Degradation, Foraging and Depletion of Mucus Sialoglycans 
by the Vagina-Adapted Actinobacterium Gardnerella vaginalis 

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Background: Mucus degradation is hypothesized to be important in bacterial vaginosis (BV), but mechanisms require investigation.

Results: We characterize a Gardnerella vaginalis pathway that performs digestion/catabolism of mucus sialoglycans.

Conclusion: G. vaginalis participates in mucosal sialic acid depletion in BV.

Significance: G. vaginalis is the first example of a BV-associated organism that recapitulates a measurable clinical phenotype of mucus degradation in an animal model.

Summary

Bacterial vaginosis (BV) is a polymicrobial imbalance of the vaginal microbiota associated with reproductive infections, preterm birth, and other adverse health outcomes. Sialidase activity in vaginal fluids is diagnostic of BV and sialic acid-rich components of mucus have protective and immunological roles. However, while mucus degradation is believed to be important in the etiology and complications associated with BV, the role(s) of sialidases and the participation of individual bacterial species in the degradation of mucus barriers in BV have not been investigated. Here we demonstrate that the BV-associated bacterium Gardnerella vaginalis uses sialidase to break down and deplete sialic-acid-containing mucus components in the vagina. Biochemical evidence using purified sialoglycan substrates supports a model in which 1) G. vaginalis extracellular sialidase hydrolyzes mucosal sialoglycans, 2) liberated sialic acid (N-acetylneuraminic acid) is transported into the bacterium, a process inhibited by excess N-glycolylneuraminic acid, and 3) sialic acid catabolism is initiated by an intracellular aldolase/lyase mechanism. G. vaginalis engaged in sialoglycan foraging in vitro, in the presence of human vaginal mucus, and in vivo, in a murine vaginal model, in each case leading to depletion of sialic acids. Comparison of sialic acid levels in human vaginal specimens also demonstrated significant depletion of mucus sialic acids in women with BV compared to women with a “normal” lactobacilli-dominated microbiota. Taken together, these studies show that G. vaginalis utilizes sialidase to support the degradation, foraging, and depletion of protective host mucus barriers, and that this process of mucus barrier degradation and depletion also occurs in the clinical setting of BV.

One in three women have bacterial vaginosis (BV) at any point in time (1), a microbial imbalance of the female reproductive tract characterized by a lack of healthy bacterial vaginal flora (mainly

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lactobacilli). Instead, the vaginal flora in BV consists of a complex community dominated by Gram-negative anaerobes, *Actinobacteria*, and other bacteria. Women with BV are at increased risk of pelvic inflammatory disease, postsurgical infections, sexually transmitted infections, and serious pregnancy complications such as intrauterine infection and preterm birth (2-17). Recent advances in DNA sequencing technologies and other molecular tools have provided an unprecedented view of the diversity and longitudinal variability of the vaginal bacterial microbiota (18-21). Such modern microbiome studies confirm and extend previous culture-based approaches, further deepening our view of the polymicrobial nature of BV (7,22,23). BV is common, and asymptomatic from the patient perspective, but often displays characteristic clinical features (also known as Amsel criteria) including increased pH, thinning of vaginal fluid secretions (or ‘abnormal discharge’), a fishy odor upon potassium hydroxide treatment of vaginal specimens, and the presence of “clue” cells (epithelial cells studded with bacteria) in wet mounts (24,25). Recent work has demonstrated some correlations between clinical features of BV and the presence of particular BV-associated bacteria (26). However, experimental models are required to examine how individual bacterial species or strains interact with the host.

Glycosylated proteins that display outermost sialic acid residues (sialoglycoproteins) are major constituents of mucus and participate in the exclusion of potential pathogens from mucosal surfaces (27). Mucins are heavily glycosylated mucus proteins, consisting of up to 16% sialic acids by weight, and they are thought to provide a thick lubricated physical barrier that prevents close contact of pathogens with epithelial cells (28,29). Immunoglobulins are another example of secreted sialoglycoproteins with mucosal immune functions (30,31). A number of studies suggest that degradation of mucus components may be a key factor in the etiology of BV and BV-associated adverse health outcomes. BV has been associated with the presence of multiple hydrolytic activities in vaginal fluids, especially glycosidases (32-34). In fact, sialidase enzyme activity in vaginal fluids is now used as a diagnostic indicator of BV (14,35-38) and has been independently correlated with risk of ascending infections and preterm birth (6,39-41). However, while current evidence strongly suggests that BV-associated bacteria produce hydrolytic enzymes that degrade protective mucus barriers, the mechanisms of mucus degradation employed by individual species or communities of bacteria in BV have not been characterized.

*Gardnerella vaginalis* is the most frequently isolated bacterium associated with BV, and produces a sialidase hypothesized to participate in the degradation of mucus (32,34,42,43). However, relatively little is known at the molecular level about the relationship between *G. vaginalis* and its human host (44,45). *G. vaginalis* was the first bacterium isolated from women with BV, although at that time the condition was referred to as “nonspecific vaginitis,” and *G. vaginalis* was mistakenly identified as a Gram-negative bacterium “*Haemophilus vaginalis*” (46). More than half a century later, we know that this organism is actually a member of the phylum *Actinobacteria* (also known as the high-GC Gram-positives). Studies showed that recovery of *G. vaginalis* from vaginal fluids was 92% sensitive and 69% specific in identifying women with BV, as diagnosed by Amsel criteria (3 of 4 subjective measures) (23). Many other studies have reproduced this strong correlation between overgrowth of *G. vaginalis* and BV. However, the potential role of *G. vaginalis* in the etiology of BV remains controversial because women with apparently “normal” microbiota at the time of sampling can also be carriers of *G. vaginalis* (23,47). Consistent with the role of *G. vaginalis* as a potential pathogen, culture-based studies recovered the bacterium from placentas of 26% of women delivering preterm with histological evidence of chorioamnionitis (9). In vitro studies have further described the pathogenic potential of *G. vaginalis* in cell adhesion and entry, cytolytic toxin production and biofilm formation (2,48,49) and computational studies revealed that that the presence of *G. vaginalis* is strongly correlated with clinical phenotypes of BV (26). Taken together, these studies support the hypothesis that *G. vaginalis* is an active participant as opposed to an innocent bystander in BV. However, further experimental study is required to demonstrate...
active participation of *G. vaginalis* in phenotypes associated with BV.

Here we present biochemical, cellular, and *in vivo* investigations of *G. vaginalis*, demonstrating that the bacterium can engage in the active degradation and foraging of mucus substrates, using sialidase to release sialic acid residues from mucus sialoglycoproteins, and subsequently capturing and catabolizing the free monosaccharide. We show that the hydrolysis and metabolism of sialic acid residues by *G. vaginalis* occurs in both *in vitro* and *in vivo* models and that *G. vaginalis* is sufficient to induce a sialoglycan-depleted state in a murine vaginal infection model. These experiments provide the first evidence of an individual BV-associated bacterium that participates in mucus degradation, a process believed to underlie the increased susceptibility to ascending uterine infections in women with BV. These studies also demonstrate that *G. vaginalis*, a species of previously controversial significance in BV, is in fact sufficient to hydrolyze mucus sialoglycans and deplete the vaginal mucosa of sialic acids during infection. Additional evidence strongly suggests that this process of sialoglycan foraging also leads to sialic acid depletion in the clinical condition of BV, further underscoring the potential clinical relevance of these findings.

**Experimental Procedures**

**Isolation of *G. vaginalis* strains from clinical specimens.** Vaginal swabs were obtained from women enrolled in the Washington University Contraceptive CHOICE Project (IRB ID# 201108155) and transported from the clinic to the lab using Port-A-Cul™ pre-reduced anaerobic transport media tubes (Becton Dickinson). Within 24 hours, tubes were brought to a hydrolyze mucus sialoglycans and deplete the vaginal mucosa of sialic acids during infection. Additional evidence strongly suggests that this process of sialoglycan foraging also leads to sialic acid depletion in the clinical condition of BV, further underscoring the potential clinical relevance of these findings.

