelial cells. Overnight cultures of *G. vaginalis* were incubated for 30 minutes

Figure 26. Aggregation of *G. vaginalis* is not affected by the anti-BapL antibody. An overnight *G. vaginalis* culture was standardized to ensure equal numbers of bacteria and either treated with PBS as a control, anti-BapL antibody or preimmune antibody. OD_{600} readings were taken ever 15 minutes for 120 minutes. The measurements were graphed, a line plotted and the slopes compared. The slopes were similar, ranging from -0.0130 to -0.0134, indicating that addition of the antibodies did not affect aggregation.

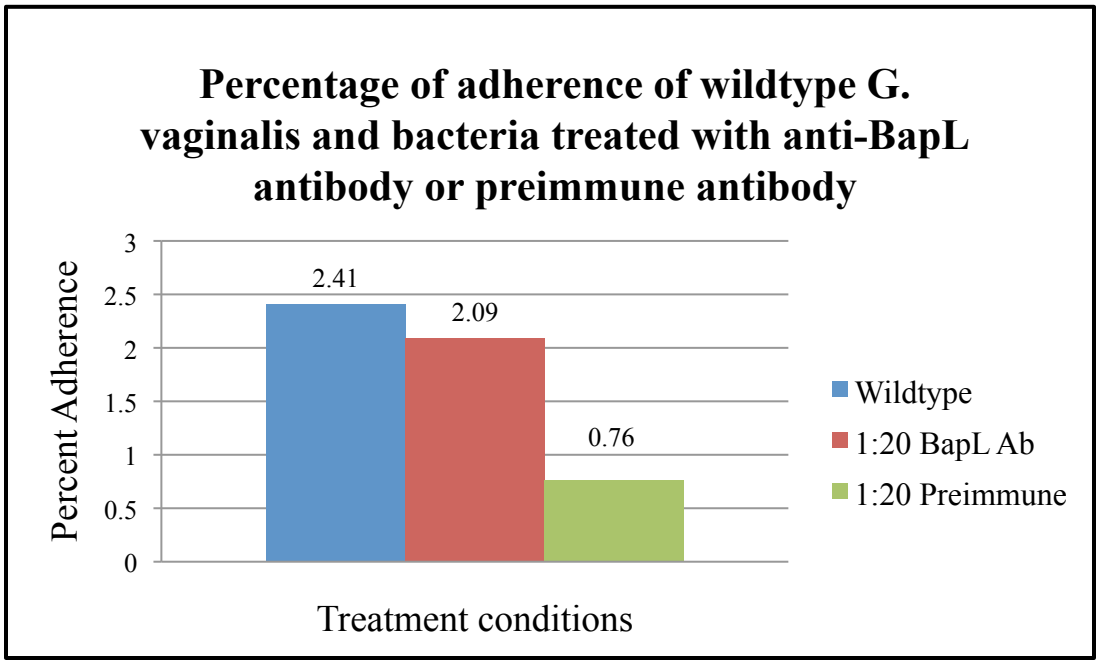

Aggregation of wildtype G. vaginalis , G. vaginalis treated with anti-BapL antibody and preimmune antibody
Optical density readings at 600 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
50
100
150
Minutes
Wildtype G. vaginalis
Preimmune antibody
Anti-BapL antibody
Linear(Wildtype G. vaginalis)
Linear(Preimmune antibody)
Linear(Anti-BapL antibody)

at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody, standardized to ensure equal numbers of bacteria then added to confluent monolayers of ME180 cervical epithelial cells. The plates were centrifuged to maximize interaction between the bacteria and epithelial cells and incubated at 37ºC for 15 minutes in 5% CO_2. After the incubation, the wells were washed to remove nonadherent bacteria and harvested with addition of 5% saponin. The bacteria were then diluted 10-fold, plated on BPS plates and incubated for 48 hours, before plates were analyzed and colonies counted. **Figure 27A** shows the percent adherence for wildtype *G. vaginalis*, *G. vaginalis* treated with 1:20 BapL antibody and *G. vaginalis* treated with 1:20 preimmune antibody. Addition of anti-BapL antibody did not significantly affect adherence of the bacteria to cervical epithelial cells. Although the percent adherence of *G. vaginalis* treated with preimmune antibody was relatively lower than either the wildtype bacteria or bacteria treated with anti-BapL antibody, this could have been due to clumping of the bacteria during plating. Adherence was also analyzed by confocal microscopy (**Figure 27B**) and it was seen that adherence did not differ significantly between wildtype *G. vaginalis* and *Gardnerella* treated with anti-BapL antibody or preimmune antibody. However, when *G. vaginalis* was pretreated with proteinase K prior to the adherence assay, the bacteria were unable to adhere to the cervical epithelial cells (**Figure 28**). This suggests that *G. vaginalis* may utilize a cell wall-anchored protein, potentially the BapL protein, for adherence to cervical epithelial cells

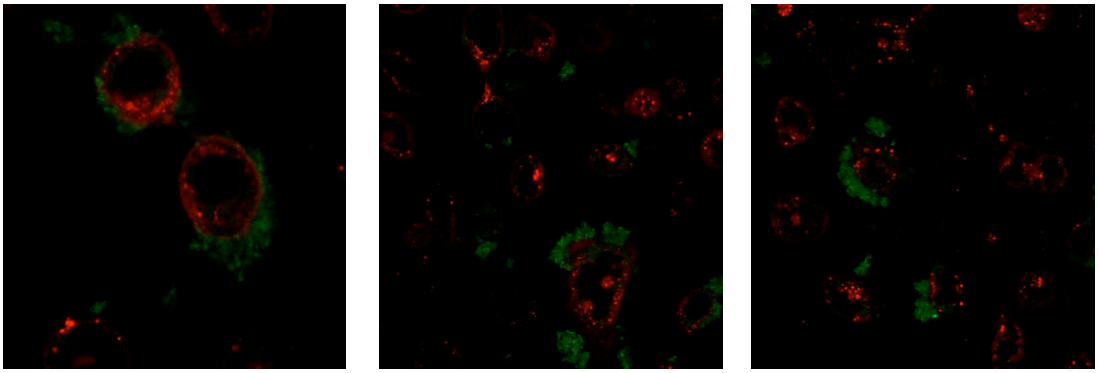
Anti-BapL antibody does not affect *G. vaginalis* interaction with ECM proteins

Figure 27. Adherence of *G. vaginalis* to cervical epithelial cells is not affected by addition of anti-BapL antibody. Adherence of wildtype *G. vaginalis*, bacteria treated with anti-BapL antibodies and bacteria treated with preimmune antibodies was compared. (A) Percent adherence calculated from CFUs and (B) Confocal images of adherence.

A.



B.



Wildtype *G. vaginalis* | *G. vaginalis* BapL Ab | *G. vaginalis* Preimmune Ab

Figure 28. Adherence of proteinase K treated *G. vaginalis* to cervical epithelial cells. Bacteria were grown in sBHIG anaerobically at 37 °C for 24 hrs. *G. vaginalis* was first treated with 20 µg/ml of proteinase K for an hour, pelleted and washed to remove residual enzyme. Bacteria (green) were standardized to ensure equal numbers, added to vaginal epithelial cells (red) and incubated for 15 minutes before washing to remove nonadherent bacteria. Qualitative estimates of adherence were ascertained by confocal microscopy

Proteinase K treated GV adherence

Wildtype *G. vaginalis* adherence

A surface adhesin, SgrA, involved in biofilm formation in hospital-acquired *Enterococcus faecium* strains, has recently been shown to play a role in binding to extracellular matrix molecules nidogen 1, nidogen 2 and fibrinogen (Hendrickx, van Luit-Asbroek et al. 2009). Perhaps the *G. vaginalis* BapL protein is acting as a microbial surface component recognizing adhesive matrix molecules (MSCRAMM), allowing the bacteria to interact with extracellular matrix molecular like fibrinogen or fibronectin. To test this, overnight cultures of *G. vaginalis* were diluted 1:10 in PBS and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. 200 µl of each culture was added to a 96-well plate that had been previously incubated and immobilized with the ECM proteins fibronectin, fibrinogen, hyuluronic acid, elastin, and collegan IV. The plates were centrifuged to maximize interaction between the bacteria and ECM proteins then incubated at 37ºC for 10 minutes. After the 10 minute incubation, the wells were washed to remove nonadherent bacteria then stained with safranin to ascertain the interaction between bacteria and ECM proteins. **Figure 29** is an image of the scanned 96-well plate with *G. vaginalis* allowed to interact with the ECM proteins. No difference is seen between wildtype *G. vaginalis* and *G. vaginalis* treated with the anti-BapL antibody or preimmune antibody. This data suggests that the anti-BapL antibody is unable to inhibit interaction of *G. vaginalis* with ECM proteins.

