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Identification and characterization of NanH2 and NanH3, enzymes responsible for sialidase activity in the vaginal bacterium *Gardnerella vaginalis*

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***Gardnerella vaginalis* is abundant in bacterial vaginosis (BV), a condition associated with adverse reproductive health. Sialidase activity is a diagnostic feature of BV and is produced by a subset of *G. vaginalis* strains. Although its genetic basis has not been formally identified, sialidase activity is presumed to derive from the *sialidase A* gene, named here *nanH1*. In this study, BLAST searches predicted two additional *G. vaginalis* sialidases, NanH2 and NanH3. When expressed in *Escherichia coli*, NanH2 and NanH3 both displayed broad abilities to cleave sialic acids from α2-3- and α2-6-linked *N*- and *O*-linked sialoglycans, including relevant mucosal substrates. In contrast, recombinant NanH1 had limited activity against synthetic and mucosal substrates under the conditions tested. Recombinant NanH2 was much more effective than NanH3 in cleaving sialic acids bearing a 9-*O*-acetyl ester. Similarly, *G. vaginalis* strains encoding NanH2 cleaved and foraged significantly more Neu5,9Ac$_2$ than strains encoding only NanH3. Among a collection of 34 *G. vaginalis* isolates, *nanH2*, *nanH3*, or both were present in all 15 sialidase-positive strains but absent from all 19 sialidase-negative isolates, including 16 strains that were *nanH1*-positive. We conclude that NanH2 and NanH3 are the primary sources of sialidase activity in *G. vaginalis* and that these two enzymes can account for the previously described substrate breadth cleaved by sialidases in human vaginal specimens of women with BV. Finally, PCRs of *nanH2* or *nanH3* from human vaginal specimens had 81% sensitivity and 78% specificity in distinguishing between *Lactobacillus* dominance and BV, as determined by Nugent scoring.**

Bacterial vaginosis (BV)[2] is a common condition in which the vagina contains few "healthy" lactobacilli and is overpopulated by diverse anaerobes (1, 2). BV has been associated with a wide array of adverse health outcomes, including increased risks of sexually transmitted infections, placental and amniotic fluid infections, and preterm birth (3–6). Several bacterial enzymes have been proposed to be virulence factors in BV, including phospholipases, cytolysins, proteases, and sialidases (7–10). In particular, sialidase (also referred to as neuraminidase, E.C.3.2.1.18) activity in vaginal fluids is considered a hallmark of BV (10–12). Sialidases act on glycan chains capped with sialic acid residues (13), which are abundant at mucosal surfaces, including the reproductive tract. Sialidase activity has been used as a diagnostic marker for BV (14, 15) and has been independently associated with adverse pregnancy outcomes, including ascending intrauterine infection and preterm birth (16–18). Sialidase production by isolates of BV-associated bacteria suggests that the enzyme activity in vaginal fluids is bacterial in origin (10, 19). It is widely believed that mucus degradation by BV bacteria contributes to the characteristically "thin" consistency of vaginal fluid in BV (9, 20) and has been postulated to contribute to the increased risks of sexually transmitted and ascending infections in women with BV (10, 16, 19, 21, 22).

Gardnerella vaginalis is one of the most common bacterial species to overgrow in BV (2, 23–25). Consistent with the notion that *G. vaginalis* is a pathogen, this bacterium has been isolated from invasive perinatal infections (26, 27) and, in one study, was found in 26% of infected placentas from cases of preterm birth (28). The pathogenic potential of *G. vaginalis* isolates has also been demonstrated *in vitro* (*e.g.* cell adhesion and invasion, cytolytic toxin production/pore formation, and biofilm formation) (29–32). We have shown that a *G. vaginalis* strain isolated from a woman with BV is sufficient to elicit several features of BV (or health complications that have been

associated with BV) in mouse models of vaginal infection (33–35). These features include vaginal sialidase activity, evidence of mucus degradation, absence of overt histological inflammation, epithelial exfoliation, and the presence of "clue-like" epithelial cells with attached bacteria in vaginal washes (33, 34). Health complications that can be reproduced in murine models by administering *G. vaginalis* include ascending uterine infection with *G. vaginalis* and other potential pathogens as well as recurrent urinary tract infection caused by *Escherichia coli* (33, 35).

Although many strains of *G. vaginalis* do not produce sialidase activity under laboratory conditions, others express sialidase activity that is both surface-bound as well as secreted (34). These strains are able to liberate sialic acids from glycoproteins in culture medium and then deplete the resulting free monosaccharide. In contrast, sialidase-negative strains cannot liberate or consume sialic acids provided in the bound form. For years, it has been assumed that the sialidase activity in *G. vaginalis* is encoded by the gene originally annotated in strain ATCC 14019 as "*sialidase A*" (34, 36–40). In support of this idea, a recent report demonstrated activity of recombinant sialidase A protein against a synthetic substrate *in vitro* (41). However, the lack of genetic tools in *Gardnerella* has prevented the construction of sialidase mutants to formally test the extent to which sialidase A contributes to the sialidase activity observed in cultured strains. Although *sialidase A* appears to be found in all sialidase-positive strains of *G. vaginalis*, the intact ORF is also present in many sialidase-negative isolates. This inconsistency has prompted multiple research groups to question whether sialidase A accounts for the enzyme activity observed in *G. vaginalis* cultures (37, 39, 42). Here we describe two previously unappreciated sialidases in *G. vaginalis*, NanH2 and NanH3, and show that these enzymes exhibit a broad range of activity not only against synthetic substrates but also against mucosal glycoproteins relevant to the human vaginal environment. Moreover, we show that the presence of *nanH2* or *nanH3* in the genomes of *G. vaginalis* strains perfectly reflects their ability to produce sialidase activity in culture. Thus, we conclude that NanH2 and NanH3 are the main sources of sialidase activity in *G. vaginalis*.

Results

Here we set out to identify the genetic basis for sialidase activity in *G. vaginalis*. Given that *sialidase A* is present in many strains that do not produce sialidase activity in culture, we suspected that genes other than *sialidase A* might encode the activity produced by sialidase-positive isolates. Therefore, we performed BLASTp searches of the predicted *G. vaginalis* proteome to identify additional sialidase homologs. To our knowledge, *Bifidobacterium longum* is the species most closely related to *G. vaginalis* in which a sialidase has been functionally characterized. The NanH2 sialidase of *B. longum* subsp. *infantis* strain ATCC15697 cleaves sialic acids in both α2-3 and α2-6 linkages and is active against milk oligosaccharides (43). Using *B. longum* ATCC15697 NanH2 as a query sequence, a BLASTp search of the proteome of *G. vaginalis* JCP8151B (34), a sialidase-positive strain, revealed two sialidase homologs in addition to sialidase A. The first result (accession number

WP_016798291) was 65% identical over 349 residues to *B. longum* NanH2 and was subsequently designated NanH2. The second result (accession number WP_016792322) was 60% identical to *B. longum* NanH2 over 372 residues and was designated NanH3. Aligning the three NanH homologs revealed that the regions of high identity centered around the sialidase domain of each protein (Fig. S1). Within JCP8151B, NanH2 and NanH3 displayed 49% identity over 572 residues, whereas sialidase A was only 29% identical over 251 residues to NanH2 and 24% identical over 292 residues to NanH3. *B. longum* ATCC15697 encodes another sialidase, NanH1, which can also cleave sialic acids in α2-3 and α2-6 linkages but is more than 100-fold less active than NanH2 (43). A BLASTp search of the JCP8151B proteome with the ATCC15697 NanH1 sequence identified sialidase A as the first result, with 43% identity between them. Thus, we propose renaming *G. vaginalis* sialidase A NanH1 and will refer to it as such from this point forward.

To test whether *G. vaginalis* *nanH1*, *nanH2*, or *nanH3* encode active sialidases, the genes were cloned from JCP8151B and expressed as His$_6$-tagged proteins in *E. coli*. Kinetic assays on IPTG-induced cultures demonstrated that both NanH2 and NanH3 were able to cleave the fluorogenic substrate 4-MU-*N*-acetylneuraminic acid (*N*-acetylneuraminic acid or Neu5Ac is the most common type of sialic acid found in nature). In contrast, NanH1 activity was undetectable under these conditions (Fig. 1*A*). The absence of NanH1 activity could not be attributed to a lack of protein expression or stability because Western blot analysis with anti-His$_6$ monoclonal antibodies revealed a prominent band at the expected molecular mass of 100 kDa (Fig. S2).