**Sialidase activity assays.** *G. vaginalis* isolates were grown anaerobically in NYC-III media overnight at 37°C and OD₆₀₀ was measured in a spectrophotometer. Whole cultures or culture supernatants were then diluted 20-fold into 100 mM sodium acetate pH 5.5 containing 460 μM 4-methylumbelliferyl-(4MU)-Neu5Ac. Substrate hydrolysis was monitored using the fluorescence of 4MU in a Tecan M200 plate reader every 2 minutes as previously described (52). Relative sialidase activities (RFU/second) were normalized to bacterial density.
Measurement of total and free sialic acids by DMB-HPLC. Derivatization and quantitation of sialic acids by HPLC was carried out as we have previously described (27,52-57)(see Figure S1 for example raw data of bovine submaxillary mucin sialic acids and N-acetyleneuraminic acid standards). For measurement of total sialic acids, (including bound and free), mild acetic acid hydrolysis of samples or specimens (2N acetic acid for 3 hrs at 80 °C) was performed prior to derivatization with DMB (1,2-diamino 4,5-methylenedioxybenzene) as described below. Alternatively, free sialic acid levels present in samples or specimens were measured by DMB derivatization without prior acid hydrolysis. Thus, the concentration of bound sialic acids is measured indirectly (Bound = Total – Free). All samples and specimens were filtered prior to derivatization over a 10,000 molecular weight cutoff centrifugal filtration device (Vivaspin). Reaction conditions for DMB derivatization were 7mM DMB, 22mM sodium thiosulfite, 0.75M 2-mercaptoethanol, and 1.4M acetic acid for 2 hours at 50 °C. Derivatized samples were injected into a Waters HPLC equipped with a reverse-phase C18 column (Tosoh Bioscience) and were eluted using isocratic conditions at 0.9ml/min using 8% methanol, 7% acetonitrile in water. An on-line fluorescence detector (Waters) was set to excite at 373nm and detect emission at 448nm. Peak integrations were used to quantitate sialic acid content by referencing a standard curve of pure sialic acid (Neu5Ac, Sigma) derivatized in parallel. Unless otherwise noted in experiments using Neu5Gc, quantitation of “sialic acid” refers to Neu5Ac.

Biochemical analysis of G. vaginalis hydrolysis and utilization of sialoglycan substrates. G. vaginalis strains were grown anaerobically overnight in 5 ml NYCIII medium at 37°C. NYCIII contains horse serum. Neu5Gc is a minor contributor to total sialic acids in NYCIII (about 10%). Bacteria were pelleted and washed in 1 ml 100 mM sodium acetate pH 5.5. The cells were then resuspended and diluted in acetate buffer to give an OD600 of 3.2. The bacterial suspensions were supplemented with human myeloma serum immunoglobulin A-IgA (Kent Laboratories) or bovine submaxillary mucin–BSM (Sigma) at a final concentration of 400 µg/ml. This mixture was then dispensed in 50 µL aliquots to each of 10 tubes and incubated at 37°C. At each time point, aliquots were centrifuged and 35 µL supernatant was removed for subsequent DMB derivatization. Samples from each experiment were derivatized in parallel to assess free and total sialic acid content.

Analysis of free sialic acid uptake and utilization. G. vaginalis strains were grown anaerobically overnight, washed, and diluted to OD 3.2 in 100 mM sodium acetate pH 5.5. Free Neu5Ac was added to 10 µM, and 50 µL aliquots were distributed to fresh tubes for incubation at 37°C. At each time point, bacteria were pelleted and 35 µL supernatant was collected for DMB derivatization and HPLC.

G. vaginalis sialic acid aldolase/lyase activity assays. G. vaginalis strains were grown anaerobically overnight in 8 ml NYC-III broth and washed once in 1ml 100mM sodium acetate pH5.5. Following resuspension in 450 µL sodium acetate buffer, the bacteria were sonicated six times for 10 seconds in a Sonic Dismembrator (Fisher Scientific) at 20% amplitude using a Microtip with 15 second cooling intervals on ice. Intact cells were pelleted by centrifugation and the clarified supernatant was transferred to a fresh tube. This process was repeated twice more to remove any remaining debris. To test for lyase activity, lysates were supplemented with Neu5Ac at 100-200 µM and incubated at 37°C in 40 µL aliquots. At each time point, an aliquot was removed, diluted 10-fold into 100 mM sodium acetate pH 5.5, and stored at -20°C prior to HPLC. To test for aldolase activity, lysates were mixed with pyruvate and N-acetylmannosamine, both at 1 mM. Aliquots were incubated at 37 °C and removed at each timepoint for sialic acid quantification by HPLC.

Biochemical analysis of G. vaginalis hydrolysis and utilization of sialoglycan substrates in human and mouse vaginal mucus. To monitor vaginal mucus sialic acid utilization by G. vaginalis, reactions were set up essentially as described above for IgA and BSM. For these assays, eluted human specimens with Nugent scores of 0-3 were validated as having no
detectable level of sialidase activity (to eliminate the rare possibility low sialidase activity in the absence of Nugent-defined BV). For mouse vaginal mucus, material was obtained by washing vaginas of uninfected mice with 50 µL phosphate buffered saline (PBS) and confirming absence of sialidase activity as for human samples.

**Murine model of *G. vaginalis* vaginal infection.** Animal infection studies were performed in accordance with approved protocols from the Washington University Division of Comparative Research. Female C57/Bl6 mice (6-8 weeks) were injected intraperitoneally with 0.5 mg β-estradiol in 100 µL filter-sterilized sesame oil three days prior to, and on the day of, inoculation. Mice were anaesthetized with isofluorane and inoculated vaginally with ~5x10^7 colony forming units of *G. vaginalis* in 20 µL sterile PBS (OD_{600}=5.0). Vaginal washes were collected by flushing the vaginas of anaesthetized mice with 50 µL sterile PBS, pipetting up and down 10 times at the vaginal orifice, followed by rinsing into an additional 10 µL PBS in a sterile 1.5 mL tube using a P200 pipet. Vaginal washes were then analyzed for the presence of free and total sialic acid levels as described above. Recovery of *G. vaginalis* in vaginal washes was confirmed by anaerobic culture on Gardnerella semi-selective media, followed by colony PCR using *G. vaginalis* specific primers as described above.

**Clinical specimen handling and analysis.** Vaginal swabs (Starplex) were collected as part of the Contraceptive CHOICE project (58) according to protocols approved by the Washington University Institutional Review Board (IRB ID# 201108155) and were stored at -80°C until use. Specimens underwent Nugent scoring using published methods as previously described (52,59). Swabs were thawed on ice and eluted in 400-1000 µL 100 mM pH 5 sodium acetate buffer in deep 96-well plates in a biosafety cabinet at room temperature. Early experiments (including total sialic acid analyses in vaginal specimens) employed 400 µL elutions, while later experiments, including free sialic acid analyses, employed 1000 µL elutions. In general, we found that the larger elution volume increased the total recoverable biological material from the swabs. Elution was allowed to proceed for approximately 40 minutes with gentle agitation. Eluted vaginal fluid was used directly in derivatization reactions for analysis of free sialic acid or hydrolyzed with mild acetic acid for analysis of total sialic acid as described below.

**Sequence accession numbers.** See Table 1.

**Institutional review board approval.** Vaginal specimens were collected in accordance with IRB-approved protocols (IRB ID# 201108155). Written informed consent was received from participants as part of the Contraceptive CHOICE project (IRB ID# 201101982) prior to inclusion in this study.