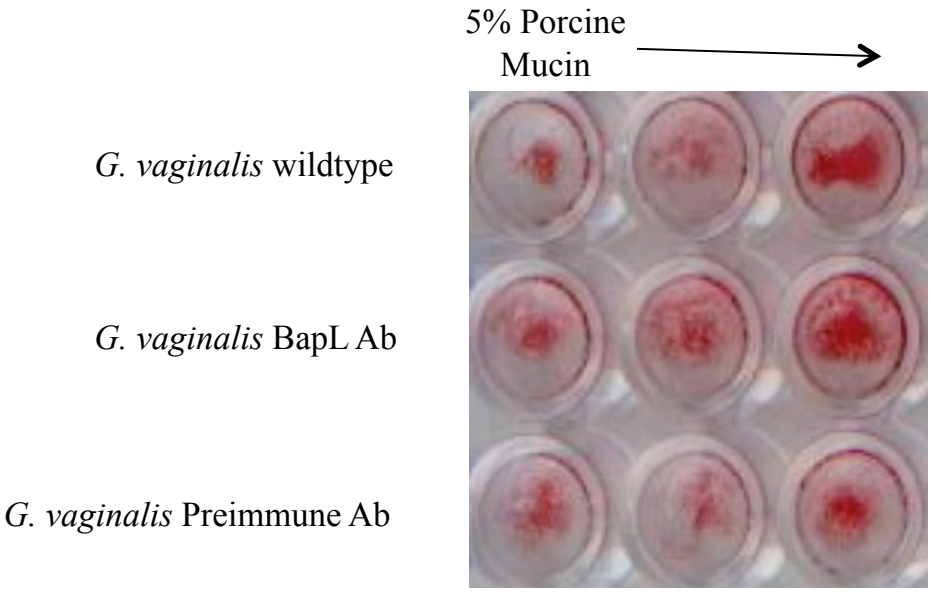
Adherence of *G. vaginalis* to mucus is not affected by the anti-BapL antibody

Figure 29. Adherence of *G. vaginalis* to ECM proteins is not affected by the anti-BapL antibody. Overnight cultures of *G. vaginalis* were diluted 1:10 in PBS and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or preimmune antibody. 200 µl of each culture was added to a 96 well plate that had been precoated with ECM proteins. The plates were centrifuged, incubated, wells washed and stained with safranin . Red color indicates attached bacterial cells.

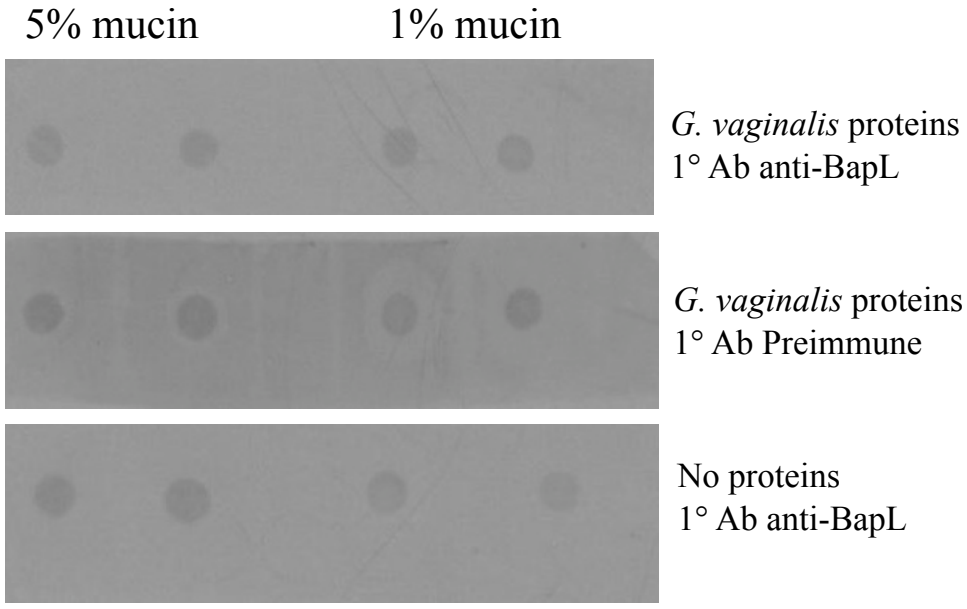
Homology searches show that the BapL protein has homology to *the Lactobacillus fermentum* protein Mlp, which in turn is similar to the mucus binding protein, Mub, from *Lactobacillus reuteri* (Roos and Jonsson 2002; Turner, Hafner et al. 2003). The Mub protein is a 358 kDa protein with 14 approximately 200 amino acid repeats and features typical of other cell surface proteins of Gram positive bacteria (Roos and Jonsson 2002). We next analyzed whether the BapL protein was playing a role in mucus binding activity of *G. vaginalis*. Overnight cultures of *G. vaginalis* were diluted 1:10 in PBS and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or 400 µg of preimmune antibody. 200 µl of each culture was added to a 96-well plate that had been previously incubated and immobilized with mucin. The plate was centrifuged to maximize interaction between the bacteria and mucin then incubated at 37ºC for 10 minutes. After the 10 minute incubation, the wells were washed to remove nonadherent bacteria then stained with safranin to ascertain the interaction between bacteria and mucin. **Figure 30A** demonstrates that there was no difference in mucus binding ability between wildtype *G. vaginalis* and *G. vaginalis* treated with anti-BapL antibody or preimmune antibody, suggesting that the anti-BapL antibody is unable to inhibit interaction of *G. vaginalis* with mucus. A mucin dot blot utilizing 5% and 1% dilutions of mucin on a nitrocellulose membrane, probed with purified surface proteins from *G. vaginalis* demonstrates that the BapL protein is not binding to porcine mucin (**Figure 30B**).

Figure 30. Adherence of *G. vaginalis* to mucus is not affected by the anti-BapL antibody. (A) Overnight cultures of *G. vaginalis* were diluted 1:10 in PBS and incubated for 30 minutes at room temperature on a rotator with either 400 µg of anti-BapL antibody or preimmune antibody. 200 µl of each culture was added to a 96 well plate that had been precoated with mucin. The plate was centrifuged, incubated, wells washed and stained with safranin. Red color indicates attached bacterial cells. (B) Mucin dot blot of adherence of G. vaginalis proteins.

A.


5% Porcine Mucin
G. vaginalis wildtype
G. vaginalis BapL Ab
G. vaginalis Preimmune Ab

B.


5% mucin
1% mucin
G. vaginalis proteins
1° Ab anti-BapL
G. vaginalis proteins
1° Ab Preimmune
No proteins
1° Ab anti-BapL

Opsonophagocytosis assays demonstrate that the anti-BapL antibody acts as an effective opsonin.