Bacterial sialidase domains typically contain an N-terminal RIP (Arg-Ile/Leu-Pro) motif, four or five aspartate box repeats (Ser/Thr-*X*-Asp-*X*-Gly-*X*-Thr-Trp/Phe), and seven conserved active site residues (44). These features are present in the amino acid sequences of all three *G. vaginalis* JCP8151B NanH proteins, with the exception of NanH3, which lacks the C-terminal auxiliary glutamate residue (Fig. 1*B*). NanH1 has an N-terminal concanavalin A–like lectin domain and a C-terminal sialidase domain but lacks predicted secretion signals, transmembrane regions, or cell wall anchoring motifs. In contrast, the 96-kDa NanH2 protein displays a predicted 51-residue N-terminal Sec-dependent signal peptide, a region with homology to Sec-independent translocases, and a C-terminal transmembrane α-helix, suggesting that NanH2 may be secreted but remain tethered to the bacterial surface (Fig. 1*B*). Homology modeling of NanH2 based on the sialidase crystal structure from *Micromonospora viridifaciens* (PDB code 1WCQ) (45), which also belongs to the Actinobacteria, revealed a β propeller fold characteristic of sialidases. The model positioned amino acid side chains to create a predicted active site consisting of conserved catalytic residues, including Glu-407 and Tyr-515, in addition to positively charged arginine residues (Arg-206, Arg-423, and Arg-487), likely responsible for binding negatively charged sialic acid (Fig. 1*C*). Corresponding putative active-site residues were also found in NanH3. Sequence analysis of the 80-kDa JCP8151B NanH3 protein revealed a predicted C-terminal membrane
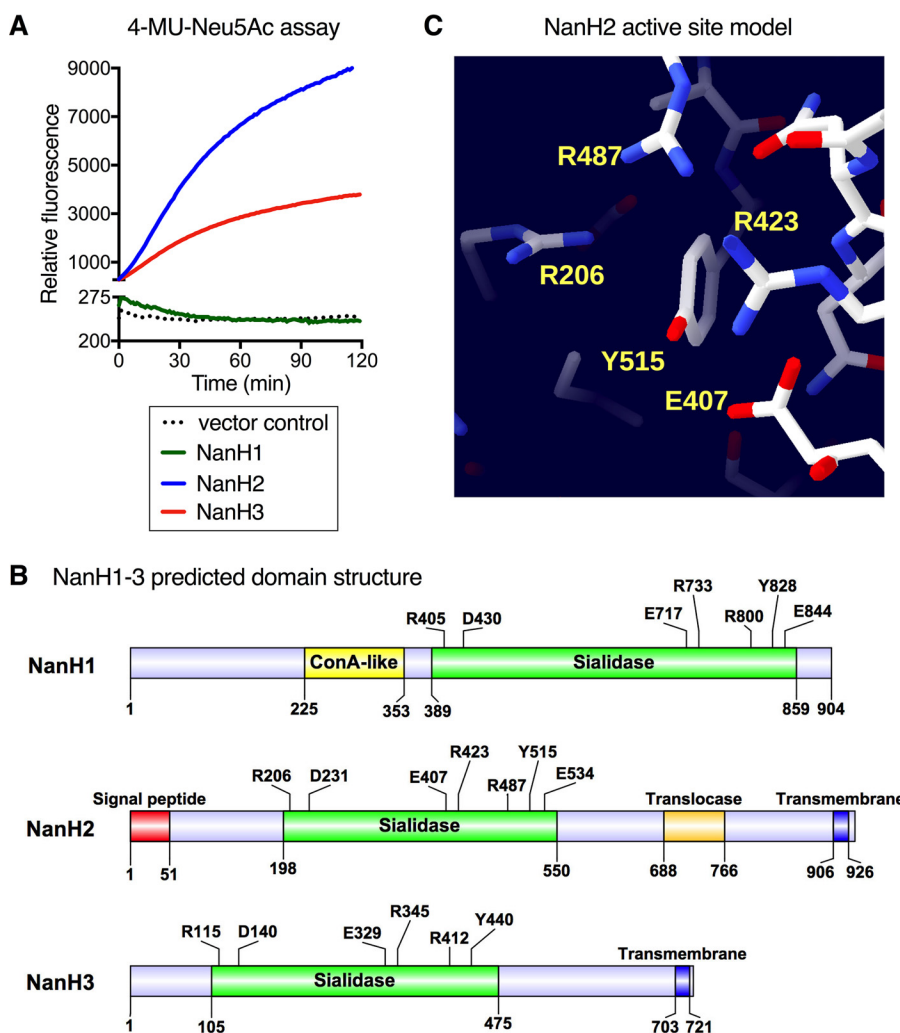
Figure 1. Sialidase activity and domain structure of *G. vaginalis* NanH1, NanH2, and NanH3. *A*, 4-MU-Neu5Ac assay of whole *E. coli* cultures expressing recombinant JCP8151B NanH1, NanH2, NanH3, or empty vector control. *B*, predicted domain structure of JCP8151B NanH1, NanH2, and NanH3. Putative catalytic residues are indicated above each protein. *ConA*, concanavalin A. *C*, JCP8151B NanH2 sialidase structural model showing conserved active-site residues, produced with Swissmodel and Deepview Swiss PDB viewer based on the sialidase crystal structure from *M. viridifaciens* (PDB code 1WCQ) (45).

helix but failed to identify an N-terminal signal peptide or other secretion signals.

Bacterial sialidase genes are often found near genes encoding proteins involved in sialic acid uptake and catabolism (46, 47). In JCP8151B, *nanH1* is found adjacent to such a gene cluster encoding putative enzyme activities involved in sialic acid foraging, including GlcNAc-6-phosphate deacetylase (*nagA*), a glucosamine-6-phosphate deaminase (*nagB*), three ABC transporter subunits, and an *N*-acetylneuraminate lyase (*nanA*). In contrast, the genes flanking *nanH2* and *nanH3* appear to encode functions unrelated to glycan degradation or sialic acid catabolism (Fig. S3).

NanH2 and NanH3 act on sialoglycans relevant to the vaginal mucosa

Given the high sialidase activity of NanH2 and NanH3 against 4-MU-Neu5Ac in *E. coli* cultures, we next investigated the substrate specificity of these two proteins. Previous analyses of the sialidase activity in vaginal specimens from women with BV demonstrated a broad capacity for cleaving sialic acids in many different contexts, including $\alpha2$-3– and $\alpha2$-6–linked sialic acids present within both *N*-linked and *O*-linked glycan substrates (48). To determine whether NanH2 and NanH3 could account for this broad range of activity, we incubated preparations of the recombinant sialidases with several different substrates and measured the resulting free sialic acids by fluorescent derivatization and HPLC. To ensure similar amounts of activity between NanH2 and NanH3, dilutions of the two enzymes were normalized in 4-MU-Neu5Ac assays before each experiment. NanH2 and NanH3 both cleaved 3′- and 6′-sialyllactose, and neither enzyme exhibited a marked preference for one linkage over the other (Fig. 2). Both sialidases also cleaved sialic acid from secretory IgA (SIgA, Fig. 3A), which contains mostly *N*-linked sialoglycans, as well as mucin from bovine submaxillary gland (BSM), which contains mostly *O*-linked sialoglycans (Fig. 3B). In addition to removing Neu5Ac from these substrates, NanH2 and NanH3 were also effective in liberating *N*-glycolylneuraminic acid from BSM (Fig. 3C). We also assessed the ability of NanH2 and NanH3 to cleave $\alpha2$-3–linked sialic acids on the capsular polysaccharide
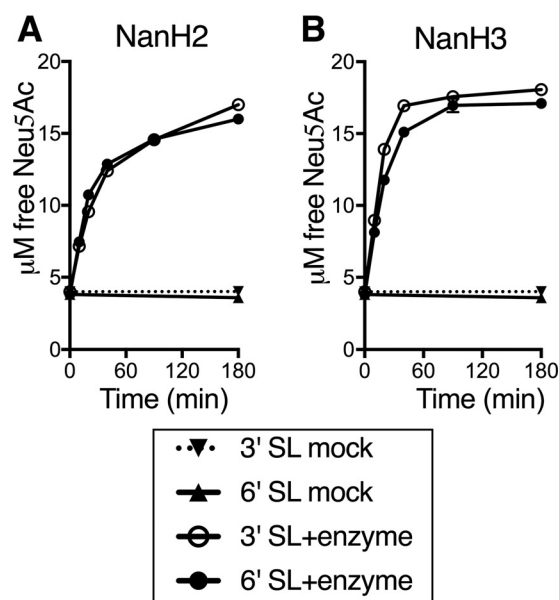
Figure 2. NanH2 and NanH3 cleave 3′- and 6′-sialyllactose. *A* and *B*, preparations of NanH2 (*A*) or NanH3 (*B*) were mixed with 20 μM 3′-sialyllactose (*3′ SL*) or 6′-sialyllactose (*6′ SL*) and incubated at 37 °C for 3 h. At each time point, aliquots of the reaction mixtures were removed and analyzed by DMB-HPLC for free Neu5Ac.