**Results**

Genomic analysis of the fully sequenced *G. vaginalis* type strain ATCC14019 revealed a putative sialidase and a nearby gene cluster encoding homologs of sialic acid catabolic enzymes (see supplemental Figure S2). However, ATCC14019 did not produce sialidase activity in our hands when grown in vitro (Figure 1A). Thus, to study sialidase-producing strains of *G. vaginalis*, and their ability to catabolize sialic acids from mucosal sialoglycans, we isolated new fresh clinical strains of *G. vaginalis*. Briefly, women who were enrolled in the Contraceptive CHOICE Project at Washington University provided self-collected vaginal swabs, which were transported to the Center for Women’s Infectious Disease Research under anaerobic conditions. *G. vaginalis* strains were isolated as described in the Experimental Procedures, and their identities were confirmed by PCR using primers reported to be specific for genomic DNA encoding the Gardnerella 16S ribosome (50). Sequencing of approximately 1400 base pairs of the 16S ribosomal DNA gave fifteen strains with 98.5-100% identity to the *G. vaginalis* reference strain ATCC14019 (Table 1). A single strain of *Bifidobacterium* was also isolated using this approach with 93% identity to the 16S sequence of *G. vaginalis* (Table 1).

Kinetic analysis of 4-methyl-umbelliferyl(4MU)-sialic acid hydrolysis using whole bacterial cultures revealed that nine of these *G. vaginalis* strains produced high levels of sialidase activity (+++, ++, or +), one strain produced
intermediate levels of sialidase activity (+), and five strains produced very low or undetectable levels of sialidase (-) when cultured anaerobically in NYCIII media (Figure 1A, Table 1). These strains producing little or no sialidase activity are hereafter referred to as “sialidase-negative.” Of the strains that produced sialidase, most of the enzyme activity was cell-associated and thus removed from the culture by pelleting bacteria (Figure 1B). However, a fraction of the activity remained in the bacterial supernatant (Figure 1B). Consistent with the quantitative analysis of enzyme activity, a chromogenic SiaX substrate analogous to the familiar X-gal substrate revealed blue staining of bacterial colonies (Figure S3). Staining also diffused through NYCIII agar plates surrounding \textit{G. vaginalis} colonies, further supporting the conclusion that a portion of the \textit{G. vaginalis} sialidase is also secreted (Figure S3). Limited proteolysis with subtilisin released the cell-associated sialidase activity into the soluble supernatant, demonstrating that the enzyme activity is resistant to mild proteolysis and further supporting the conclusion that sialidase is localized on the surface of \textit{G. vaginalis} (Figure S3C, arrowhead). However, there are no canonical Gram-positive signal sequences in the amino acid sequence of the predicted sialidase according to SignalP4.1, nor are there any LPXTG motifs.

We measured the ability of \textit{G. vaginalis} isolates to consume sialic acids during anaerobic growth in NYCIII media, which contains free sialic acids (~17%) as well as bound sialoglycans (~83%). Total sialic acid levels in the bacterial cultures were measured using previously described methods of fluorescent derivatization and HPLC separation (52,53,56). Compared to uninoculated media incubated in parallel, growth of the sialidase-positive strains resulted in progressive depletion of sialic acids from the culture media (Figure 2A-B). On average, sialidase-positive strains consumed about half of the total sialic acids in culture media by 8 hours and about 75% of sialic acids were consumed by 24 hours post-inoculation. In contrast, sialidase-negative isolates consumed little or no sialic acid (Figure 2A-B). Differences in growth between strains did not account for differences in sialic acid foraging under these conditions (Figure S4). These data demonstrate that sialidase-positive \textit{G. vaginalis} isolates are able to liberate and consume sialic acids present as bound sialoglycans in the extracellular environment.

To define the sequence of events in \textit{G. vaginalis} sialoglycan hydrolysis and sialic acid consumption, we examined processing of the purified sialoglycoprotein substrate immunoglobulin A (IgA) by washed bacteria resuspended in acetate buffer, comparing the sialidase-positive strain JCP8151B to the sialidase-negative strain JCP8481A. Strains were mixed with human IgA and the levels of free sialic acids in the soluble extracellular compartment were measured over time. Free sialic acid in the soluble supernatant was evident within minutes, indicating extracellular hydrolysis of IgA sialic acid residues (Figure 3A). The concentration of free sialic acid peaked at 40 minutes and progressively declined with very little free sialic acid remaining (<1 µM) after 120 minutes (Figure 3A). These results clearly show that sialic acids on IgA are hydrolyzed outside the cell by \textit{G. vaginalis} sialidase prior to their uptake and catabolism. Additional studies examined the ability of \textit{G. vaginalis} isolates to consume free sialic acids when the requirement for sialidase was bypassed. Consistent with the data using IgA as a substrate, sialidase-positive strains readily consumed the provided free sialic acid. In contrast, sialidase-negative strains were unable to efficiently take up and catabolize free sialic acids (Figure 3B). The lack of sialidase and catabolic activity consistently observed among “sialidase-negative” \textit{G. vaginalis} isolates suggests that these strains fail to express the uptake and/or catabolic mechanisms downstream of sialidase, which are expressed in sialidase-positive strains under these conditions.

Much of the sialic acid at mucosal surfaces is found in mucins, heavily O-glycosylated proteins that are cell-surface associated, or secreted by specialized cells found at all mucosal sites (60-62). To investigate the utilization of mucosal sialic acids, we measured \textit{G. vaginalis} hydrolysis and utilization of a purified preparation of mucin from bovine submaxillary glands. Surprisingly, \textit{G. vaginalis} was much less capable of consuming sialic acid (Neu5Ac) when it was presented in the context of bovine submaxillary mucin (BSM) as...
compared to human IgA (Figure 4A). This was not due to an inability to hydrolyze sialic acids from O-glycans, since free sialic acid levels displayed similar kinetics for BSM and IgA during the first 20 minutes after exposure to G. vaginalis washed whole cells (Figure 4B).

We hypothesized that the reduced ability of G. vaginalis to consume sialic acids on BSM may have something to do with its bovine origin. BSM displays approximately equal amounts of Neu5Ac and 5-N-glycolyl neuraminic acid (Neu5Ge), a hydroxylated form of 5-N-acetyl neuraminic acid (Neu5Ac) that is found in all mammals except humans (63-65). Analysis of Neu5Ge during sialic acid catabolism by G. vaginalis revealed similar kinetics of release compared to Neu5Ac; however, the levels of free Neu5Ge remained high throughout the 4 hour duration of the assay (Figure 4B), demonstrating that G. vaginalis efficiently liberates but inefficiently consumes the nonhuman sialic acid Neu5Ge. To test the hypothesis that Neu5Ge may impair the catabolism of Neu5Ac, we performed G. vaginalis sialic acid utilization assays using human IgA, (which contains only Neu5Ac), in the presence or absence of excess purified Neu5Ge. Consistent with their behavior in culture, sialidase-negative G. vaginalis isolates were instead incubated with excess ManNAc and pyruvate, Neu5Ac production was observed (Figure 5B). We emphasize that the amount of Neu5Ac produced in these experiments was small compared to the input of pyruvate and ManNAc. However, the data provide strong evidence that the Actinobacterium G. vaginalis uses a similar pathway of Neu5Ac catabolism as previously described for Gram-negative bacteria—a retro-aldol mechanism mediated by a Neu5Ac lyase/aldolase (66). The uptake of free Neu5Ac by G. vaginalis was markedly reduced in the presence of Neu5Ge (Figure 5C), whereas investigation of sialate lyase (Figure 5A) and sialidase (Figure 5D) activities revealed no inhibition of activity in the presence of excess Neu5Ge.