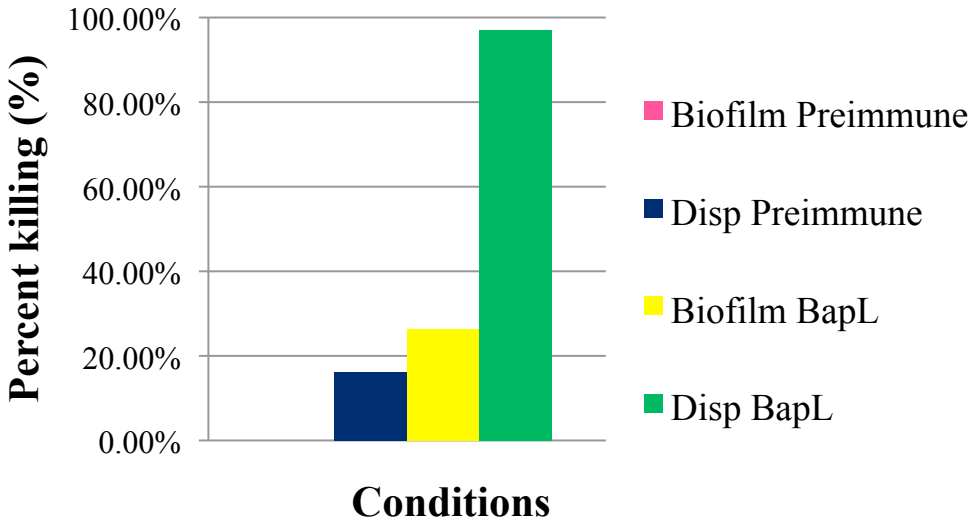
We compared phagocyte killing by the anti-BapL antibody and preimmune antibody for biofilm and dispersed biofim cultures of *G. vaginalis*. It is known that biofilm bacteria exhibit increased resistance to phagocytosis; therefore, we hypothesized that the percent killing seen with biofilm bacteria compared to dispersed biofilm bacteria would be low. **Figure 31** shows that 100% of biofilm bacteria survived when leukocytes, preimmune antibody, and complement were added; however, 26.3% of biofilm bacteria were killed when preimmune antibody was replaced with the anti-BapL antibody. In contrast, dispersed biofilm bacteria were rapidly killed by leukocytes when the anti-BapL antibody was added (97% killing). This data suggests that dispersed biofilm *G. vaginalis* bacteria are more sensitive to opsonophagocytosis and the anti-BapL antibody is acting as an effective opsonin.

Far Western analysis failed to demonstrate an interaction between BapL and ME180 cervical epithelial protein

Since we demonstrated that *G. vaginalis* adheres robustly to ME180 cervical epithelial cells, we analyzed whether the BapL protein was interacting with a structure on the surface of the cervical epithelial cells. Proteins were extracted from confluent monolayers of ME180 cervical epithelial cells and separated by SDS-PAGE. The gels were then transferred to PVDF membrane and incubated with surface proteins that had been purified from *G. vaginalis* by sonication. The membranes were then washed, probed

Figure 31. Opsonophagocytic activity of anti-BapL antibody and preimmune antibody against biofilm and dispersed biofilm cultures of *G. vaginalis*. G. vaginalis biofilms were grown on glass beads. Glass beads were sonicated for 10 sec to produce dispersed biofilm cultures. Cultures were subjected to opsonophagocytic assays utilizing leukocytes, baby bunny complement and either anti-BapL antibody or preimmune antibody. The assays were incubated for 90 mins then dilution plated for CFU counts. Percent killing was determined as described in the text.

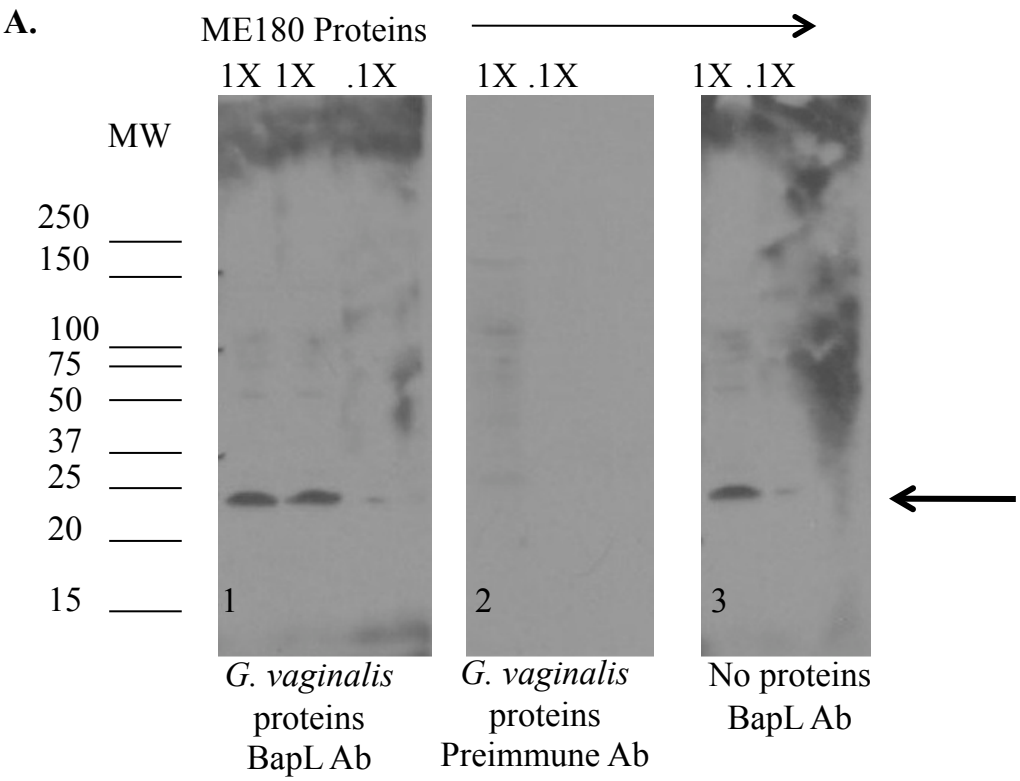
Opsonophagocytosis killing between *G. vaginalis* biofilm and dispersed biofilm using anti-BapL antibody or preimmune antibody

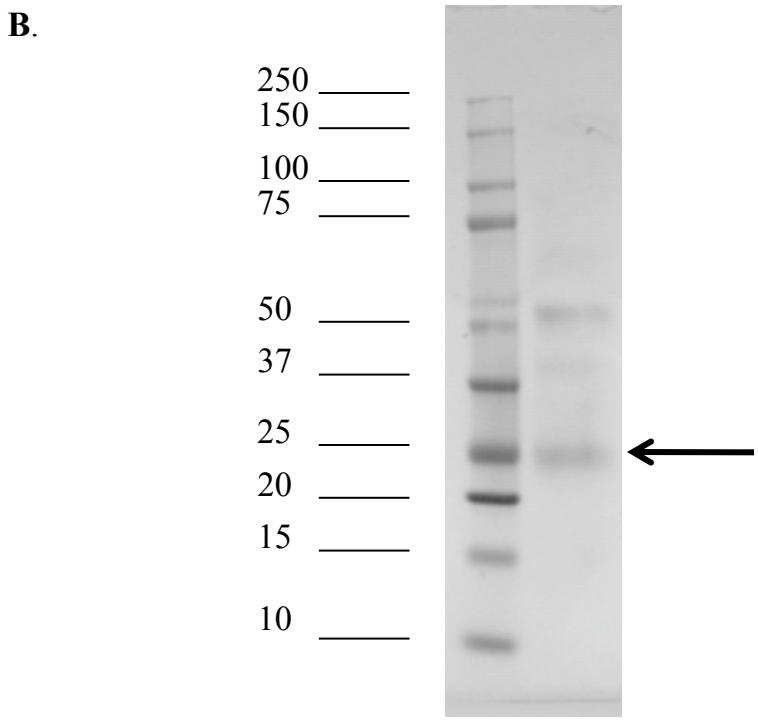


with anti-BapL antibody followed by HRP-labeled goat anti-rabbit antibody, and analyzed by film. **Figure 32A** shows three membranes, one which was probed with *G. vaginalis* proteins and anti-BapL antibody, the second was probed with *G. vaginalis* proteins and preimmune antibody and the third was an antibody control membrane only probed by the anti-BapL antibody. A 23 kDa band is seen on the membrane probed with *G. vaginalis* proteins with the anti-BapL antibody; however, the exact same band is seen in the antibody control membrane which was not probed with *G. vaginalis* proteins. This suggests that the anti-BapL antibody is binding nonspecifically to a ME180 cervical epithelial cell protein.

To confirm these results, we continued with an immunoprecipitation assay utilizing the anti-BapL antibody coupled to a protein G column to pull-down BapL-ME180 protein complexes. Anti-BapL antibody was coupled to protein G beads and allowed to incubate with 10 mg of *G. vaginalis* surface proteins and 10 mg of ME180 cervical epithelial cell proteins. After a two hour incubation, the mixture was placed in a column and washed 4X with PBS. The bound proteins were eluted by addition of 0.2M Glycine/HCl pH 2.5 and neutralized with 1M Tris/HCl pH 9.0. The proteins were separated by SDS-PAGE and the gel stained with Imperial protein reagent to visualize proteins. **Figure 32B** shows the immunoprecipitated proteins; only the 23 kDa cervical epithelial cell protein and a few contaminating proteins are seen on the gel, the 325 kDa BapL protein is missing. This suggests that the 23 kDa protein is actually interacting nonspecifically with the anti-BapL antibody and is not interacting with the BapL protein.