Figure 3. NanH2 and NanH3 act on *N*-linked and *O*-linked sialoglycan substrates relevant to the host mucosa. *A–D*, sialoglycans included SIgA from human colostrum (*A*), BSM (*B* and *C*), and GBS whole cells (*D*). Preparations of NanH2 or NanH3 were mixed with each substrate and incubated at 37 °C for 4 h. AUS was used as a positive control. At each time point, aliquots of the reaction mixtures were removed and analyzed for Neu5Ac or N-glycolylneuraminic acid (*Neu5Gc*) by DMB-HPLC.
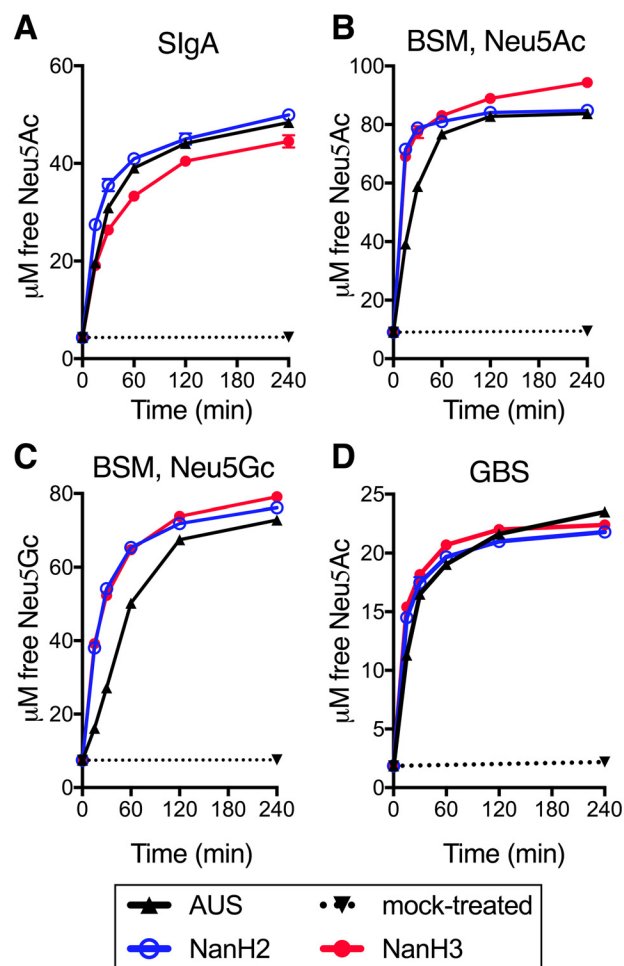
of the vaginal bacterium group B *Streptococcus* (GBS). GBS is an important potential pathogen during pregnancy (49) and is often found colonizing the vagina in women with high Nugent scores (50), a method of laboratory diagnosis for BV (23). As with the other sialoglycan substrates, both NanH2 and NanH3 cleaved Neu5Ac from the GBS capsule (Fig. 3*D*). Based on these experiments, we conclude that *G. vaginalis* NanH2 and NanH3 can cleave sialic acids from several substrates relevant to the vaginal mucosa.

NanH2 is more effective than NanH3 at cleaving 9-O-acetylated sialic acid

Sialic acids on mucosal sialoglycans may be modified with *O*-acetyl esters at carbon positions 7, 8, and 9. *O*-acetylated sialic acids are known to resist the action of many sialidases, with the extent of the inhibition depending on the position of the modification (51–53). To determine whether NanH2 or NanH3 could cleave *O*-acetylated sialic acids, we incubated each sialidase with BSM or intact GBS cells, both of which contain sialoglycan chains modified with 7-*O*- and 9-*O*-acetyl esters. For these experiments, we used a GBS strain with high levels of *O*-acetylation resulting from an active site mutation in the *neuA* esterase gene (54). In time course assays, NanH2 and NanH3 released similar quantities of the 7-*O*-acetylated sialic acid *N*-acetyl-7-*O*-acetylneuraminic acid (Neu5,7Ac$_2$) from both substrates (Fig. 4, *A* and *B*). However, NanH2 was far more effective than NanH3 in releasing the 9-*O*-acetylated sialic acid *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) from both BSM and GBS cells (Fig. 4, *C* and *D*).

Activity of purified NanH1

In a study characterizing the NanH1 and NanH2 sialidases of *B. longum* subsp. *infantis*, Sela *et al.* (43) reported that NanH1 was much less active than NanH2 against α2-3 and α2-6

sialyllactosyl 4-methylumbelliferol and against a library of *p*-nitrophenol–tagged sialylgalactosides. However, when used at a 80- to 160-fold higher concentration than NanH2, purified *B. longum* NanH1 exhibited detectable activity against these substrates. Therefore, in a final attempt to examine possible sialidase activity of *G. vaginalis* NanH1, we used nickel purification to isolate large quantities of His$_6$-tagged JCP8151B NanH1. When used at 10 μg/ml in 4-MU-Neu5Ac assays, NanH1 was able to cleave the fluorescent substrate at low levels *in vitro* (Fig. 5*A*).

We next wanted to compare the activity of purified recombinant NanH1 with that of purified NanH2 and NanH3. However, in our initial experiments, recombinant NanH2 was localized to the supernatant of *E. coli* cultures, whereas NanH3 was found in the cytoplasm, and neither protein could be isolated in significant quantities using nickel affinity resin. We surmised that the soluble fraction of each protein had lost its C-terminal transmembrane region and His$_6$ tag, whereas proteins retaining the tag were likely insoluble because of the hydrophobic transmembrane regions of both NanH2 and NanH3. Thus, we generated additional *nanH2* and *nanH3* constructs lacking the
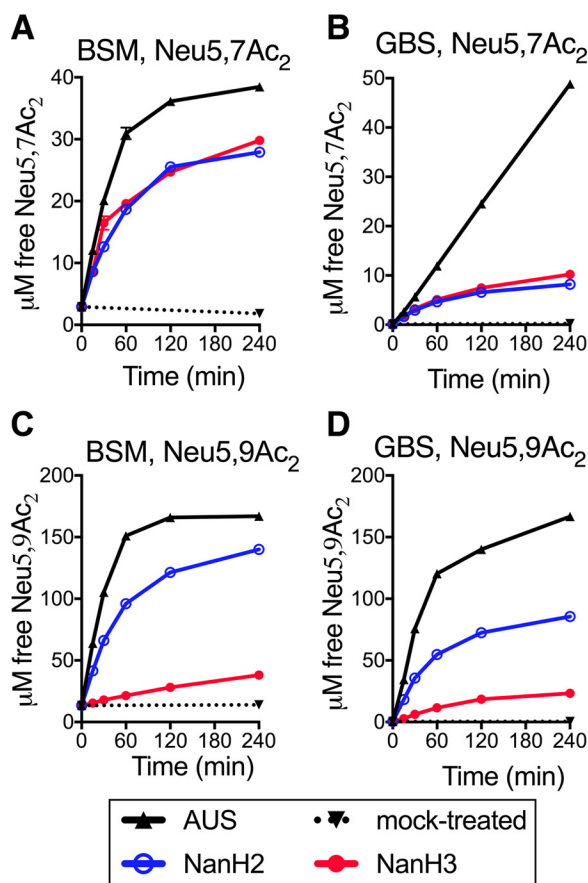
Figure 4. NanH2 and NanH3 release *O*-acetylated sialic acids. *A–D*, preparations of NanH2, NanH3, or AUS were incubated with BSM or GBS whole cells and incubated at 37 °C for 4 h. Release of BSM Neu5,7Ac$_2$ (*A*), GBS Neu5,7Ac$_2$ (*B*), BSM Neu5,9Ac$_2$ (*C*) or GBS Neu5,9Ac$_2$ (*D*) was determined by DMB-HPLC.

Figure 5. Sialidase activity of isolated recombinant NanH proteins. *A*, 4-MU-Neu5Ac assay on JCP8151B NanH1 isolated from *E. coli* lysates. *B*, normalization of recombinant NanH1, NanH2, and NanH3 by SDS-PAGE and Coomassie staining. *MW*, molecular weight. *C*, end point assay showing release of sialic acids from 4-MU-Neu5Ac, BSM, colostrum SIgA, and sialyllactose in the presence of NanH1, NanH2, or NanH3. Samples were incubated at 37 °C for 2 h, and free sialic acids were measured by DMB-HPLC.

transmembrane regions but retaining the C-terminal His$_6$ tags. The putative signal sequence of NanH2 was also removed, allowing all three proteins to be isolated from the *E. coli* cytoplasm using the same method. The truncated versions of NanH2 and NanH3 were expressed in *E. coli*, and protein was enriched by nickel purification similar to NanH1. SDS-PAGE followed by staining with Coomassie Blue revealed expected bands of ~100 kDa for NanH1 and NanH2 and 77 kDa for NanH3 (Fig. 5*B*). Next we incubated the three sialidases with 4-MU-Neu5Ac, BSM, colostrum IgA, or sialyllactose and measured the liberation of free sialic acids by DMB-HPLC. Although the amount of each enzyme was roughly equivalent, as determined by Coomassie staining and BCA assays, only NanH2 and NanH3 released large quantities of sialic acid from these substrates (Fig. 5*C*). NanH1 released little Neu5Ac from 4-MU-Neu5Ac, BSM, or colostrum IgA and was completely inactive against BSM Neu5,7Ac$_2$, BSM Neu5,9Ac$_2$, or sialyllactose under the conditions tested. Because some sialidases require divalent cations for full activity, we also performed sialic acid release assays on 4-MU-Neu5Ac and colostrum IgA in the presence of 1 mM CaCl$_2$ or MgCl$_2$, but NanH1 showed no increase in activity in the presence of divalent cations (Fig. S4). Additional experiments ruled out the possibility that NanH1 preparations were contaminated with *N*-acetylneuraminate lyase activity (present in all *E. coli* strains), which could theo-

retically degrade liberated Neu5Ac and mask sialidase activity in NanH1-treated samples analyzed by HPLC.[3]