To determine whether G. vaginalis sialoglycan foraging occurs within the physiologically relevant context of human vaginal fluid, we next investigated the kinetics of G. vaginalis sialoglycan hydrolysis and sialic acid catabolism following incubation of bacteria directly in buffered human vaginal specimens. For these experiments, we used human vaginal mucus pooled from 14 women with a “normal” lactobacilli-dominated microbiota as verified by Nugent score (0-3) and negative sialidase activity assay. Washed pellets of sialidase-positive and sialidase-negative G. vaginalis isolates were incubated with human vaginal fluid, followed by analysis of free and total sialic acid levels. Consistent with their behavior in culture, sialidase-positive G. vaginalis isolates were incubated with human vaginal fluid, followed by analysis of free and total sialic acid levels. Consistent with their behavior in culture, sialidase-negative G. vaginalis isolates were incubated with human vaginal fluid, resulting in a pool of free sialic acids that was evident within minutes and destroyed over time (Figure 6A). Depletion of the free sialic acid pool by G. vaginalis was mirrored by reductions in the total sialic acid, with virtually no intact mucus sialoglycan remaining after 120 minutes (Figure 6B). In contrast, mucus sialoglycans remained intact in the presence of sialidase-negative G. vaginalis (Figure 6A-B). These data suggest that
the degradation and catabolism of human vaginal sialoglycans proceeds through the same pathway as observed for the purified human sialoglycan substrate IgA (Figures 3A&4).

To test the potential for sialic acid catabolism by \textit{G. vaginalis} in the context of the mouse vagina, isolates were first incubated with mucus derived from pooled mouse vaginal washes. After a four-hour incubation, sialidase-positive isolates reduced the total sialic acid pool by about 75% (Figure 6C). Sialidase-negative strains had a negligible impact on total sialic acid levels in mouse vaginal mucus. Next, we tested whether \textit{G. vaginalis} is sufficient to degrade, catabolize, and deplete sialic acids from the vaginal mucosa \textit{in vivo}. Sialidase-positive \textit{G. vaginalis} strain JCP8151B was inoculated into the vaginas of C57/BL6 mice, followed by measurement of free and total sialic acids in vaginal washes as described in the Experimental Procedures. Control groups of mice received an equal volume of vehicle alone and were washed in parallel with \textit{G. vaginalis}-infected mice at 24 and 72-hours post-infection. At both time points, pinpoint colonies of \textit{G. vaginalis} were re-isolated from murine vaginal washes by plating on semi-selective media, cultured under anaerobic conditions, and confirmed by PCR. Resident bacteria also grew on “Gardnerella semi-selective” agar, resistant to colistin, nalidixic acid, trimethoprim-sulfamethoxazole, and other antibiotics, obscuring an absolute estimation of \textit{G. vaginalis} vaginal titers. We note that previously published models of \textit{G. vaginalis} vaginal infection did not address this potentially confounding factor (67,68).

Biochemical analyses of vaginal washes at 24 hours post infection revealed significantly higher levels of free Neu5Ac in the \textit{G. vaginalis}-infected mice compared to mock-infected animals, indicating that the active breakdown of vaginal mucus sialoglycans by \textit{G. vaginalis} occurs \textit{in vivo} (Figure 7A). By the 72-hour time point, \textit{G. vaginalis}-infected mice exhibited significantly lower levels of bound Neu5Ac (Figure 7B), in vaginal washes compared to mock-infected animals. Together these data show that \textit{G. vaginalis} participates in the hydrolysis and foraging of vaginal mucus sialoglycans \textit{in vivo}, depleting more than half of the bound Neu5Ac content in vaginal washes (mean 19.61µM confidence interval 13.96-25.25) compared to mock-infected animals (mean 41.38 µM 95% confidence interval 25.73-57.03).

To investigate the evidence that sialic acid hydrolysis, foraging, and depletion occurs in the clinical setting of BV, we performed biochemical analyses of eluted vaginal fluids from women with bacterial vaginosis (Nugent score 7-10) compared to women with a “normal” lactobacilli-dominated microbiota (Nugent score 0-3). Measurements of free and total sialic acid levels in these specimens revealed that women with BV had >3-fold higher free sialic acid levels (mean 20.7 µM, 95% confidence interval 11.3-30.0 µM) compared to normal controls (mean 5.8 µM, 95% confidence interval 3.7-7.8 µM) (Figure 8A). These data are consistent with a steady release of free sialic acids in vaginal mucus due to bacterial sialidase activity associated with BV. In contrast, measurements of total sialic acids in vaginal fluids revealed that women with BV had >3-fold lower levels of total sialic acid (mean 48 µM, 95% confidence interval 32.7-63.4 µM) compared to women with a “normal” lactobacilli-dominated microbiota (mean 158 µM, 95% confidence interval 104.5-212.1 µM) (Figure 8B). These data strongly suggest that the hydrolysis, catabolism, and depletion of vaginal mucus sialic acids observed in our experimental models are also occurring in women with BV.

**Discussion**

“Dysbiosis” refers to the idea of an imbalance between “beneficial” and “harmful bacteria” at mucosal surfaces, a concept that has increasing support in gut pathologies such as inflammatory bowel disease (69-71) and vaginal conditions such as bacterial vaginosis.

Here we provide the first report of a BV-associated bacterium, \textit{G. vaginalis}, which actively participates in the degradation of protective vaginal mucus barriers. The data presented here also demonstrate Nugent-defined BV as the first
example, to our knowledge, of a microbial imbalance or dysbiosis in humans in which protective mucus sialoglycan barriers are measurably degraded and depleted from affected mucosal specimens compared to normal controls.

We show that *G. vaginalis* sialic acid foraging was sufficient to cause mucosal sialic acid depletion in a mouse vaginal infection model. The degree of sialic acid depletion observed in clinical specimens in the current study is consistent with our previous work on BV specimens showing that sialic acids provided exogenously in various contexts (e.g. α2-3 and α2-6-linked sialic acids found on both O-linked and N-linked sialoglycoproteins) are effectively cleaved by sialidases present in BV vaginal fluids, but not by healthy controls (52). The data further demonstrate that sialidase-positive *G. vaginalis* strains isolated from women with BV are capable of hydrolyzing vaginal sialoglycans, including IgA and mucin, and *G. vaginalis* was sufficient to result in depletion of vaginal sialic acids within 72 hours in a murine model. *G. vaginalis* secretion and surface presentation of sialidase enzyme allowed for the extracellular hydrolysis of bound Neu5Ac-containing vaginal mucus sialoglycans, followed by transport of Neu5Ac into the intracellular compartment (see model, Figure 9). Following uptake, catabolism of sialic acid proceeded without accumulation of intracellular Neu5Ac (intracellular Neu5Ac was detectable in low concentrations in our hands) through a mechanism involving a sialic acid aldolase/lyase reaction. While total levels of sialic acid were lower in BV vaginal fluids, the levels of free sialic acid in BV clinical specimens were often elevated, consistent with the ‘release and capture’ biochemical pathway demonstrated *in vitro* and *in vivo* with *G. vaginalis*.

The machinery for bacterial sialic acid catabolism has been described in several other systems, mostly in aerobic Gram-negative *Proteobacteria* (72-77). Sialic acid catabolism has also been recently demonstrated to support growth of the Gram-positive bacterium Group B *Streptococcus* in a vaginal model of infection(78). Interestingly, this bacterium does not encode a putative sialidase or express sialidase activity when grown in culture, suggesting that it may have a competitive advantage in the BV vaginal environment where we show (Figure 8) free sialic acid levels are much higher than in a lactobacilli-dominated vaginal environment.

**Foraging on sialic acid in the vagina.** Metabolic machinery for sialic acid hydrolysis and utilization has been implicated in gastrointestinal, respiratory, and periodontal pathogenesis of several bacterial species, including *Vibrio cholerae* (79), *Streptococcus pneumoniae* (80), and *Tannerella forsythia* (81,82). The “normal” commensal microbiota of the adult oral gastrointestinal mucosa such as *Streptococcus oralis* and *Bacteroides thetaiotaomicron* have also been shown to hydrolyze sialic acids and utilize host mucus under certain conditions (83-86). Other studies suggest that in the infant gut, the beneficial commensal *B. longum* subspecies infantis utilizes a sialidase to hydrolyze sialic acid-containing oligosaccharide ‘prebiotics’ secreted by the maternal mammary gland (87,88). However, to our knowledge, this is the first description of sialylglycan foraging by a sialidase-expressing vaginal-adapted bacterium. In contrast to oral and gastrointestinal environments, where sialidase activity is ubiquitous, BV presents a unique opportunity for the study of disease-associated sialidase activity. Detectable sialidase activity in vaginal fluids occurs almost universally in BV, and rarely if ever in normal controls with vaginal microbiota dominated by lactobacilli (14,35-38).