Figure 32. Immunoprecipitation assays demonstrate that the anti-BapL antibody, not BapL, interacts with a ME180 cervical epithelial protein. Me180 cervical epithelial proteins were harvested and separated by SDS-PAGE. The gel was transferred to PVDF membrane and incubated with surface proteins from *G. vaginalis*. The membrane was probed with either anti-BapL antibody or preimmune Ab. (A) PVDF membrane incubated with *G. vaginalis* proteins probed with anti-BapL antibody, membrane incubated with *G. vaginalis* proteins probed with preimmune antibody or membrane incubated with no protein and probed with anti-BapL antibody as a control. (B) Anti-BapL antibody was coupled to a protein G column and 10 mg of *G. vaginalis* surface proteins and 10 mg of ME180 cervical epithelial cell protein were ran onto column, bound proteins were eluted by 0.2M Glycine/HCl pH 2.5. Eluted proteins were separated by SDS-PAGE and the gel was stained overnight with Imperial protein stain. Arrow indicates the approximatedly 23 kDa protein that interacts nonspecifically with the anti-BapL antibody.


A.
ME180 Proteins
1X 1X .1X
1X .1X
1X .1X
MW
250
150
100
75
50
37
25
20
15
1
2
3
G. vaginalis proteins BapL Ab
G. vaginalis proteins Preimmune Ab
No proteins BapL Ab


B.
250
150
100
75
50
37
25
20
15
10

EMS mutagenesis did not produce a *bapL* mutant of *G. vaginalis*

Since no genetic system is available in *G. vaginalis*, we utilized random mutagenesis in order to produce a *bapL* mutant. Ethyl methanesulfonate or EMS is a mutagenic, teratogenic and possibly carcinogenic organic compound that produces random mutations in genetic material by nucleotide substitution. EMS reacts with guanine in DNA, forming 0-6-ethylguanine, which impairs the ability of DNA polymerase to accurately incorporate a cytosine opposite of guanine. An EMS kill curve was performed and 50 µl of EMS for 30 minutes was found to kill 90% of *G. vaginalis*. This amount of EMS and 30 minutes was used to mutate *G. vaginalis*. The mutagenized *G. vaginalis* culture was allowed to recover for 48 hours in BHIG before being stained with anti-BapL antibody and an Alexa-488 labeled goat anti-rabbit secondary antibody. These stained bacteria were then sorted by fluorescence activated cell sorting (FACS) and low fluorescent intensity bacteria were collected. The bacteria with low fluorescence intensity could potentially have a defect in the *bapL* gene, producing fewer BapL proteins on the surface of the bacteria and thereby causing a lower amount of fluorescence intensity due to a lower amount of anti-BapL antibody binding. Bacteria with the lowest fluorescence intensity were collected in individual wells of a 96-well plate and allowed to incubate anaerobically for 48 hours at 37°C.

Approximately 20 potential *G. vaginalis* mutants were found after the 48 hour incubation. Because we hypothesized that BapL may be playing a role in biofilm formation, we utilized biofilm assays to determine if any of the 20 potential mutants had a defect in biofilm formation. Unfortunately, no defect in biofilm formation was seen in any

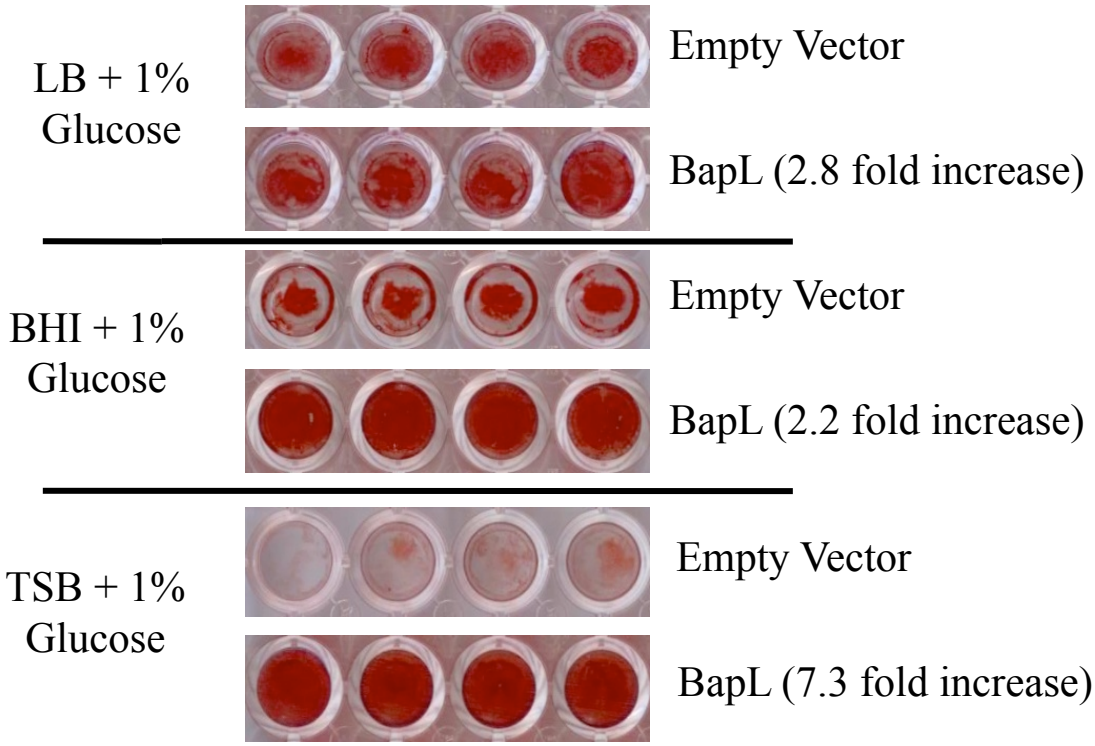
of the potential mutants tested. Western blot analysis of surface proteins from each of the potential mutants was also inconclusive, in that no mutant was definitively BapL negative. These experiments should be repeated, since thousands of potential mutants would need to be screened in order to cover the whole *G. vaginalis* genome.

Heterologous expression of BapL in *S. aureus* caused an increase in biofilm formation but this was found to be due to an increase in PNAG production

Finally, the *bapL* gene was cloned into the *E. coli/S. aureus* shuttle vector pRB473 for heterologous expression in the poor-biofilm producing strain of *S. aureus* RN4220. The *bapL* gene was PCR amplified using the primers BapSbfFWD and BapSbfReV, which had been designed from previous sequence analysis to introduce Sbf1 restriction sites on either end of the gene. The PCR product was subcloned into the plasmid pCR2.1®Topo to construct the recombinant plasmid pCR2.1-Topo-*bapL*. This plasmid was used to transform CH3 blue chemically competent *E. coli* cells, resultant colonies were pooled and plasmid purified, and plasmid was digested with Sfb1 to drop out the *bapL* PCR product. This digested *bapL* PCR product was then ligated into Pst1-linearized *E. coli/S. aureus* shuttle vector pRB473, producing plasmid pRB473-*bapL*, and used to transform CH3 blue chemically competent *E. coli*. Plasmid was purified from individual *E. coli* transformants and screened for correct insert by digestion with Pst1. Plasmid with correct insert size was used to transform *S. aureus* strain RN4220, producing strain RN4220-*bapL*.

Strain RN4220-*bapL* and RN4220 with empty pRB473 were analyzed for their ability to form a biofilm. **Figure 33** shows the biofilms produced by strain RN4220-*bapL*

Figure 33. Heterologous expression of BapL in *S. aureus* RN4220 caused an increase in biofilm formation. The *bapL* gene was PCR amplified and cloned into the *E. coli/S. aureus* shuttle vector pRB473. Plasmid *pRB473-bapL* and empty vector were transformed into *S. aureus* RN4220, which is a poor-biofilm producing strain, by electroporation. Cultures were grown in LB + 1% glucose, BHI + 1% glucose and TSB + 1% glucose and analyzed for biofilm formation by washing and staining with safranin.