Release of Neu5,9Ac$_2$ by G. vaginalis strains suggests NanH2 expression

Given the *in vitro* data showing that NanH2 was better than NanH3 at cleaving 9-*O*–acetylated sialic acids, we hypothesized that if *nanH2* is expressed in *G. vaginalis*, then NanH2-encoding strains might be better able to liberate 9-*O*–acetylated sialic acids than strains encoding only NanH3. To test this hypothesis, we used NCBI sequence data to identify *G. vaginalis* strains encoding NanH2 (JCP8151A, JCP8151B, JCP8522, and GED7760B; designated "*nanH2+*") and strains encoding NanH3 but not NanH2 (JCP7276, JCP8017, JCP8066, and JCP8070; designated "*nanH3* only"). A *nanH3* gene is also present in three of the four *nanH2+* strains (JCP8151A, JCP8151B, and JCP8522), whereas GED7760B is *nanH3*-negative. *G. vaginalis* isolates were grown overnight in NYCIII medium supplemented with BSM as a source of *O*-acetylated sialic acids. Supernatants from spent cultures were then analyzed by DMB-HPLC to determine how much of each sialic acid species was liberated. A separate portion of each sample was treated with *Arthrobacter ureafaciens* sialidase (AUS) to release and measure sialic acids that remained bound to gly-

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[3] L. S. Robinson, unpublished data.
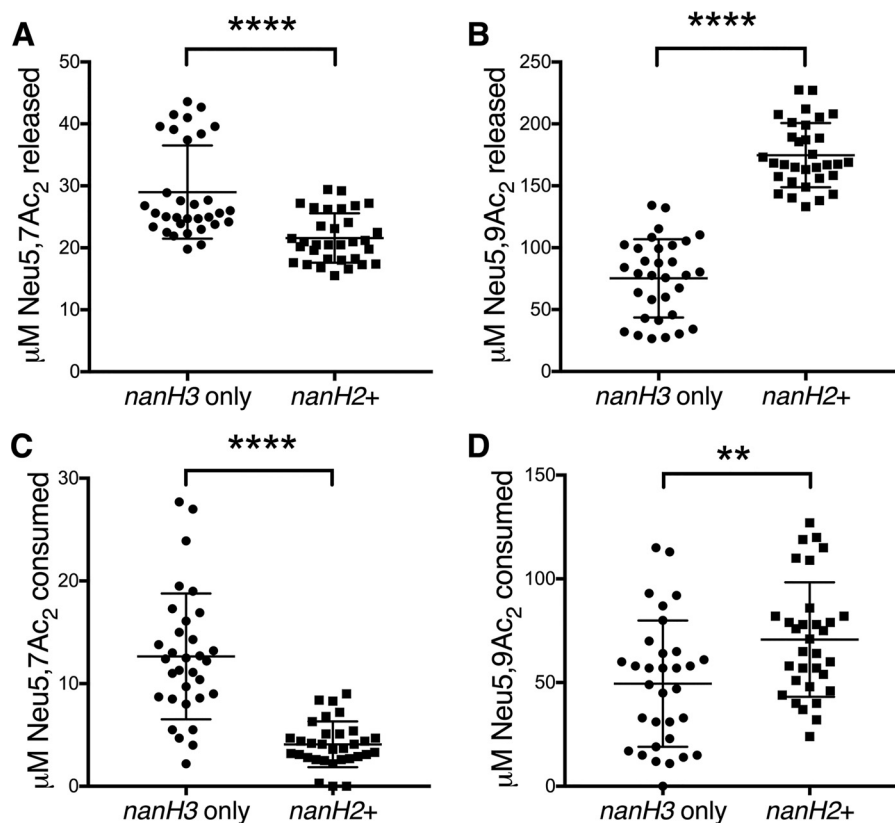
Figure 6. Release and consumption of acetylated sialic acids by *nanH3*-only or *nanH2*+ strains of *G. vaginalis*. Following overnight growth of *G. vaginalis* clinical isolates in NYCIII medium supplemented with BSM, supernatants were analyzed by DMB-HPLC for free and total acetylated sialic acids. Released Neu5,7Ac$_2$ (*A*) and Neu5,9Ac$_2$ (*B*) were calculated by subtracting the concentration of bound sialic acid in the spent culture supernatants from that of the uninoculated medium control. Consumed Neu5,7Ac$_2$ (*C*) and Neu5,9Ac$_2$ (*D*) were calculated by subtracting the concentration of total sialic acid in each culture supernatant from the total sialic acid in the uninoculated medium control. ****, $p < 0.0001$; **, $p < 0.008$ in two-tailed Mann–Whitney tests.

can substrates following overnight growth while preserving their acetylation patterns (55). As expected, all eight *G. vaginalis* strains were able to release and consume Neu5Ac and *N*-glycolylneuraminic acid from the medium (Fig. S5). The *nanH3*-only strains released and consumed significantly more Neu5,7Ac$_2$ than the *nanH2*+ strains (Fig. 6, *A* and *C*). In contrast, and consistent with the *in vitro* enzyme data, *nanH2*+ strains released and consumed more Neu5,9Ac$_2$ than the *nanH3*-only strains (Fig. 6, *B* and *D*), supporting the interpretation that NanH2 was expressed and secreted under these conditions.

The presence of nanH2 or nanH3 accounts for sialidase activity in G. vaginalis cultures

In light of our findings that recombinant NanH1 had only weak sialidase activity *in vitro*, we investigated the possibility that *nanH2* and *nanH3* may better account for culture sialidase activity among the 34 *G. vaginalis* isolates in our strain repository (Table 1). Many of these strains have been tested previously for sialidase activity (34). Additional strains were tested for activity against 4-MU-Neu5Ac in kinetic assays. In total, 19 strains were sialidase-negative, and 15 were sialidase-positive (Fig. S6A). Next we used NCBI sequence data to identify *nanH1*, *nanH2*, and *nanH3* in the genomes of published isolates. PCR with primers specific for each *nanH* gene was used to determine their presence or absence in

strains that had not been sequenced previously as well as in sequenced strains whose genomes are not closed (Fig. S6B). In each case where PCR and genome sequences were available, we found that both strategies gave complimentary results. None of the 19 sialidase–negative strains encoded either NanH2 or NanH3, although most of them possessed *nanH1* (Table 2). Conversely, all 15 sialidase–positive strains encoded NanH2, NanH3, or both. We therefore conclude that *nanH2* and *nanH3* account for the sialidase activity observed in cultured *G. vaginalis*.

The presence of nanH2 or nanH3 in vaginal specimens from women with and without bacterial vaginosis

In a final set of experiments, we wanted to find out whether the presence of *nanH2* or *nanH3* in vaginal samples might also have diagnostic applications. To test this, we used genomic DNA isolated from a subset of a previously described cohort of women (56) as a template for PCR as described under "Experimental procedures." We evaluated a total of 67 specimens from women with BV (Nugent 7–10, $n = 21$), "no BV" (Nugent 0–3, $n = 23$), and intermediate phenotypes (Nugent 4–6, $n = 24$). We performed two distinct PCR assays for *nanH2* and *nanH3* with appropriate controls, including no template or genomic DNA from *G. vaginalis* isolates with neither *nanH2* nor *nanH3* (JCP8108), *nanH2* only (GED7760B), or *nanH3* only (JCP8066). We then categorized each sample in a blinded fashion by the