**Sialidase status of *G. vaginalis* isolates.** Other papers have shown that some *G. vaginalis* strains are sialidase-positive, while others are negative (34,89,90), a finding we confirm among our *G. vaginalis* isolates (Figure 1, Table 1). One recent study that investigated sialidase activity and the presence or absence of a gene encoding a putative sialidase open reading frame (by PCR) concluded that sialidase-negative strains lack the sialidase gene (89). Another similar study concluded that phenotypically sialidase-negative strains can encode a predicted sialidase, while failing to express the active enzyme in culture (90). Among our *G. vaginalis* strains, we confirmed the presence of a ~3 kilobase open reading frame encoding a putative sialidase homolog, including phenotypically-sialidase-negative JCP8108 and JCP7275 (Figure S5). This finding is consistent...
with our observation that strain ATCC14019 encodes the genes for sialidase and sialic acid catabolism (Figure S2), while being phenotypically sialidase-negative. It is possible that sialidase and other components of the pathway may be differentially regulated among “sialidase-positive” and “sialidase-negative” strains of G. vaginalis. Further studies are required to define and understand the behaviors of G. vaginalis isolates under different environmental conditions.

Neu5Gc as an inhibitor of G. vaginalis Neu5Ac uptake. Two major forms of sialic acids differing by a single oxygen atom are found in mammals – Neu5Ac and Neu5Gc. The hydroxylated acetyl neuraminic acid Neu5Gc is found alongside Neu5Ac in all mammals examined, except humans, due to a genetic event that inactivated the enzyme responsible for synthesis of Neu5Gc from Neu5Ac. G. vaginalis sialidase does not appear to have strong preferences between Neu5Ac and Neu5Gc as substrates. However, the uptake and catabolism of sialic acids was substrate dependent and occurred much more slowly and incompletely in the context of significant amounts of Neu5Gc. The presence of Neu5Gc during Neu5Ac uptake lead to higher concentrations of free extracellular Neu5Ac, showing that Neu5Ac is indeed released, but taken up more slowly. Addition of excess Neu5Gc did not inhibit the liberation of Neu5Ac by G. vaginalis sialidase or the catabolism of Neu5Ac by cell lysates, showing that Neu5Gc acts downstream of sialic acid hydrolysis, yet upstream of the catabolic machinery, beginning with the intracellular lyase enzyme, together suggesting that Neu5Gc inhibits Neu5Ac transport into G. vaginalis. These results are similar to a previous observation that Neu5Gc inhibits the uptake of Neu5Ac in a distantly related Gram-positive bacterium Streptococcus oralis (91). Addition of exogenous glucose dramatically increased the rate of Neu5Ac uptake and catabolism (Figure S6), suggesting that transport of Neu5Ac in G. vaginalis may be an active process. We also note that at the completion of our uptake assays, the remaining extracellular Neu5Ac levels are low (around 1 µM). Taken together, these data strongly suggest a high-affinity transport system in G. vaginalis with a preference for Neu5Ac.

There are over 50 structurally distinct sialic acids reported and it is possible that other structural variations of sialic acid may influence whether G. vaginalis sialidase or uptake machinery are effective on these molecules. In particular, O-acetylation of the 3-carbon sialic acid side chain has been known to influence that biological properties of sialic acids and to be resistant to cleavage by many sialidases (57,92,93). In fact, our studies strongly suggest that O-acetylation protects sialic acids from cleavage by G. vaginalis sialidase (data not shown).

Foraging of sialoglycans: a normal or pathologic process? The depletion of mucosal sialic acid in BV has a number of interesting biological, immunological, and clinical implications. Mucus is widely held as a first-line of defense that protects mucosal epithelial surfaces from direct contact with bacteria. Mucus contains a high concentration of glycoproteins, including immunoglobulins and mucins, which have defined roles in the exclusion of potential pathogens from mucosal surfaces. Vaginal sialidase activity has been correlated with adverse obstetric and gynecological outcomes in several clinical studies, suggesting that sialic acid foraging by BV-associated bacteria may lead to increased risk of reproductive host pathology. In most cases that have been studied so far, bacterial sialidases and/or sialic acid catabolic enzymes do contribute to virulence and/or colonization by bacterial pathogens. However, as described above, there are several examples of commensal bacteria that seem to strike a balance with the host, foraging on mucus sialic acid residues without damage to host tissues. Further studies should examine whether and how mucosal depletion of sialic acids may contribute to the general risk of secondary infection associated with BV.

Sialoglycan depletion as a community activity? We emphasize that other BV-associated bacteria or combinations of BV-associated bacteria may also be capable of the hydrolysis and depletion of mucosal sialic acids. In fact, nearly all vaginal isolates from the phylum Bacteroidetes can produce sialidase enzyme, including isolates from the BV-associated genus Prevotella (32,34,94).
The observation that women with BV have significantly lower levels of vaginal mucus sialic acids is likely the result of a milieu of enzymes produced by distinct communities of bacteria in individual women. It may be of interest in future studies to examine potential relationships between community composition, sialidase levels, and the extent of sialic acid depletion. Sialidase action on mucus sialoglycans may also promote further mucus degradation by revealing underlying substrates that can be cleaved by other glycosidases produced by vaginal bacteria. Evidence of enhanced glycan degradation in bacterial dysbiosis has been reported in human vaginal fluids (52) and fecal specimens (95). These studies provide a template for integrating knowledge gained from clinical correlations and microbiome studies together with experimental models aimed at understanding the potential contributions of different community members to the degradation of protective mucus barriers in BV.

Finally, recent studies have demonstrated correlations between particular BV-associated bacteria and certain clinical features of BV (26). However, causal relationships between individual bacterial species and measurable clinical phenotypes of BV have been elusive. Here we demonstrate the consequence of an accepted diagnostic feature of BV – vaginal sialidase activity – on mucosal sialoglycans of women with this condition. The data presented here show that sialic acid residues are both liberated and depleted in BV compared to normal controls, demonstrate that G. vaginalis is sufficient to elicit this phenotype in an animal model, and moreover, provide a detailed understanding of G. vaginalis sialic acid foraging at the biochemical level. These findings demonstrate mucosal sialic acid depletion as a new clinical feature of BV and provide the first example of a BV-associated bacterium, G. vaginalis, which plays an active role in the degradation of protective vaginal mucus barriers.