LB + 1% Glucose
Empty Vector
BapL (2.8 fold increase)
BHI + 1% Glucose
Empty Vector
BapL (2.2 fold increase)
TSB + 1% Glucose
Empty Vector
BapL (7.3 fold increase)

and empty vector under different media conditions. *S. aureus* RN4220-*bapL* was able to produce a thicker biofilm than RN4220 with empty vector under the media conditions tested, producing a 2.8 fold increase in biofilm in LB + 1% glucose, a 2.2 fold increase in biofilm in BHI + 1% glucose and a 7.3 fold increase in TSB + 1% glucose.

S. aureus has a very efficient stress response system; therefore, expression of foreign proteins can upregulate production of PNAG. To ensure that the introduction of the *bapL* gene had not led to upregulation of PNAG production, a dot blot assay for PNAG was employed. Standardized amounts of overnight cultures of RN4220-*bapL* and empty vector were centrifuged to pellet the cells and resuspended in 0.5 M EDTA. The samples were boiled, supernatants diluted, and blotted onto nitrocellulose membrane. The membranes were then dried, blocked with BSA, and probed with anti-PNAG antibody followed by ant-goat HRP-labeled secondary antibody. **Figure 34** shows the PNAG dot blot of two colonies of RN4220-*bapL* and RN4220 with empty vector; bacteria harboring the plasmid pRB473-*bapL* had a significant increase in production of PNAG compared to bacteria with empty vector. This suggests that the increase in biofilm formation seen in bacteria with pRB473-*bapL* is due to an increase in PNAG production and is not directly related to production of the BapL protein.

To circumvent this problem of increased production of PNAG, the pRB473-*bapL* plasmid was transduced into *S. aureus* strain RN4220Δ*ica,* which is deficient in the genes required to produce PNAG. Biofilm formation was then assessed without the interference of increased production of PNAG. **Figure 35** shows the biofilms produced by RN4220Δ*ica* with pRB473-*bapL* and empty vector in different media conditions. No

Figure 34. Dot blot analysis of PNAG production by wildtype *S. aureus* RN4220 and *S. aureus* RN4220-*bapL* and empty vector. Overnight cultures of RN4220, RN4220-*bapL* and RN4220 empty vector were centrifuged to pellet the cells and resuspended in 0.5 M EDTA. The samples were boiled to extract PNAG, diluted and blotted onto a nitrocellulose membrane. The membranes were probed with anti-PNAG antibody and HRP-labeled secondary antibody before being analyzed by x-ray film.

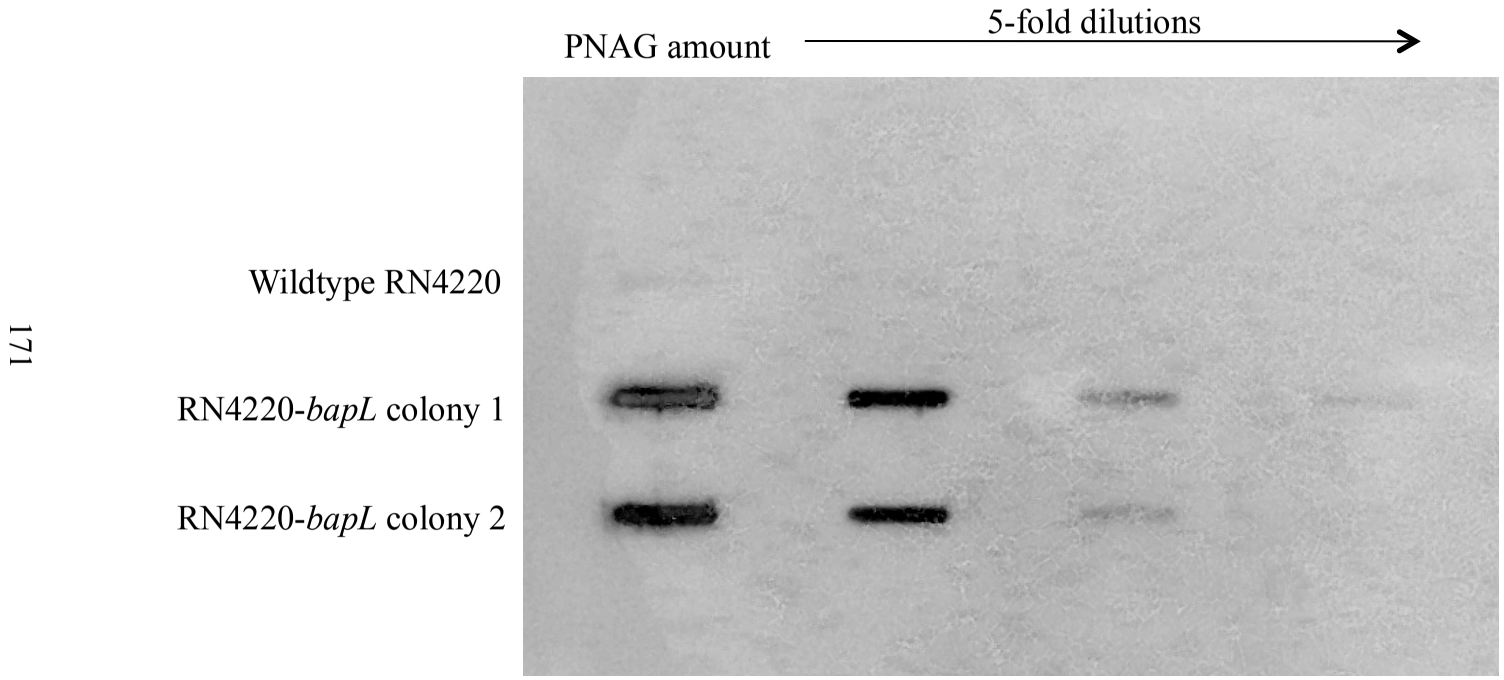
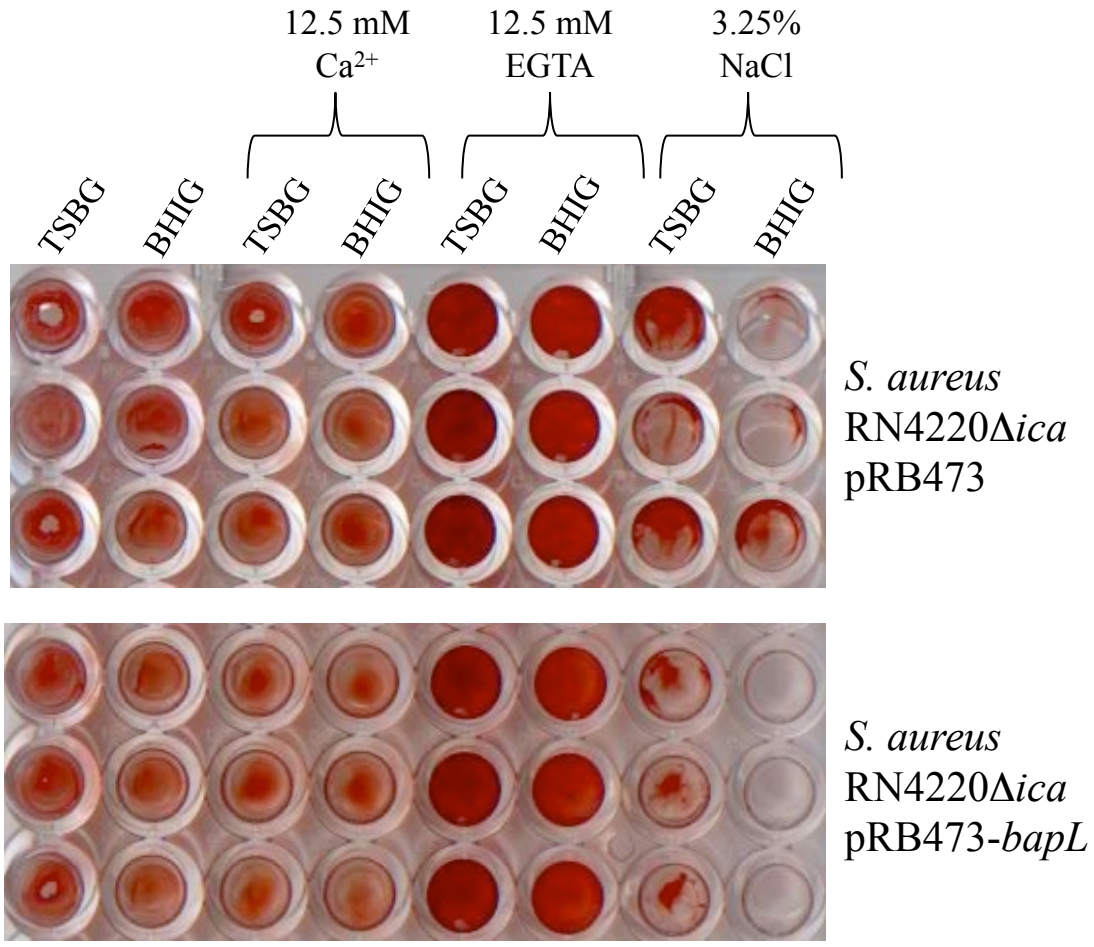