Table 1
Strains, plasmids, and primers used in this study

Name	Description or sequence	Source or reference
Strains		
G. vaginalis JCP7275	Clinical isolate	34, 88
G. vaginalis JCP7276	Clinical isolate	34, 88
G. vaginalis JCP7659	Clinical isolate	34, 88
G. vaginalis JCP7672	Clinical isolate	34, 88
G. vaginalis JCP7719	Clinical isolate	34, 88
G. vaginalis JCP8017	Clinical isolate	34, 88
G. vaginalis JCP8066	Clinical isolate	34, 88
G. vaginalis JCP8070	Clinical isolate	34, 88
G. vaginalis JCP8108	Clinical isolate	34, 88
G. vaginalis JCP8151A	Clinical isolate	34, 88
G. vaginalis JCP8151B	Clinical isolate	34, 88
G. vaginalis JCP8481	Clinical isolate	34, 88
G. vaginalis JCP8522	Clinical isolate	34, 88
G. vaginalis GED7275B	Clinical isolate	89
G. vaginalis GED7760B	Clinical isolate	89
G. vaginalis ATCC 14019	Type strain	ATCC
G. vaginalis ATCC 49145	Type strain	ATCC
G. vaginalis NML 060420	Clinical isolate	90
G. vaginalis UMB0105	Clinical isolate	91
G. vaginalis UMB0233	Clinical isolate	91
G. vaginalis 007/03D MASH	Clinical isolate	92
G. vaginalis 1500E	Clinical isolate	92
G. vaginalis 61/19V5	Clinical isolate	92
G. vaginalis 64/20B	Clinical isolate	92
G. vaginalis 64/20LIT	Clinical isolate	92
G. vaginalis 505v3	Clinical isolate	This study
G. vaginalis 508v2	Clinical isolate	This study
G. vaginalis 514v5	Clinical isolate	This study
G. vaginalis 525v8	Clinical isolate	This study
G. vaginalis 530v3	Clinical isolate	This study
G. vaginalis 533v3	Clinical isolate	This study
G. vaginalis 589v6	Clinical isolate	This study
G. vaginalis 600v6	Clinical isolate	This study
G. vaginalis 667v6	Clinical isolate	This study
E. coli Top10	Cloning strain	Invitrogen
E. coli BL21(DE3)	Expression strain	Scott Hultgren
E. coli LSR4	MG1655 Δ*nanA*	75
Plasmids		
pET101/D-Topo	*E. coli* expression vector	Invitrogen
pGvagSia	ATCC14019 *nanH1* in pET101/D-Topo	This study
pTrc99A	*E. coli* expression vector	Scott Hultgren
pLR34	JCP8151B *nanH2 his* in pTrc	This study
pLR35	JCP8151B *nanH3 his* in pTrc	This study
pLR37	JCP8151B *nanH1 his* in pTrc	This study
pLR43	JCP8151B *nanH2 his* truncate in pTrc	This study
pLR44	JCP8151B *nanH3 his* truncate in pTrc	This study
Primers		
G. vag tuf F1	ATGGCAAAGGAAAAGTACGAGCG	This study
G. vag tuf R1	TCAAGCCCTCTTCCATTGCG	This study
G. vag sia f	CACCATGGAACGTCGTTCAACG	This study
G. vag sia R	AATGTCTCTTCCATGTTGGC	This study
8151B nanH1 F Nco	TTTTCCATGGCAGAACGTCGTTCAACGAAGACG	This study
8151B nanH1 his R Bam	TTTTGGATCCTTAGTGGTGGTGGTGGTGGTG GTGAATGTCTCTTCCATTTTGGCTCC	This study
8151B nanH2 F Nco	CCATGGCAATTTTTACAATGCTGTGGAATTCATGT	This study
8151B nanH2 his R Bgl2	TTTTAGATCTTAGTGGTGGTGGTGGTGGTGTGCAATCTTATTGCGCATTGC	This study
8151B nanH3 F Nco	AAAACCATGGCAACTGTTTGGCCAACTAAAAGTAATCTCAC	This study
8151B nanH3 his R Pst	TTTCTGCAGTTAGTGGTGGTGGTGGTGGTGATATTTCATATTTTTTAATTTCATTAATGCTGTAGAGATAGC	This study
8151B nanH2 A51F Nco	TTTTCCATGGCTACTTCAGAAAAATCCAGTAAACC	This study
8151B nanH2 S908 his R Bgl2	TTTTAGATCTTAGTGGTGGTGGTGGTGGTGTGAACTTCCCGTTGAAACTTTAC	This study
8151B nanH3 T702 his R Pst	TTTCTGCAGTTAGTGGTGGTGGTGGTGGTGAGTTGTGTTGATTCCAGTTTTTTGC	This study
G. vag sia universal F3	CGAGGCAAAATGATTAACGAAGG	This study
G. vag sia universal R1	TTGGCTCCTTTCAGTTCGC	This study
G. vag nanH2 qPCR F	AGGAGTGCGTATGCGTAGAAG	This study
G. vag nanH2 qPCR R	CCGCACTGCTGAGTTTCAC	This study
G. vag nanH3 qPCR F	CAGTTCCAATGGAAGTGTGC	This study
G. vag nanH3 qPCR R	AGCATCTGGGAATGCTCTTG	This study
G. vag nanH2 MP F1	CAGCGTTGATCAGTTGGATAAATC	This study
G. vag nanH2 MP R1	AAGCATTACAACCGCAAGAAC	This study

presence or absence of *nanH2* or *nanH3* bands in at least two independent PCR experiments. Women were then classified as being positive for either *nanH2* or *nanH3* or as having neither *nanH2* nor *nanH3*. Comparison of these results in relation to Nugent categories (0–3, 4–6, and 7–10) revealed a striking pattern. Although 17 of 21 women with BV were positive for *nanH2* or *nanH3*, only 5 of 23 *Lactobacillus*-dominant samples showed the presence of *nanH2* or *nanH3*. Interestingly, among the intermediate samples, 13 of 24 were positive for *nanH2* or *nanH3* (χ^2 p = 0.0004). Considering women with BV or no BV,

Table 2

The presence of *nanH2* or *nanH3* corresponds with culture sialidase activity in *G. vaginalis* clinical isolates

The ability of each strain to produce sialidase activity is shown, as is the presence or absence of the three *nanH* genes. There are five (boxed) genotype–phenotype relationships.

Strain	Sialidase activity	*nanH1*	*nanH2*	*nanH3*
GED7760B	+	+	+	-
JCP8151A	+	+	+	+
JCP8151B	+	+	+	+
JCP8522	+	+	+	+
600v6	+	+	+	+
GED7275B	+	+	-	+
JCP7276	+	+	-	+
JCP7659	+	+	-	+
JCP7672	+	+	-	+
JCP7719	+	+	-	+
JCP8017	+	+	-	+
JCP8066	+	+	-	+
JCP8070	+	+	-	+
589v6	+	+	-	+
667v6	+	+	-	+
JCP7275	-	+	-	-
JCP8108	-	+	-	-
ATCC 14019	-	+	-	-
ATCC 49145	-	+	-	-
NML 060420	-	+	-	-
UMB0105	-	+	-	-
UMB0233	-	+	-	-
1500E	-	+	-	-
007/03Dmash	-	+	-	-
61/19V5	-	+	-	-
505v3	-	+	-	-
508v2	-	+	-	-
514v5	-	+	-	-
525v8	-	+	-	-
530v3	-	+	-	-
533v3	-	+	-	-
64/20LIT	-	-	-	-
64/20B	-	-	-	-
JCP8481	-	-	-	-

this PCR test had 80.95% sensitivity (95% confidence interval, 60–92.33) and 78.26% specificity (95% confidence interval, 58.1–90.34) compared with the Nugent score for BV diagnosis. Thus, molecular testing of *nanH2* and *nanH3* may have diagnostic potential.

Discussion

Sialidase activity is consistently observed in the vaginal fluid of women with BV, and the enzyme could benefit vaginal microorganisms in several ways. First, sialidases provide a carbon source to *Gardnerella* by liberating terminal sialic acid residues from vaginal glycoproteins, thus allowing their uptake and catabolism (34). Second, sialidase activity in the vagina may reveal cryptic receptors for adhesins and toxins by uncapping underlying sugars, such as galactose residues, as they do for

bacteria in the mouth and airway (57–59). Third, vaginal sialidases could alter the physical properties of mucus, allowing more intimate association with the epithelium, as occurs with viral sialidases (60). Finally, sialidase activity could have immunomodulatory consequences for receptors on mammalian cells. Sialic acids serve as ligands for receptors called Siglecs expressed on innate immune cells, and removal of sialic acids changes the inflammatory potential of these immune cells (61, 62). One or more of these mechanisms could promote the growth or colonization of sialidase-expressing vaginal bacteria, including *Prevotella* and *Bacteroides* as well as *G. vaginalis* (9, 10, 19). In addition to influencing interactions between sialidase producers and the host, such functions could predispose the vaginal microbiome toward dysbiosis by shifting the physical and/or immunological milieu to favor BV-associated bacteria.

Here we report three lines of evidence that NanH2 and NanH3 (and not sialidase A, which we renamed NanH1) are the major contributors to sialidase activity in *G. vaginalis* cultures. First, NanH2 and NanH3 were much more similar in sequence to the enzymatically active *B. longum* sialidase NanH2 than was NanH1, whereas *G. vaginalis* NanH1 was more similar to the relatively inactive (43) *B. longum* NanH1. Second, although *G. vaginalis* NanH1 had minimal activity against numerous sialic acid substrates even at high concentrations, NanH2 and NanH3 were able to cleave sialic acids in many different molecular contexts, such as α2-3– and α2-6–linked sialic acids as well as *N*- and *O*-linked sialoglycans found on SIgA and mucin. Thus, both NanH2 and NanH3 have a sufficient range of substrate specificity to account for the full breadth of sialidase activities observed previously in BV specimens (48). Finally, among 34 *G. vaginalis* clinical isolates, the ability to cleave sialic acids corresponded with the presence of *nanH2* or *nanH3* in 100% of cases, whereas the presence of *nanH1* in the genome was observed in many sialidase-negative strains.