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**References**

1. Allsworth, J. E., and Peipert, J. F. (2007) *Obstetrics and gynecology* **109**, 114-120
2. Gelber, S. E., Aguilar, J. L., Lewis, K. L., and Ratner, A. J. (2008) *Journal of bacteriology* **190**, 3896-3903
3. Donders, G. G., Bosmans, E., Dekeersmaecker, A., Vereecken, A., Van Bulck, B., and Spitz, B. (2000) *Am J Obstet Gynecol* **182**, 872-878
4. Hay, P. E., Lamont, R. F., Taylor-Robinson, D., Morgan, D. J., Ison, C., and Pearson, J. (1994) *BMJ* **308**, 295-298
5. Svare, J. A., Schmidt, H., Hansen, B. B., and Lose, G. (2006) *Bjog* **113**, 1419-1425
6. McGregor, J. A., French, J. I., Jones, W., Milligan, K., McKinney, P. J., Patterson, E., and Parker, R. (1994) *Am J Obstet Gynecol* 170, 1048-1059; discussion 1059-1060
7. Holst, E., Goffeng, A. R., and Andersch, B. (1994) *J Clin Microbiol* 32, 176-186
8. Hillier, S. L., Nugent, R. P., Eschenbach, D. A., Krohn, M. A., Gibbs, R. S., Martin, D. H., Cotch, M. F., Edelman, R., Pastorek, J. G., 2nd, Rao, A. V., and et al. (1995) *N Engl J Med* 333, 1737-1742
9. Hillier, S. L., Martius, J., Krohn, M., Kiviat, N., Holmes, K. K., and Eschenbach, D. A. (1988) *N Engl J Med* 319, 972-978
10. Hendler, I., Andrews, W. W., Carey, C. J., Klebanoff, M. A., Noble, W. D., Sibai, B. M., Hillier, S. L., Dudley, D., Ernest, J. M., Leveno, K. J., Wapner, R., Iams, J. D., Varner, M., Moawad, A., Miodovnik, M., O'Sullivan, M. J., and Van Dorsten, P. J. (2007) *Am J Obstet Gynecol* 197, e481-e485
11. Hitti, J., Hillier, S. L., Agnew, K. J., Krohn, M. A., Reisner, D. P., and Eschenbach, D. A. (2001) *Obstetrics and gynecology* 97, 211-219
12. Rezeberga, D., Lazdane, G., Kroica, J., Sokolova, L., and Donders, G. G. (2008) *Acta Obstet Gynecol Scand* 87, 360-365
13. Silver, H. M., Sperling, R. S., St Clair, P. J., and Gibbs, R. S. (1989) *Am J Obstet Gynecol* 161, 808-812
14. Zhang, X., Xu, X., Li, J., Li, N., Yan, T., and Ju, X. (2002) *Zhonghua fu chan ke za zhi* 37, 588-590
15. Brotman, R. M., Klebanoff, M. A., Nansel, T. R., Yu, K. F., Andrews, W. W., Zhang, J., and Schwebke, J. R. (2010) *J Infect Dis* 202, 1907-1915
16. Cohen, C. R., Lingappa, J. R., Baeten, J. M., Ngayo, M. O., Spiegel, C. A., Hong, T., Donnell, D., Celum, C., Kapiga, S., Delany, S., and Bukusi, E. A. (2012) *PLoS medicine* 9, e1001251
17. Watts, D. H., Krohn, M. A., Hillier, S. L., and Eschenbach, D. A. (1990) *Obstetrics and gynecology* 75, 52-58
18. Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schutte, U. M., Zhong, X., Koenig, S. S., Fu, L., Ma, Z. S., Zhou, X., Abdo, Z., Forney, L. J., and Ravel, J. (2012) *Science translational medicine* 4, 132ra152
19. Fredricks, D. N. (2011) *Anaerobe* 17, 191-195
20. Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., McCulle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., Brotman, R. M., Davis, C. C., Ault, K., Peralta, L., and Forney, L. J. (2011) *Proc Natl Acad Sci U S A* 108 Suppl 1, 4680-4687
21. Srinivasan, S., Liu, C., Mitchell, C. M., Fiedler, T. L., Thomas, K. K., Agnew, K. J., Marrazzo, J. M., and Fredricks, D. N. (2010) *PLoS One* 5, e10197
22. Hillier, S. L., Krohn, M. A., Rabe, L. K., Klebanoff, S. J., and Eschenbach, D. A. (1993) *Clin Infect Dis* 16 Suppl 4, S273-S281
23. Krohn, M. A., Hillier, S. L., and Eschenbach, D. A. (1989) *Journal of clinical microbiology* 27, 1266-1271
24. Amsel, R., Totten, P. A., Spiegel, C. A., Chen, K. C., Eschenbach, D., and Holmes, K. K. (1983) *Am J Med* 74, 14-22
25. Eschenbach, D. A., Hillier, S., Critchlow, C., Stevens, C., DeRouen, T., and Holmes, K. K. (1988) *Am J Obstet Gynecol* 158, 819-828
26. Srinivasan, S., Hoffman, N. G., Morgan, M. T., Matsen, F. A., Fiedler, T. L., Hall, R. W., Ross, F. J., McCoy, C. O., Bumgarner, R., Marrazzo, J. M., and Fredricks, D. N. (2012) *PloS one* 7, e37818
27. Lewis, A. L., and Lewis, W. G. (2012) *Cellular microbiology* 14, 1174-1182
28. Slomiany, B. L., Murty, V. L., Piotrowski, J., and Slomiany, A. (1996) *General pharmacology* 27, 761-771
29. Moran, A. P., Gupta, A., and Joshi, L. (2011) *Gut* 60, 1412-1425
30. Anthony, R. M., and Ravetch, J. V. (2010) *Journal of clinical immunology* 30 Suppl 1, S9-14
31. Yoo, E. M., and Morrison, S. L. (2005) *Clin Immunol* **116**, 3-10
32. Briselden, A. M., Monela, B. J., Stevens, C. E., and Hillier, S. L. (1992) *Journal of clinical microbiology* **30**, 663-666
33. Howe, L., Wiggins, R., Soothill, P. W., Millar, M. R., Horner, P. J., and Corfield, A. P. (1999) *International journal of STD & AIDS* **10**, 442-447
34. Olmsted, S. S., Meyn, L. A., Rohan, L. C., and Hillier, S. L. (2003) *Sex Transm Dis* **30**, 257-261
35. Smayevsky, J., Canigia, L. F., Lanza, A., and Bianchini, H. (2001) *Infectious diseases in obstetrics and gynecology* **9**, 17-22
36. Wiggins, R., Crowley, T., Horner, P. J., Soothill, P. W., Millar, M. R., and Corfield, A. P. (2000) *Journal of clinical microbiology* **38**, 3096-3097
37. Sumeksri, P., Koprasert, C., and Panichkul, S. (2005) *Journal of the Medical Association of Thailand = Chotmaihet thangphaet* **88 Suppl 3**, S7-13
38. Myziuk, L., Romanowski, B., and Johnson, S. C. (2003) *Journal of clinical microbiology* **41**, 1925-1928
39. Cauci, S., and Culhane, J. F. (2011) *Am J Obstet Gynecol* **204**, 142 e141-149
40. Cauci, S., McGregor, J., Thorsen, P., Grove, J., and Guaschino, S. (2005) *Am J Obstet Gynecol* **192**, 489-496
41. Bradshaw, C. S., Morton, A. N., Garland, S. M., Horvath, L. B., Kuzevska, I., and Fairley, C. K. (2005) *Journal of clinical microbiology* **43**, 1304-1308
42. Moncla, B. J., Braham, P., and Hillier, S. L. (1990) *Journal of clinical microbiology* **28**, 422-425
43. von Nicolai, H., Hammann, R., Salehnia, S., and Zilliken, F. (1984) *Zentralbl Bakteriol Mikrobiol Hyg A* **258**, 20-26
44. Oakley, B. B., Fiedler, T. L., Marrazzo, J. M., and Fredricks, D. N. (2008) *Appl Environ Microbiol* **74**, 4898-4909
45. Srinivasan, S., Liu, C., Mitchell, C. M., Fiedler, T. L., Thomas, K. K., Agnew, K. J., Marrazzo, J. M., and Fredricks, D. N. *PLoS One* **5**, e10197
46. Gardner, H. L., and Dukes, C. D. (1954) *Science* **120**, 853
47. Briselden, A. M., and Hillier, S. L. (1990) *Journal of clinical microbiology* **28**, 2761-2764
48. Harwich, M. D., Jr., Alves, J. M., Buck, G. A., Strauss, J. F., 3rd, Patterson, J. L., Oki, A. T., Girerd, P. H., and Jefferson, K. K. (2010) *BMC genomics* **11**, 375
49. Patterson, J. L., Stull-Lane, A., Girerd, P. H., and Jefferson, K. K. (2010) *Microbiology* **156**, 392-399
50. Fredricks, D. N., Fiedler, T. L., Thomas, K. K., Oakley, B. B., and Marrazzo, J. M. (2007) *Journal of clinical microbiology* **45**, 3270-3276
51. Marrs, C. N., Knobel, S. M., Zhu, W. Q., Sweet, S. D., Chaudhry, A. R., and Alcendor, D. J. (2012) *Microbes and infection / Institut Pasteur* **14**, 500-508
52. Lewis, W. G., Robinson, L. S., Perry, J., Bick, J. L., Peipert, J. F., Allsworth, J. E., and Lewis, A. L. (2012) *The Journal of biological chemistry* **287**, 2079-2089
53. Lewis, A. L., Cao, H., Patel, S. K., Diaz, S., Ryan, W., Carlin, A. F., Thon, V., Lewis, W. G., Varki, A., Chen, X., and Nizet, V. (2007) *The Journal of biological chemistry* **282**, 27562-27571
54. Lewis, A. L., Desa, N., Hansen, E. E., Knirel, Y. A., Gordon, J. I., Gagneux, P., Nizet, V., and Varki, A. (2009) *Proceedings of the National Academy of Sciences of the United States of America* **106**, 13552-13557
55. Lewis, A. L., Lubin, J. B., Argade, S., Naidu, N., Choudhury, B., and Boyd, E. F. (2011) *Applied and environmental microbiology* **77**, 5782-5793
56. Lewis, A. L., Nizet, V., and Varki, A. (2004) *Proceedings of the National Academy of Sciences of the United States of America* **101**, 11123-11128
57. Weiman, S., Daresh, S., Carlin, A. F., Varki, A., Nizet, V., and Lewis, A. L. (2009) *Glycobiology* **19**, 1204-1213
58. Secura, G. M., Allsworth, J. E., Madden, T., M ullersman, J. L., and Peipert, J. F. (2010) *Am J Obstet Gynecol* **203**, 115 e111-117
59. Nugent, R. P., Krohn, M. A., and Hillier, S. L. (1991) *Journal of clinical microbiology* **29**, 297-301
60. Singh, P. K., and Hollingsworth, M. A. (2006) *Trends in cell biology* **16**, 467-476
61. Hattrup, C. L., and Gendler, S. J. (2008) *Annual review of physiology* **70**, 431-457
62. McGuckin, M. A., Linden, S. K., Sutton, P., and Florin, T. H. (2011) *Nature reviews. Microbiology* **9**, 265-278
63. Chou, H. H., Hayakawa, T., Diaz, S., Krings, M., Indriati, E., Leakey, M., Paabo, S., Satta, Y., Takahata, N., and Varki, A. (2002) *Proceedings of the National Academy of Sciences of the United States of America* **99**, 11736-11741
64. Varki, A. (2001) *American journal of physical anthropology Suppl 33*, 54-69
65. Chou, H. H., Takematsu, H., Diaz, S., Iber, J., Nickerson, E., Wright, K. L., Muchmore, E. A., Nelson, D. L., Warren, S. T., and Varki, A. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 11751-11756
66. Vimr, E. R., Kalivoda, K. A., Deszo, E. L., and Steenbergen, S. M. (2004) *Microbiology and molecular biology reviews : MMBR* **68**, 132-153
67. Trinh, H. T., Lee, I. A., Hyun, Y. J., and Kim, D. H. (2011) *Planta medica* **77**, 1996-2002
68. Xavier, R. J., and Podolsky, D. K. (2007) *Nature* **448**, 427-434
69. Severi, E., Randle, G., Kivlin, P., Whitfield, K., Young, R., Moxon, R., Kelly, D., Hood, D., and Thomas, G. H. (2005) *Molecular microbiology* **58**, 1173-1185
70. Marion, C., Burnaugh, A. M., Woodiga, S. A., and King, S. J. (2011) *Infection and immunity* **79**, 1262-1269
71. Almagro-Moreno, S., and Boyd, E. F. (2009) *Infection and immunity* **77**, 3807-3816
72. Byers, H. L., Tarelli, E., Homer, K. A., and Beighton, D. (1999) *Glycobiology* **9**, 469-479
73. Marcobal, A., Barboza, M., Sonnenburg, E. D., Pudlo, N., Martens, E. C., Desai, P., Lebrilla, C. B., Weimer, B. C., Mills, D. A., German, J. B., and Sonnenburg, J. L. (2011) *Cell host & microbe* **10**, 507-514
74. Sela, D. A., Chapman, J., Adeuya, A., Kim, J. H., Chen, F., Whitehead, T. R., Lapidos, A., Rokhsar, D. S., Lebrilla, C. B., German, J. B., Price, N. P., Richardson, P. M., and Mills, D. A.
(2008) *Proceedings of the National Academy of Sciences of the United States of America* **105**, 18964-18969