PNAG amount
5-fold dilutions
Wildtype RN4220
RN4220-*bapL* colony 1
RN4220-*bapL* colony 2

Figure 35. Heterologous expression of BapL in a PNAG negative strain did not cause an increase in biofilm formation. The *bapL* gene was PCR amplified and cloned into the *E. coli/S. aureus* shuttle vector pRB473. Plasmid *pRB473-bapL* and empty vector were transduced into *S. aureus* RN4220Δ*ica*, which is deficient in the genes required to produce PNAG. Cultures were grown in TSB + 1% glucose, 12.5 mM calcium, 12.5 mM EGTA and 3.25% NaCl and BHI + 1% glucose, 12.5 mM calcium, 12.5 mM EGTA and 3.25% Nacl and analyzed for biofilm formation by washing and staining with safranin.


12.5 mM
Ca2+
12.5 mM
EGTA
3.25%
NaCl
TSBG
BHIG
TSBG
BHIG
TSBG
BHIG
TSBG
BHIG
S. aureus
RN4220Δica
pRB473
S. aureus
RN4220Δica
pRB473-bapL

significant difference is seen between the biofilms produced by the bacteria with pRB473-*bapL* or empty vector in any of the eight media conditions tested. This could be suggestive of a few things: the BapL protein may not be surface exposed or functional in the heterologous host, the protein may not be translated in the host, the BapL protein may not be important in biofilm formation or it may need to act in concert with other proteins, like pilin, to produce biofilm.

In order to elucidate the function of the BapL protein, heterologous expression was attempted in a number of different organisms, including *Bifidobacterium longum*, which is the closest known relative of *G. vaginalis*, *Lactococcus lactis* and *Bacillus subtilis*. Unfortunately, technical limitations related to the very large size of BapL prevented expression in the other host strains.

Discussion

Recently, Swidsinski et al. found that *G. vaginalis* is able to form an adherent biofilm on the vaginal epithelium of women with BV and that this biofilm may contribute to the pathogenesis and recurrence of BV (Swidsinski, Mendling et al. 2005). Supporting the argument that the biofilm phenotype of *Gardnerella* leads to the recurrence seen with BV are the follow-up papers by Swidsinski et al. and Patterson et al. that observed a resurgence of a dense an active bacterial biofilm consisting primarily of *G. vaginalis* after cessation of treatment and biofilms of *G. vaginalis* were resistant to high amounts of lactic acid and hydrogen peroxide (Patterson, Girerd et al. 2007; Swidsinski, Mendling et al. 2008). It has also been shown that *G. vaginalis* produces a number of virulence properties,

including the ability to form a biofilm, produce cytotoxic changes to mammalian cells and adhere to vaginal epithelial cells, that many of the other BV-associated organisms do not possess; this lends precedence to the theory that *G. vaginalis* may be the most important organism in the pathogenesis of BV. We choose to study biofilm formation by *G. vaginalis* in order to better understand the bacteria's role in BV and improve available treatment methods.

We identified a protein in *G. vaginalis* that has significant homology to the BAP (biofilm-associated protein) family of proteins. The first member of the BAP family to be described was found in *Staphylococcus aureus* and many have since been described in a number of other pathogenic bacteria, including *Salmonella* sp., *Enterococcus* sp, and most recently *Acinetobacter baumannii* (Cucarella, Solano et al. 2001; Toledo-Arana, Valle et al. 2001; Latasa, Roux et al. 2005; Latasa, Solano et al. 2006; Loehfelm, Luke et al. 2008). All BAP proteins are high molecular weight, surface proteins with a core of internal repeats involved in biofilm formation (Latasa, Solano et al. 2006). The staphylococcal Bap protein is involved in primary attachment of the bacteria to a surface, biofilm maturation, and production of a persistant infection (Cucarella, Solano et al. 2001; Latasa, Solano et al. 2006).

The newly identified *G. vaginalis* protein, which we call BapL for Bap-like, is a high molecular weight protein, with a predicted molecular mass of 325 kDa. It is a highly repetitive protein, with multiple copies of repeat domains. We hypothesize that the extensive repeat region seen in the *G. vaginalis* BapL protein could serve to promote interactions between the protein and abiotic surfaces or with other BapL proteins on

neighboring bacteria, thus leading to biofilm formation and maturation. The BapL protein is exposed on the surface of *G. vaginalis*, as seen by confocal microscopy and western blot analysis. This is consistent with the sequencing data, which shows that the BapL protein has a carboxy-terminal segment containing an LPxTG motif, which is typical of cell wall-anchored surface proteins in gram-positive bacteria (Navarre and Schneewind 1994). The *bapL* gene is also highly expressed in biofilm cultures, as analyzed by reverse-transcriptase PCR.

An antibody was generated in rabbits against a small, antigenic peptide of the BapL protein. This antibody was utilized in antibody inhibition assays against biofilm formation, initial adherence, adherence to cervical epithelial cells, and adherence to ECM proteins and mucin. However, the anti-BapL antibody was unable to inhibit any of these properties of *G. vaginalis*. This does not conclusively demonstrate that BapL is not involved in any of these properties. The antibody was designed against a small, antigenic peptide of the BapL protein and it therefore may bind in such a way that it does not inhibit the active site of the protein thereby making the antibody inefficient in inhibiting the role of BapL or perhaps there is redundancy within the BapL protein and other sites on the protein can be used to elicit a function. Another reason why the anti-BapL antibody may not block biofilm formation is that there may be other proteins, carbohydrates or nucleic acids involved in biofilm formation that the anti-BapL antibody is unable to block.

Next, we analyzed whether the BapL protein was interacting with a receptor on the surface of ME180 cervical epithelial cells to cause adherence. ME180 cervical epithelial cell proteins were separated and transferred to a PVDF membrane and incubated with *G.*

vaginalis surface proteins. Upon film exposure, a 23 kDa protein was visible on the membrane incubated with *G. vaginalis* proteins and probed with anti-BapL antibody. Unfortunately, the control membrane, which was not incubated with protein but was probed with anti-BapL antibody, also showed that 23 kDa protein. This suggests that the anti-BapL antibody interacts nonspecifically with a ME180 cervical epithelial cell protein. However, this does not rule out the fact that BapL could be interacting with a ME180 cervical epithelial cell protein; perhaps the BapL protein has become nonfunctional during the purification process and is unable to bind to is receptor or the receptor did not retain fuction after electrophoresis and electroblotting.

To further elucidate whether the BapL protein was interacting with a receptor on the surface of ME180 cervical epithelial cells, immunoprecipitation assays were conducted. Anti-BapL antibody was coupled to protein G beads and used to pull down BapL:ME180 protein complexes. The protein was eluted from the column, separated by SDS-PAGE and stained overnight to visualize proteins. Outside of a few contaminanting proteins, the only relevant protein on the gel was the 23 kDa protein that interacts nonspecifically with the anti-BapL antibody; no BapL protein was seen on the gel. This suggests that the BapL protein may not be the protein that allows for adherence of *G. vaginalis* to ME180 cervical epithelial cells.

When EMS was used to mutagenize the *G. vaginalis* genome, no *bapL* mutants were found. However, this could be due to the limited numbers of mutants that have been screened or analysis of a phenotype that is not associated with inactivation of the *bapL* gene. Although there are many possible pitfalls associated with producing a mutant using

EMS, it is the most efficient mechanism of obtaining mutant bacteria when a genetic system is lacking.