We also demonstrate that a preliminary PCR assay targeting *nanH2*/*nanH3* in human vaginal specimens performs reasonably well in BV diagnosis based on the gold standard method of laboratory diagnosis (based on the Nugent method). Our data show that PCR detection of *nanH2*/*nanH3* in human vaginal specimens has ~80% sensitivity and specificity to predict BV diagnosis. Nugent scoring is widely used for laboratory BV diagnosis, and it has brought the field forward in several important ways. However, it is a crude metric that many in the field are trying to replace with more objective and quantitative molecular methods (24, 63, 64). Because the PCR assays presented here focus on genes that likely encode virulence functions, they may have advantages over Nugent scoring in the potential prediction of adverse gynecological and obstetric outcomes. Previous studies have shown that vaginal sialidase activity is associated with greater risks of pregnancy loss, preterm birth, and placental infection (16–18).

NanH2 and NanH3 are similar to each other and other functionally characterized sialidases in many ways, but the two proteins also have some important differences. For example, most bacterial sialidases are either freely secreted into the extracellular environment or secreted and retained at the cell surface, where they can access host sialoglycans (65). Sialidases typically

have an N-terminal signal peptide (66) that directs secretion through the Sec translocase (67). All four *G. vaginalis* NanH2 proteins in the NCBI database have a 51-amino acid signal peptide, as predicted by SignalP, a program that identifies putative signal sequences and distinguishes them from likely transmembrane regions (68), and are thus likely to be extracellular. Consistent with this prediction, when full-length JCP8151B NanH2 was expressed in *E. coli*, the majority of the sialidase activity was found in the culture supernatant. The NanH2 amino acid sequence also contains a 78-residue PRK00708 domain downstream of the sialidase domain. PRK00708 domains are associated with Sec-independent translocases (69), suggesting that NanH2 secretion may involve additional mechanisms besides that provided by the Sec machinery. In contrast, the JCP8151B NanH3 protein does not have a predicted signal peptide, nor do the NanH3 proteins in the other *nanH2+*, *nanH3+* strains, JCP8151A and JCP8522. However, the NanH3 proteins in most *G. vaginalis* strains lacking *nanH2* have longer N termini that include signal peptides according to SignalP, suggesting that NanH3 may be secreted in some *G. vaginalis* strains and intracellular or unexpressed in others.

Both NanH2 and NanH3 have predicted transmembrane α-helices at their C termini. Our initial attempts to purify recombinant NanH2 and NanH3 from *E. coli* involved full-length, C-terminal His-tagged versions of the two proteins. However, we were unable to isolate significant quantities of either enzyme from the soluble fraction of *E. coli* lysates or supernatants using Ni^{2+} affinity resin despite the presence of sialidase activity in these fractions (and its absence from fractions derived from *E. coli* with vector alone). We suspect that the full-length polypeptides were insoluble because of the C-terminal hydrophobic α-helices but that some percentage of molecules was proteolytically cleaved upstream of the transmembrane domain, separating them from the His_6 tag and releasing them into solution. Indeed, plasmids encoding His-tagged NanH2 or NanH3 lacking the C-terminal transmembrane regions yielded soluble protein that readily bound to nickel affinity resin. These findings suggest that, following secretion, some portion of membrane-bound NanH2 and NanH3 may be released into the environment because of proteolytic sensitivity upstream of their putative transmembrane α-helices. This is consistent with our previous observation that a significant fraction of *G. vaginalis* sialidase activity is found in culture supernatants (34). Similarly, the membrane-bound NanA sialidase of *Streptococcus pneumoniae* can be proteolytically cleaved upstream of its LP*X*TG cell-anchoring motif without appreciable loss of activity (70).

The one significant functional difference we noted between NanH2 and NanH3 was that NanH2 was much more effective than NanH3 at cleaving 9-*O*–acetylated sialic acids on BSM and GBS whole cells. This *in vitro* difference was also evident *in vivo*, as *G. vaginalis* strains encoding NanH2 were better able to release and consume 9-*O*–acetylated sialic acids than strains encoding NanH3 only. Although GBS is a common vaginal commensal and often has high levels of *O*-acetylated sialic acids, elutions from human vaginal swabs had little, if any, 9-*O*–

acetylated sialic acid.[4] However, the ability to liberate 9-*O*–acetylated sialic acids may help *nanH2+ G. vaginalis* strains to colonize niches outside of the vagina. For example, in men, women, and children, *G. vaginalis* has been reported to be an inhabitant of the distal gastrointestinal tract (anal swabs) (71–73), where 9-*O* acetylation of sialic acid reaches high levels (colon, rectum, and anus) (52, 53). Given that 9-*O* acetylation impedes the activity of many bacterial sialidases (74, 75), NanH2-expressing strains of *G. vaginalis* may have a competitive advantage in the rectum because of an expanded capacity to forage on 9-*O*–acetylated sialic acids.

NanH3 may be the protein reported by von Nicolai *et al.* (76), who described a *G. vaginalis* membrane-bound sialidase that was released into the soluble fraction of bacterial suspensions by sonication and was active against a broad range of substrates, including sialyllactose, fetuin, and BSM. Gel chromatography revealed a molecular mass of around 75 kDa, which is only slightly smaller than full-length *G. vaginalis* JCP8151B NanH3 (80 kDa) and almost identical in size to NanH3 lacking its putative transmembrane helix (77 kDa, Fig. 5*B*). To our knowledge, the *G. vaginalis* strain used in this previous study has not been sequenced, precluding efforts to confirm the presence of *nanH3* in its genome, but the similarities in molecular mass, cellular localization, and enzymatic activity suggest that the purified protein was indeed NanH3.

Our data strongly suggest that NanH1 is not responsible for the sialidase activity observed in *G. vaginalis*. At least three other research groups have also found that the presence of *nanH1* does not predict culture sialidase activity in many *G. vaginalis* strains (37, 39, 42). In particular, one study reported that fewer than half of 77 *nanH1+* strains produced detectable sialidase activity (42). Nevertheless, to our knowledge, *nanH1* is present in all sialidase-positive strains of *G. vaginalis* examined to date. A recent investigation speculated that the lack of sialidase activity observed in many *nanH1+* strains could be due to transcriptional regulation of *nanH1* (41). Although not an unreasonable hypothesis, we were unable to find a published analysis of transcription or upstream sequence differences to support this idea.

Interestingly, the apparent deficit in NanH1 sialidase activity is not easily explained by its amino acid sequence alone. The protein contains five aspartate boxes and all seven catalytic residues typical of bacterial sialidases as well as the conserved RIP motif at the N terminus of the sialidase domain. Furthermore, the persistence of an intact *nanH1* ORF in at least 20 distinct *G. vaginalis* isolates points toward an important function for this gene. NanH1 lacks a predicted N-terminal signal sequence, implying intracellular localization. In mammals, intracellular sialidases function in metabolism and regulation of inflammatory states (61). In some bacteria (*e.g. Bifidobacterium*), these sialidases are thought play a purely metabolic role, such as cleavage of oligosaccharides after they are transported into the cytoplasm (43). Alternatively, intracellular sialidases sometimes escape into the extracellular environment through cell lysis. For example, the *Clostridium perfringens* NanH sialidase

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[4] A. L. Lewis, unpublished data.

lacks a predicted signal sequence and accumulates intracellularly in log phase cultures (77) but can be found in the supernatant of death phase cultures. This mechanism is especially plausible in the case of *G. vaginalis* because of its thin cell wall (78).

The NanH1 sialidase of *B. longum* may provide insight into the function of *G. vaginalis* NanH1. The two proteins are 44% identical, and both have an N-terminal concanavalin A–like domain while lacking discernable secretion sequences and membrane-anchoring regions (43). Concanavalin A is a lectin, and therefore the N-terminal domain is likely involved in substrate binding. Also, both *nanH1* open reading frames are found adjacent to a predicted catabolic gene cluster. Consistent with our finding that *G. vaginalis* NanH1 is a relatively inactive sialidase, *B. longum* NanH1 was shown to have a 175-fold lower k_{cat} than *B. longum* NanH2 in the presence of $\alpha 2$-6–linked sialyllactosyl 4-MU and a 140-fold lower k_{cat} in the presence of $\alpha 2$-3–linked sialyllactosyl 4-MU (43). However, sialidases containing lectin domains can exhibit a K_m for polyvalent substrates that is 100-fold lower than their K_m for monovalent derivatives (79). Although JCP8151B NanH1 failed to release significant quantities of sialic acid from the two polyvalent substrates used in this study (colostrum IgA and BSM), it may be more active against a different polyvalent sialoside that is recognized by NanH1's putative lectin domain.

Several other Gram-positive bacteria encode multiple sialidases their genomes. *S. pneumoniae* has three sialidases, NanA, NanB, and NanC, all of which are secreted and have N-terminal lectin domains (80–83). *C. perfringens* also encodes three sialidases, NanH, NanI, and NanJ (84), and *Tannerella forsythia* encodes at least two sialidases, SiaHI and NanH (85). In each of these species, one sialidase accounts for the majority of the observed sialidase activity, and one sialidase is relatively inactive under the conditions tested (86, 87). Thus, *G. vaginalis* is not unique in having a sialidase homolog of unknown function. Future work should focus on defining the specific roles of the three *G. vaginalis* sialidases. Such work would be greatly aided by the development of effective genetic tools for this organism.

Experimental procedures

Sequence analyses

Conserved domains and active-site residues in protein sequences were predicted using RPS-BLAST (NCBI). Sequence alignments were performed with Clustal Omega (EMBL-EBI). SignalP and Phobius (EMBL-EBI) were used to identify putative signal peptides and transmembrane regions.