88. Sela, D. A., Li, Y., Lerno, L., Wu, S., Marcobal, A. M., German, J. B., Chen, X., Lebrilla, C. B., and Mills, D. A. (2011) *The Journal of biological chemistry* **286**, 11909-11918

89. Santiago, G. L., Deschaght, P., El Aila, N., Kiama, T. N., Verstraeten, H., Jefferson, K. K., Temmerman, M., and Vaneechoutte, M. (2011) *Am J Obstet Gynecol* **204**, e451-457

90. Pleckaityte, M., Janulaitiene, M., Lasickiene, R., and Zvirbliene, A. (2012) *FEMS immunology and medical microbiology* **65**, 69-77

91. Byers, H. L., Homer, K. A., Tarelli, E., and Beighton, D. (1999) *Journal of medical microbiology* **48**, 375-381

92. Chowdhury, S., and Mandal, C. (2009) *Biotechnology journal* **4**, 361-374

93. Schauer, R. (2009) *Current opinion in structural biology* **19**, 507-514

94. Nakayama-Imaohji, H., Ichimura, M., Iwasa, T., Okada, N., Ohnishi, Y., and Kuwahara, T. (2012) *The journal of medical investigation : JMI* **59**, 79-94

95. Hoskins, L. C., and Boulding, E. T. (1981) *The Journal of clinical investigation* **67**, 163-172
| Patient | Strain Name | BV status | Nugent Score | Sialidase Activity | # of identical 16S bases/total sequenced | % identity to reference strain | GenBank Accession number |
|---------|-------------|-----------|--------------|-------------------|-----------------------------------------|-------------------------------|------------------------|
| N/A     | ATCC14019   | unknown   | N/A          | -                 | reference                              | 100.0%                        | --                     |
| 047275  | JCP7275     | positive  | 10           | -                 | 1408/1409                              | 99.9%                         | JX860309               |
| 047276  | JCP7276     | intermediate | 5           | +++               | 1411/1411                              | 100.0%                        | JX860310               |
| 047659  | JCP7659     | positive  | 8            | ++                | 1397/1407                              | 99.3%                         | JX860311               |
| 047672  | JCP7672     | negative  | 3            | +/−               | 1412/1412                              | 100.0%                        | JX860312               |
| 047719  | JCP7719     | positive  | 8            | +                 | 1385/1397                              | 99.1%                         | JX860313               |
| 048017  | JCP8017A    | positive  | 8            | +++               | 1398/1409                              | 99.2%                         | JX860314               |
| 048017  | JCP8017B    | positive  | 8            | +++               | 1403/1413                              | 99.3%                         | JX860315               |
| 048066  | JCP8066     | negative  | 0            | ++++              | 1382/1402                              | 98.6%                         | JX860316               |
| 048070  | JCP8070     | positive  | 8            | +++               | 1400/1412                              | 99.2%                         | JX860317               |
| 048108  | JCP8108     | positive  | 8            | -                 | 1390/1396                              | 99.6%                         | JX860318               |
| 048151  | JCP8151A    | positive  | 10           | +++               | 1377/1394                              | 98.8%                         | JX860319               |
| 048151  | JCP8151B    | positive  | 10           | +++               | 1394/1408                              | 99.0%                         | JX860320               |
| 048481  | JCP8481A    | positive  | 10           | -                 | 1389/1410                              | 98.5%                         | JX860322               |
| 048481  | JCP8481B    | positive  | 10           | +++               | 1397/1409                              | 99.1%                         | JX860323               |
| 048522  | JCP8522     | positive  | 8            | -                 | 1332/1428                              | 93.3%                         | JX860308               |

*Bifidobacterium* isolate

| Patient | Strain Name | BV status | Nugent Score | Sialidase Activity | # of identical 16S bases/total sequenced | % identity to reference strain | GenBank Accession number |
|---------|-------------|-----------|--------------|-------------------|-----------------------------------------|-------------------------------|------------------------|
| 047499  | JCP7499     | positive  | 8            | -                 | 1332/1428                              | 93.3%                         | JX860308               |
Figure Legends

Figure 1. *Gardnerella vaginalis* clinical isolates produce cell-associated and secreted sialidase. (A) *G. vaginalis* isolates were grown anaerobically in NYCIII media and samples were analyzed for sialidase activity, normalized to the optical density of cultures as described in the Experimental Procedures. (B) The proportion of cell-associated versus secreted sialidase activity was determined by pelleting bacteria and comparing activity remaining in the supernatant with that of the whole culture suspension. Error bars represent standard deviation.