Finally, the BapL protein was expressed in a heterologous host and the host was analyzed for biofilm formation. Introduction of a plasmid carrying the *bapL* gene caused an increase in PNAG production in the *S. aureus* heterologous host RN4220. When the plasmid was moved into the PNAG deficient strain, *S. aureus* RN4220Δ*ica*, no difference was seen between biofilm formation in the bacteria harboring *bapL* versus empty vector. This could mean that BapL plays no role in biofilm formation or that the protein is not being expressed functionally in the heterologous host. Equally likely other components like carbohydrates or nucleic acid might be necessary to produce biofilm, and if not supplied by the heterologous host, no increase in biofilm formation would be seen with the addition of just the BapL protein. It has been shown in *Salmonella* sp. that curli, cellulose and the cell surface protein BapA play a role in biofilm formation; deletion of BapA caused a loss of the capacity to form a biofilm, however, overproduction of curli could compensate for the biofilm deficiency in the *bapA* mutant strain (Latasa, Roux et al. 2005; Jonas, Tomenius et al. 2007). It is known that *G. vaginalis* produces pili; perhaps pili play a role in biofilm formation along with the BapL protein (Johnson and Davies 1984; Boustouller, Johnson et al. 1987). Without a more advanced genetic system, like plasmids or a transformation protocol for *G. vaginalis*, the exact role of BapL may not be known for some time.

CHAPTER 6

Conclusions and Clinical Relevance

This work was designed to elucidate the virulence properties of *G. vaginalis* in an effort to better understand the pathogenesis of BV and improve current therapies. BV is of particular clinical relevance due to its propensity to increase transmission and acquisition of the HIV retrovirus and cause numerous pregnancy complications. These studies have addressed basic questions relating to the virulence potential of *G. vaginalis* and its ability to form a biofilm. Although it was known that *G. vaginalis* was able to form a biofilm on the vaginal epithelium of women with BV, nothing was known about the components of the biofilm matrix or what gene(s) could be involved in formation of the biofilm.

To help elucidate the role of *G. vaginalis* in BV, the virulence potential of *G. vaginalis* and other BV-associated anaerobes were compared. It was shown that *G. vaginalis* is able to form a thick, adherent biofilm, cause cytotoxicity and adhere robustly to vaginal epithelial cells (Patterson, Stull-Lane et al. 2010). The other BV-associated anaerobes tested had one of the virulence properties, but none had the ability to produce all three virulence properties. Surprisingly, only *Peptoniphilus* sp. bacteria were able to adhere to vaginal epithelial cells in a manner similar to *G. vaginalis*; although, *Peptoniphilus* adhered more homogenously to the vaginal cells whereas *G. vaginalis*

adhered as large clusters. This is the first record of the ability of *Peptoniphilus* to adhere to vaginal epithelial cells and this data warrants more research into the role *Peptoniphilus* plays in BV.

G. vaginalis has also been found to produce a pore-forming toxin called vaginolysin (Gelber, Aguilar et al. 2008). Our research has shown that vaginolysin requires direct contact with epithelial cells to exert its activity. There is precedence in the literature for cholesterol-dependent cytolysins, like vaginolysin, to be injected directly into host cells. It has been found that the Gram-positive pathogen *Streptococcus pyogenes* utilizes a type III secretion-like system called cytolysin-mediated translocation for injection of effector proteins into the host cell (Madden, Ruiz et al. 2001). Perhaps *G. vaginalis* is capable of cytolysin-mediated translocation; our data support a model in which vaginolysin is translocated into the host cell membrane via direct contact between bacteria and host cell. We hypothesize that once vaginolysin has produced a pore in the host cell membrane, effector proteins could be passed into the host cell through the vaginolysin pore and vaginolysin and the effector proteins could act synergistically to trigger cytotoxicity.

One of the most important virulence properties that *G. vaginalis* possesses is its ability to form a thick, tenacious biofilm. Biochemical characterization of the *G. vaginalis* biofilm matrix demonstrates an increase in carbohydrate content compared to planktonic cultures. Lectin blots, which utilize sugar-binding proteins that are highly specific for their sugar moieties, show that wheat germ agglutinin, which binds to N-acetylglucosamine, binds to biofilm extracts. This suggests that N-acetylglucoasmine is a major component of the biofilm matrix. However, when compounds that are able to degrade N-

acetylglucoasmine, like sodium metaperidoate, were used in degradation assays, the compound was unable to degrade *G. vaginalis* biofilms. This suggests that although the polysaccharide is a major constituent of the biofilm matrix, it is not the most important component of the biofilm in regards to structure and architecture. Other degradation assays utilizing the proteases, proteinase K and trypsin, show that the *G. vaginalis* biofilm is able to be degraded by proteolytic enzymes, suggesting that a proteinaceous adhesin is involved in biofilm formation in *G. vaginalis*. This is very important data in BV research because up until this point, nothing was known about the *G. vaginalis* biofilm. This data points to a new avenue of therapeutic research; an enzyme that specifically targets the biofilm matrix of *G. vaginalis* may prove a valuable addition to current antibiotic therapy.

Sensitivity assays demonstrated that *G. vaginalis* biofilms were more resistant to lactobacilli derived compounds and tolerated 4-fold higher concentrations of hydrogen peroxide and 8-fold higher concentrations of lactic acid than planktonic cultures. However, when the biofilm was degraded with proteinase K, this increased the susceptibility of the bacteria to hydrogen peroxide and lactic acid to planktonic levels. This alludes to the fact that BV may be better treated if the *G. vaginalis* bacteria are in the planktonic mode of growth rather than the biofilm mode of growth. A good adjuvant to antibiotic therapy would be an enzyme that could reduce biofilm mass down to its planktonic component before addition of the antibiotic.

Natural proteases were also analyzed for their ability to degrade *G. vaginalis* biofilms. The three fruit proteases, bromelain, papain and ficin, proved to be effective biofilm degraders. We hypothesize that natural proteases, like the fruit proteases tested,

could have the ability to degrade the *G. vaginalis* biofilm without overly affecting the vaginal epithelial cells. When papain was used to disperse the biofilm, it increased killing by clindamycin to 95%; without first dispersing the biofilm, only 70% of the bacteria were killed by an MIC 3 times the amount needed to kill planktonic bacteria. Perhaps, papain will eventually be used in combination with antibiotic therapy to reduce the relapse seen with BV. Future experiments should analyze the effect of the natural proteases on vaginal biopsies from women with or without BV.

Since it has been seen that many bacteria which produce a protein-based biofilm matrix utilize a family of proteins called biofilm-associated proteins or BAP, we searched a partial genome sequence of *G. vaginalis* for sequence homology to the most well characterized BAP, the staphylococcal Bap protein. Our analysis revealed an open-reading frame encoding a hypothetical protein which was of large size and contained a core of tandem repeating units. The protein had 35% homology to the staphylococcal Bap protein and although this does not seem like high sequence homology, the presence of other similar characteristics, like the large size and repeating units, make it highly likely that the hypothetical protein falls within the family of BAP proteins. The *bapL* gene is also highly expressed under biofilm conditions. Due to the protein's similarity to the staphylococcal protein and its expression in biofilm cultures, we have called the protein BapL for Bap-like.

Because the BapL protein is so large, 325 kDa, it was unrealistic to try and purify the whole protein from *E. coli* and use this to immunize rabbits for production of the anti-BapL antibody. Therefore, antibody was developed against a small, antigenic peptide of

the BapL protein. This antibody was used in western blot analysis of the expression of the BapL protein under different conditions; it was found that BapL is more highly expressed under the biofilm mode of growth than under planktonic conditions. The antibody was also used for confocal microscopy which confirmed surface expression of the BapL protein and antibiotic specificity at the species level.

Antibody inhibition assays were performed to identify the function of the BapL protein. However, the antibody failed to inhibit biofilm formation, initial adherence, aggregation, adherence to cervical epithelial cells, and adherence of the bacteria to ECM proteins and mucin. Since only a small, antigenic section of the BapL protein was used to produce antibodies, it could have produced an antibody that was able to interaction with the BapL protein, as seen in the western blot and confocal microscopy data, but failed to interact in such a way that it blocked the active site of the protein, thereby, allowing for continued function when the antibody was bound. However, the data could also suggest that the BapL protein is not involved in the functions tested or the protein is required but needs other components to fulfill its function. This could be analyzed by producing mutant bacteria lacking the BapL protein and other components, like carbohydrates or pilin, and testing their ability to produce a biofilm, adhere, etc. Since no genetic system is available for *G. vaginalis*, employment of random mutagenesis with a DNA mutatgen like EMS could potentially produce a *bapL* mutant. Although we did not find a *bapL* mutant using EMS mutagenesis, this could be due to the limited number of potential mutants that were screened.