Strains and culture conditions

Strains, plasmids, and primers used in this study are shown in Table 1. Published strains of *G. vaginalis* were isolated as described previously (34). Unpublished strains were isolated from vaginal samples collected under University of Alabama IRB Protocol F140410006 (initial approval date, June 27, 2014). Briefly, samples were collected using BD BBL™ Liquid Amies Copan CultureSwab™ swabs and transported to the laboratory for processing the same day. Samples were streaked onto *Gardnerella* Selective Agar plates (Hardy Diagnostics) and incubated at 35 °C for 24–48 h in an atmosphere containing 5% CO_2. *G. vaginalis* colonies demonstrated yellowing of the medium surrounding the colonies. Three colonies per *Gardnerella* Selective Agar plate were used to inoculate BD HBT (Human Blood Tween™) bilayer plates and incubated at 35 °C in a 5% CO_2-enriched atmosphere for 24–48 h. Small white colonies surrounded by a β-hemolytic zone with a diffuse edge were selected for further purification and testing. BD BBL oxidase reagent droppers were used to perform an oxidase test on small, translucent, β-hemolytic colonies. These colonies were also subjected to a catalase test using anaerobic catalase reagent 15% hydrogen peroxide. Oxidase- and catalase-negative specimens were Gram-stained and examined at high power ($\times 100$) under a microscope. Gram-negative to Gram-variable pleomorphic coccobacilli were selected for cryopreservation. Following isolation, *G. vaginalis* strains were grown in a vinyl anaerobic chamber (Coy Products) at 37 °C in NYCIII medium (per liter: 15 g of proteose peptone no. 3, 3.75 g of yeast extract, 5 g of NaCl, 5 g of glucose, 17 ml of 1 M HEPES, 100 ml of heat-inactivate horse serum) or on HBT agar plates (Fisher Scientific). Both with our own freezer stocks as well as those from multiple other investigators, we occasionally found that strains thought to be completely isolated had multiple colony types when streaked on solid medium. This was especially true when the stock was grown under different conditions or on a different medium type than the original isolation employed. To ensure the purity of *G. vaginalis* strains, they were streaked from −80 °C stocks onto NYCIII or HBT bilayer plates and assessed visually for consistent colony size, color, and morphology. Colonies that varied by these criteria were picked and restreaked on fresh plates until uniformity was established. Then species identity was confirmed by colony PCR using the *G. vaginalis*–specific primers G. vag tuf F1 and G. vag tuf R1.

E. coli was grown while shaking in lysogeny broth (LB) at 37 °C or as indicated, with antibiotic selection where required. *E. coli* Top10 was used for cloning putative sialidase-encoding genes into pET101/D-Topo or pTrc99A as described below. *E. coli* BL21(DE3) or LSR4 (MG1655 *nanA* (75)) were used for protein expression. For plasmid maintenance in *E. coli*, ampicillin was used at 100 μg/ml (pTrc99A- and pET101/D-Topo-based plasmids). GBS was grown standing aerobically at 37 °C in Todd Hewitt broth with 5 μg/ml erythromycin to maintain the pDCerm plasmid.

DNA manipulations

PCR products for cloning were generated with Phusion polymerase (New England Biolabs) and a purified genomic DNA template. Restriction enzymes were also from New England Biolabs. The full-length *sialidase A/nanH1* (accession number ATJH01000171), *nanH2* (accession number ATJH01000056), and *nanH3* (accession number ATJH01000033) genes from *G. vaginalis* JCP8151B were amplified with the primer pairs 8151B nanH1 F Nco/8151B nanH1 his R Bam, 8151B nanH2 F Nco/8151B nanH2 his R Bgl2, and 8151B nanH3 F Nco/8151B nanH3 his R Pst, respectively. For sialidase purification, truncated *nanH2* and *nanH3* genes lacking the predicted C-terminal transmembrane segment (both genes) and N-terminal signal peptide (*nanH2* only) were amplified with the primer pairs 8151B nanH2 A51 F Nco/8151B S908 his R Bgl2 and 8151B

nanH3 F Nco/8151B nanH3 T702 his R Pst. The resulting amplicons were cloned into the pTrc99A expression vector.

Sialidase activity assays on bacterial isolates

Most *G. vaginalis* strains were grown in NYCIII broth as described above. Strains that could not be cultivated in NYCIII were grown on HBT agar plates. For strains grown in broth, stationary phase cultures were used. Strains grown on plates were scraped off the agar, suspended in 100 mM sodium acetate buffer (pH 5.5), and adjusted to an A_{600} of 2.0. 20 μl of each sample was mixed with 100 μl of 100 mM sodium acetate buffer (pH 5.5) containing 300 μM 2-(4-methylumbelliferyl)-*N*-acetyl-neuraminic acid (4-MU-Neu5Ac, Gold Bio) in a black polypropylene assay plate (Eppendorf). Fluorescence was measured at an excitation of 365 nm (bandwidth, 9 nm) and an emission of 440 nm (bandwidth, 20 nm) every 60 s for 2 h in a Tecan Infinite M200 plate reader at 37 °C. For the experiment presented in Fig. S6, enzyme activity was calculated from the linear portion of each curve and expressed as the change in relative fluorescence units. *E. coli* BL21(DE3) expressing JCP8151B *nanH1*, *nanH2*, or *nanH3* was inoculated into 2 ml of LB containing 200 μM IPTG (Gold Biotechnology) and grown while shaking overnight at room temperature. The next day, cultures were tested for activity as above, except that 20 mM sodium acetate buffer was used. To confirm NanH1-His$_6$ expression, bacteria were lysed and analyzed by Western blotting and detection with an anti-His$_6$ mAb (Covance).

Crude recombinant NanH2 protein preparation

In cultures of *E. coli* BL21(DE3) expressing full-length NanH2 from pLR34, high sialidase activity (4-MU-Neu5Ac hydrolysis) was detected in the culture supernatant, whereas culture supernatants from BL21(DE3) containing the empty vector had no such activity. Thus, for crude NanH2 protein preparations, *E. coli* was grown while shaking in LB overnight at room temperature, and cell-free supernatants were prepared by centrifuging cultures at 12,000 × g for 10 min and passing the supernatant through a 0.22-μm filter.

Crude recombinant NanH3 protein preparation

Full-length NanH3 was primarily intracellular when expressed in *E. coli*, so clarified whole-cell lysates were used as a source of recombinant NanH3. Although *E. coli* does not encode its own sialidase, it does encode an intracellular sialate lyase (NanA) that hydrolyzes free sialic acid to *N*-acetylman-nosamine. In many experiments, we measured the generation of free sialic acid to detect sialidase activity. To prevent breakdown of free sialic acid when liberated by NanH3-containing *E. coli* lysates, the *nanH3* expression plasmid pLR35 (and a parallel empty vector control) was transformed into an *E. coli* strain lacking the *nanA* gene (LSR4) (75). Briefly, clarified *E. coli* whole-cell lysates were prepared as follows. Bacteria were grown while shaking in 200 ml LB broth at 37 °C to an A_{600} of 1.0. After addition of 200 μM IPTG, bacteria were incubated while shaking overnight at room temperature. Then cells were pelleted, washed in 30 ml PBS, resuspended in 10 ml of lysis buffer (50 mM NaH$_2$PO$_4$ (pH 7.4), 300 mM NaCl, and 10 mM imidazole), and sonicated five times for 10 s each in a Sonic

Dismembrator (Fisher Scientific) at 35% amplitude on ice. The cell lysate was clarified by centrifuging three times (with transfer of the supernatant to fresh tubes) at 15,000 × g for 10 min.

Enzyme purification

For expression of NanH1, BL21(DE3) cells carrying JCP8151B *nanH1-His$_6$* in pTrc99A were grown while shaking in 800 ml of LB at 37 °C to an OD of 0.5 and then induced with 1 mM IPTG for 4 h at 37 °C. Truncated NanH2 and NanH3 (lacking the transmembrane segments of both proteins and the signal sequence of NanH2) were expressed similarly in BL21(DE3), except cultures were induced with 0.2 mM IPTG and shaken overnight at room temperature. Following induction, cells were pelleted at 12,000 × g, washed in 120 ml of PBS, resuspended in 7.5 ml of lysis buffer, and sonicated and centrifuged as above. The clarified lysate was transferred to a 15-ml Falcon tube containing 600 μl of His-Select nickel affinity gel (Sigma), rotated for 1 h at 4 °C, applied to a 5-ml disposable polypropylene column (Thermo Scientific), and washed with 20 ml of lysis buffer. Bound proteins were eluted in 110-μl fractions of imidazole elution buffer. Fractions were evaluated for purity by SDS-PAGE, followed by staining with Coomassie G-250 or Western blotting with anti-His$_6$ antibody. Both methods revealed a prominent band at the expected molecular mass of 100 kDa in a subset of fractions. These fractions contained sialidase activity, as determined by 4-MU-Neu5Ac hydrolysis, and were used in substrate specificity assays. Protein concentration was measured using a Micro BCA Protein Assay Kit (Thermo Scientific). The absence of *N*-acetylneuraminate lyase activity, which might destroy liberated sialic acid, was confirmed by diluting each sialidase preparation 40-fold into a solution of 20 mM sodium acetate (pH 5.5) and 20 μM Neu5Ac. After a 3-h incubation at 37 °C, DBM-HPLC revealed negligible Neu5Ac degradation.