Figure 2. Sialidase-positive *G. vaginalis* strains consume sialic acid from bound sialoglycans in culture media. *G. vaginalis* isolates were cultured anaerobically in NYC-III media, and total sialic acid content of the media was measured over 24 hours post inoculation. (A) Heatmap of sialic acid in media shows reduction of starting Neu5Ac levels (red) to low levels (blue) by each *G. vaginalis* strain over 24 hours with sialidase-positive strains. Values are means of 2-4 experiments. The time point taken at 7-12 hours is reported as 10 hours. (B) Pooled data of sialidase-positive strains JCP7719, JCP7659, JCP8017A, JCP7276, JCP8066, JCP8522, JCP8151B, JCP8151A, and JCP8070 at 4, 8, and 24 hours compared to 24 hour data from media alone control and sialidase-negative strains JCP7275, JCP8108, JCP8481A, and JCP8481B. Combined data are shown from 5 independent experiments performed on different days. Each strain was analyzed in at least 2 independent experiments. Bars represent median values. Statistical significance was examined using one way ANOVA followed by Bonferroni multiple comparisons test **p<0.01 ***p<0.001.

Figure 3. (A) Extracellular hydrolysis of human IgA sialic acids by *G. vaginalis* precedes consumption. Clinical isolates JCP8151B (sialidase-positive) and JCP8481A (sialidase-negative) were incubated with purified human serum IgA in acetate buffer and the concentration of free sialic acid in the supernatant was monitored over the course of three hours. Analysis of 3 samples from a single *G. vaginalis* preparation are shown in this representative experiment. (B) *G. vaginalis* clinical isolates consume free sialic acid. *G. vaginalis* strains were washed and incubated with 10 µM free Neu5Ac in acetate buffer and sialic acid concentration was measured over a four-hour time course. Combined data from 2 independent experiments are shown. Sialidase-positive strains (JCP7719, JCP7276, JCP8066, JCP8522, JCP8151B, JCP8070) at 30, 60, and 120 minutes were compared to 120 min data from sialidase-negative strains (JCP7275, JCP8108, and JCP8481A). Bars are geometric means. Statistical significance was examined using one way ANOVA followed by Bonferroni multiple comparisons test *p<0.05 ***p<0.001, ****p<0.0001.

Figure 4. The rate of *G. vaginalis* sialic acid (Neu5Ac) consumption from sialoglycans is substrate-dependent and inhibited by N-glycolyneuraminic acid (Neu5Gc). Washed *G. vaginalis* JCP8151B was incubated with 0.2 mg/ml human IgA or 0.3 mg/ml bovine submaxillary mucin (BSM), followed by kinetic measurements of total (A) and free (B) sialic acids. The total starting concentration of Neu5Ac in each experiment was approximately 15 µM. “Ac” and “Gc” refer to quantitation of Neu5Ac or Neu5Gc respectively in reactions with BSM. (C and D) Added Neu5Gc inhibits consumption but not release Neu5Ac from IgA by *G. vaginalis*. Washed *G. vaginalis* JCP8151B was incubated with human IgA with or without 150 µM Neu5Gc (“+ free Gc”) and levels of total (C) and free (D) Neu5Ac were determined. These data are representative of 3 or more independent experiments.

Figure 5. Excess Neu5Gc interferes with Neu5Ac transport, but not sialidase or lyase activities. Cell lysates of *G. vaginalis* JCP8151B were prepared by sonication and centrifugation of cell debris, and then lysates were mixed with enzyme substrates. (A) Neu5Ac (100 µM) was consumed within hours, consistent with sialate lyase activity. (B) ManNAc and pyruvate (1 mM each) allow cell lysates to synthesize micromolar concentrations of Neu5Ac in the reverse (aldolase) reaction. (C) Uptake and catabolism of Neu5Ac by live *G. vaginalis* was performed after washing cells as described in the
Experimental Procedures. (D) *G. vaginalis* sialidase activity was measured using the fluorescent 4-MU-Sia substrate. The non-human sialic acid Neu5Gc (100 µM) “+ Gc”, slows the uptake of free Neu5Ac by *G. vaginalis*, but does not inhibit sialidase activity, or Neu5Ac lyase activity. These data are representative of three or more independent experiments.

**Figure 6.** *G. vaginalis* liberates and consumes sialic acids from human and mouse vaginal mucus *in vitro* (A and B) *G. vaginalis* sialidase-positive strains (JCP8151B, JCP8017A) and sialidase-negative strain (JCP8481A, ATCC14019) were incubated *in vitro* with vaginal mucus eluted from human vaginal swabs (pool of 14 BV-negative, sialidase-negative samples) followed by measurement of free (A) and total (B) sialic acids by HPLC as described in the Experimental Procedures. Representative time courses are shown from three independent experiments. (C) *G. vaginalis* sialidase-positive strain JCP8151B and sialidase-negative strain JCP8108 were incubated for 4 hours *in vitro* with vaginal mucus from mouse vaginal washes (pooled from uninfected C57/Bl6 mice), followed by measurement of total Neu5Ac by HPLC. Data points are shown from 3 independent experiments performed on different days. Mean values with standard deviation are shown.

**Figure 7.** *G. vaginalis* liberates and depletes sialic acids from vaginal mucus barriers *in vivo*. 1X10⁶ *G. vaginalis* isolate JCP8151B was inoculated vaginally into C57B/6 mice. Vaginal washes were collected at 24 and 72 hours post infection using 50 µl phosphate-buffered saline. Free (A) and bound (B) Neu5Ac was measured by HPLC as previously described. Free Neu5Ac was measured at 24 hours and bound Neu5Ac was measured at 72 hours post-infection. Combined data of 2-3 independent experiments are shown (n= 10 animals per group). Bars represent median values. Note log scales. * P< 0.01 *** P < 0.0001.

**Figure 8.** New biochemical features of human BV: sialic acid hydrolysis and depletion. Vaginal specimens were assigned a clinical status of ‘BV’ or ‘No BV’ based on the method of Nugent scoring of Gram stained slides (score ranges shown in parentheses). Vaginal swabs were eluted and subjected to biochemical analysis of free (A) and total (B) sialic acid (N-acetyleneuraminic acid, Neu5Ac) levels as described in the Experimental Procedures. A total of 63 individuals were studied. Median values with standard deviation are shown. Note log scales. Statistical significance was evaluated using the nonparametric Mann Whitney U-test.

**Figure 9.** ‘Release and capture’ model of *G. vaginalis* sialic acid utilization: 1) The secretion and extracellular localization of sialidase enzyme allows for the release of sialic acids from mucosal sialoglycan substrates outside the cell, 2) a transport system pumps the liberated free Neu5Ac into the cell, a process that can be inhibited by Neu5Gc, and 3) intracellular catabolism is initiated by Neu5Ac lyase/aldolase.
Figure 1. *Gardnerella vaginalis* clinical isolates produce cell-associated and secreted sialidase. (A) *G. vaginalis* isolates were grown anaerobically in NYCIII media and samples were analyzed for sialidase activity, normalized to the optical density of cultures as described in the Methods. (B) The proportion of cell-associated versus secreted sialidase activity was determined by pelleting bacteria and comparing activity remaining in the supernatant with that of the whole culture suspension.
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