Since we had seen that *G. vaginalis* adheres robustly to ME180 cervical epithelial cells, we hypothesized that the BapL protein may be playing a role in this adherence. To test this, a sandwich western blot approach was taken. ME180 cervical epithelial cells were separated by SDS-PAGE, transferred to PVDF membrane and probed with purified surface proteins from G. *vaginalis*. If the BapL protein was able to interact with a protein on the surface of the ME180 cervical epithelial cells, it should be able to bind to that band on the PVDF membrane. When probed with the anti-BapL antibody, the ME180 protein:BapL complex should be visible on x-ray film. A 23 kDa protein was seen when the ME180 protein PVDF membrane was probed with purified surface proteins from *G. vaginalis*; however, the same band was seen in an antibody control membrane. This suggests that the anti-BapL antibody is interacting with a ME180 protein. This data does not rule out the hypothesis that the BapL protein is interacting with a receptor on the surface of the ME180 cervical epithelial cells. Perhaps during purification of the surface proteins of *G. vaginalis*, the BapL protein becomes inactivated or denatured; this would inhibit binding of the protein to its ME180 protein receptor on the PVDF membrane.

Next, immunoprecipitation assays were employed to pull down any ME180 proteins that interacted with the BapL protein. The anti-BapL antibody was coupled to protein G beads and allowed to interact with *G. vaginalis* surface proteins and ME180 cervical epithelial cell proteins. After an incubation, the bound protein was eluted and separated by SDS-PAGE. The 23 kDa protein which interacted with the anti-BapL antibody was seen on the gel; however, other than a few contaminating proteins, the BapL protein is not seen on the gel. This suggests that the BapL protein is not interacting with a

ME180 cervical epithelial cell surface protein. Perhaps other yet unknown *G. vaginalis* surface proteins or carbohydrates interact with the ME180 cervical epithelial cells to allow for adherence. These results could be confirmed by production of a *bapL* mutant strain of *G. vaginalis* and testing for its ability to adhere to cell lines.

Finally, the BapL protein was expressed in the heterologous host *S. aureus* RN4220, which is a poor biofilm-producing bacterial strain. *S. aureus* RN4220-*bapL* was able to produce a thicker biofilm than RN4220 with empty vector; however, when we tested production of the biofilm polysaccharide PNAG, it was shown that RN4220-*bapL* had a significant increase in production of PNAG. Because *S. aureus* has a very efficient stress response system, it sometimes upregulates production of PNAG when large amounts of foreign DNA, such as the plasmid pRB473-*bapL*, are introduced. To circumvent this problem, the plasmid pRB473-*bapL* was transduced into the *S. aureus* strain RN4220Δ*ica*; however, in this strain, no increase in biofilm formation was seen. We hypothesized that perhaps the BapL protein was interacting with PNAG, allowing the carbohydrate to become anchored to the bacterial surface causing the increase in biofilm formation. However, a PNAG blot comparing secreted and cell surface associated PNAG from *S. aureus* RN4220Δ*ica* with empty vector or RN4220Δ*ica*-*bapL* demonstrated no difference between PNAG on the cell surface of bacteria with the *bapL* vector or empty vector control (data not shown). Other heterologous hosts, like *Bifidobacterium longum* and *B. subtilis*, were assessed for their ability to express BapL; however, no bacteria tested have been able to express a cell surface associated BapL protein. Because *B. longum* is closely related to *G. vaginalis*, a *B. longum* plasmid, pDOJHR, was assessed for its ability to replicate in *G.*

vaginalis; however, an efficient transformation protocol for *G. vaginalis* has not been identified, causing problems with detecting whether pDOJHR would be an appropriate vector for *G. vaginalis*.

Until a genetic system is developed to allow for mutational analysis of *G. vaginalis*, the only way to ascertain what role the BapL protein is playing in biofilm formation is to potentially develop an antibody to the whole protein or larger portions of the protein and employ antibody inhibition assays with a number of antibodies which span the whole protein. Also, it would be important to know which part of the protein harbors its active site; this could be accomplished by expressing truncated versions of the protein in a heterologous host and looking for a particular function, like adherence or biofilm formation. However, since we were unable to show a particular function of the BapL protein, this experiment would be a shot in the dark, utilizing a potential function that may not actually be expressed.

The scientific community is highly interested in the enzymatic degradation of bacterial biofilms, as biofilm formation is a cause of many medical, industrial and environmental problems (Chaignon, Sadovskaya et al. 2007). An agent that could disintegrate the biofilm, releasing planktonic bacterian into the environment, would allow antibiotics to eliminate the infection with improved efficiency (Chaignon, Sadovskaya et al. 2007). There is great need for an animal model of BV, since possible inflammatory effects of enzymatic treatment must first be studied before using the treatment in situ.

In conclusion, we have learned that *G. vaginalis* is more virulent than other BV-associated anaerobes tested. Its ability to produce a biofilm could play an important role in

the pathogenesis of BV, since biofilm bacteria are more resistant to chemicals like lactic acid and hydrogen peroxide. A proteinaceous adhesin is involved in biofilm formation by *G. vaginalis*. This is a significant result in that it allows researchers to focus their efforts on developing an enzyme that could be used in conjunction with antibiotic therapy, to degrade the biofilm and allow the constituent bacteria to be more susceptible to antibiotic challenge. This pretreatment with a protease before addition of antibiotic may reduce the amount of relapse seen with BV.

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APPENDIX A

Percent reduction in biofilm mass of each enzyme concentration tested.

Appendix A. Percent reduction in biofilm mass of each enzyme concentration tested.

Bromelain

Units	10	2	0.4	0.08	1.6E-2	3.2E-3	6.4E-4	1.3E-4	2.6E-5	5.1E-6
pH 7.4	67.1	52	75.6	69.4	55.9	38	13.5	12.9	14	24.4
pH 4.5	75.9	72.6	69.6	59	47.8	40.3	34.6	28.9	20.6	24.3

Papain

Units	10	2	0.4	0.08	1.6E-2	3.2E-3	6.4E-4	1.3E-4	2.6E-5	5.1E-6
pH 7.4	84.5	88.9	83.3	62.6	32.4	8	0	0	0	0.1
pH 4.5	88.5	88.6	85.9	61.7	32.3	26.4	19	13.5	12.3	15.1

Ficin

Units	5.78	1.12	2.3E-1	4.6E-2	9.3E-3	1.9E-3	3.7E-4	7.4E-5	1.4E-5	3.0E-6
pH 7.4	91.8	88.9	66.1	59.2	46.1	16.9	0	5.3	9.2	21
pH 4.5	92.7	75.2	77.8	74.5	41.1	24.5	22.2	26.9	21.1	20.6

Pepsin

Units	345	69	13.8	2.76	5.5E-1	1.1E-1	2.3E-2	4.4E-3	8.8E-4	1.8E-4
pH 7.4	34.7	19.3	26.8	22.4	19.6	13.3	10.6	7	11.5	21.4
pH 4.5	60.3	36.5	29.8	22	22.3	21	20.5	15.4	15.6	10

Values shown are percent biofilm reduction for each enzyme unit tested.

VITA

Jennifer Louise Patterson was born April 14, 1983 in Virginia Beach, Virginia. She is a citizen of the United States. She graduated from Princess Anne High School, Virginia Beach, Virginia in 2001. She received her Bachelor of Science degree in Recombinant Gene Technology from Concord University in 2005. Her interest in bacterial pathogenesis led her to Virginia Commonwealth University where she received a doctorate degree in 2010. While at VCU, she received the Mary Coleman Award, Phi Kappa Phi scholarship, C.C. Clayton Award and the Elizabeth Fries Young Investigator award. Jennifer was selected as a member of the US Delegration to the 57th Nobel Laureate Meeting in Lindau, Germany, as well as a member of the Phi Kappa Phi Honor Society. Jennifer published two first author papers in the journals American Journal of Obstetrics and Gynecology and Microbiology. She published a second author paper in the Journal of Virology. She is a member of the American Society of Microbiology and Women in Science (VCU Chapter).