Normalization of sialidase activity

In substrate specificity assays with recombinant NanH2, NanH3, and AUS (Figs. 2–4), similar amounts of enzyme activity were used to investigate their ability to cleave sialic acids from different substrates. To estimate activity, 0.5 μl of each sialidase preparation was used in a 4-MU-Neu5Ac assay as described above. After 20 min, slopes were calculated from the linear portions of the curves. Preparations with higher slopes were used in proportionally smaller quantities to ensure similar amounts of overall activity in assays testing each substrate.

Sialidase activity assays using recombinant enzymes

Assays on recombinant sialidases were carried out in 20 mM sodium acetate buffer (pH 5.5) at 37 °C. For the experiments presented in Figs. 2–4, 25 μM 3'-sialyllactose, 25 μM 6'-sialyllactose (both from Carbosynth), 1 mg/ml human colostrum IgA (Sigma product no. I1010), and 1 mg/ml bovine submaxillary mucin (BSM, Sigma product no. M3895) were used. In these experiments, NanH2 and NanH3 were used at the following approximate specific activities based on comparison with AUS using 4-MU-Neu5Ac as substrate: sialyllactose, 15 milliunits/ml; colostrum IgA, 10 milliunits/ml; BSM, 40 milliunits/
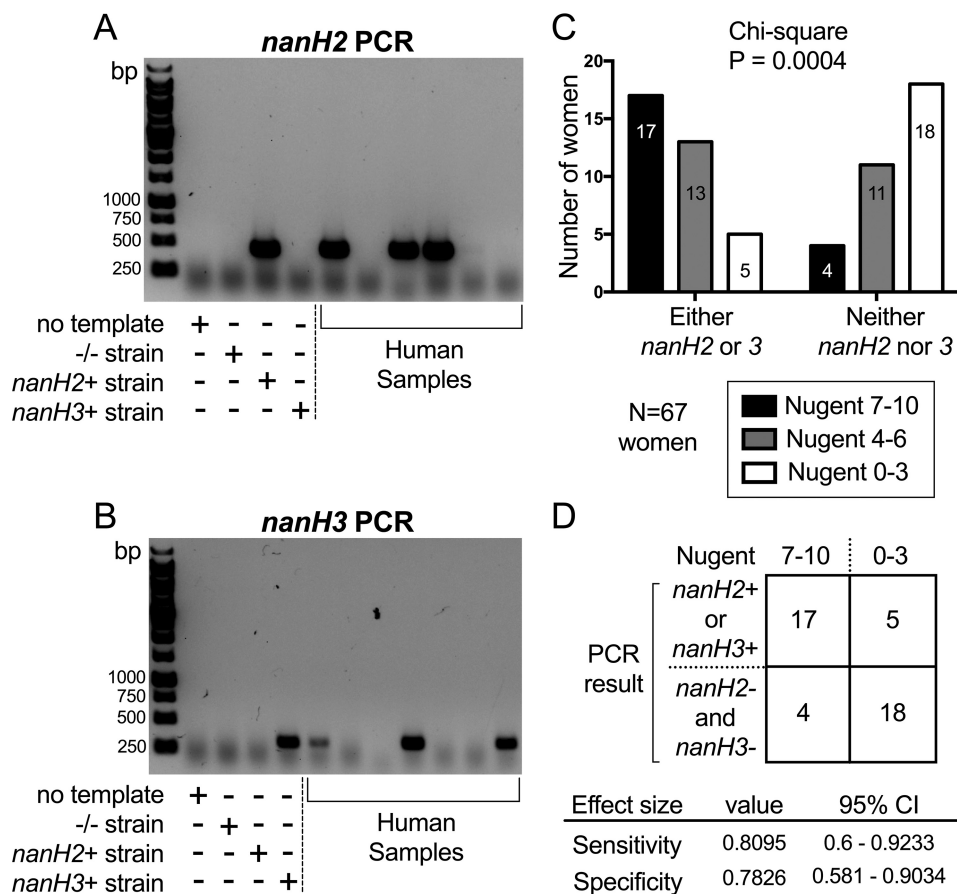
Figure 7. Amplification of *nanH2* and *nanH3* in vaginal specimens. *A* and *B*, PCR of *nanH2* (*A*) and *nanH3* (*B*) was performed on genomic DNA isolated from 67 human vaginal specimens as described under "Experimental procedures." *C* and *D*, categorical analyses was carried out to test whether the presence of *nanH2* or *nanH3* was related to bacterial vaginosis status as determined by Nugent score. *C*, a bar graph illustrates the relationship of *nanH2*/*nanH3* status across the three Nugent score categories. *D*, categorical analysis of the *nanH2*/*nanH3* PCR data *versus* the Nugent score.

ml; GBS with Neu5,7Ac$_2$ (unmigrated), 15 milliunits/ml; GBS with Neu5,9Ac$_2$ (migrated), 8 milliunits/ml. In time course assays, reactions were stopped by transferring tubes to dry ice for 5 min; samples were subsequently stored at $-80\,°C$ until derivatization for HPLC. To test the effect of divalent cations on NanH1 activity, sialidase assays with 4-MU-Neu5Ac and colostrum IgA were also performed in the presence of 1 mM CaCl$_2$ or MgCl$_2$. For assays with GBS, strain COH1 $\Delta neuA$ expressing NeuA N301A from a plasmid was used because it accumulates high levels of sialic acid *O*-acetylation that originates at the C-7 position but migrates to C-9 under slightly alkaline conditions (54). GBS was grown to an OD of 0.4 in 800 ml of Todd–Hewitt broth with 5 μg/ml erythromycin. Cells were pelleted at 12,000 $\times$ *g* for 10 min, washed three times in ice-cold 100 mM sodium acetate buffer (pH 5.5), and stored dry at $-80\,°C$. Before each experiment, cells were resuspended in 20 mM sodium acetate buffer (pH 5.5), to an OD of 30. For experiments monitoring release of 9-*O*-acetylated sialic acids, GBS cells were first resuspended in 100 mM Tris (pH 9.0) and incubated at 37 °C for 30 min to migrate *O*-acetyl groups from the 7-carbon to the 9-carbon position (74, 75). The bacteria were then washed once in 100 mM sodium acetate buffer (pH 5.5) and resuspended in 20 mM sodium acetate buffer (pH 5.5). For the end point experiment presented in Fig. 6*C*, recombi-

nant NanH1, NanH2, and NanH3 were used at $\sim$5 μg/ml. Human colostrum IgA and bovine submaxillary mucin were both used at 1 mg/ml, and 4-MU-Neu5Ac, 3′-sialyllactose, and 6′-sialyllactose were used at 1 mM.

Sialic acid measurements by DMB-HPLC

Sialic acids were derivatized and quantified by HPLC as described previously (48, 74, 75). Samples were derivatized by mixing with an equal volume of 2$\times$ DMB (1,2-diamino 4,5-methylenedioxybenzene) reagent (14 mM DMB, 44 mM sodium hydrosulfite, 1.5 M 2-mercaptoethanol, and 2.8 M acetic acid) and incubating for 2 h at 50 °C. Immediately after derivatization, samples were loaded into the temperature-controlled autoinjector of a Waters HPLC (set to keep samples at 4 °C) equipped with a reverse-phase C18 column (Tosoh Bioscience) and a Waters fluorescence detector set to excite at 373 nm and detect emission at 448 nm. The area under each peak was used to quantitate sialic acid concentrations by referring to a standard curve of Neu5Ac (Sigma) derivatized in parallel. Relative HPLC retention times using this system have been well-established by our group and others for the sialic acid species present in BSM and GBS (54, 74, 75), both of which contain Neu5Ac and *O*-acetylated species at each site of the sialic acid side chain (carbon positions 7, 8, and 9).

Identification of two sialidases in Gardnerella vaginalis

Release and consumption of O-acetylated sialic acids

Growth medium containing bound O-acetylated sialic acids was prepared by adding sterile filtered BSM to NYCIII broth to a final concentration of 1.5 mg/ml. *G. vaginalis* strains were grown overnight in standard NYCIII medium and then diluted 100-fold into fresh NYCIII supplemented with BSM. The following day, saturated cultures were centrifuged, and the supernatants were collected for DMB-HPLC analysis. Mild acetic acid is often used to release bound sialic acids for measurement with DMB, but such treatment can also cause the migration of acetyl groups on sialic acids. Therefore, a portion of each supernatant was diluted 5-fold into 100 mM sodium acetate buffer (pH 5.5) and mixed with an excess of AUS (100 milliunits/ml) for 2 h to liberate bound sialic acids before DMB derivatization. Bound sialic acid concentrations were calculated by subtracting the free from total sialic acid concentration in each sample. Sialic acid release was calculated by subtracting the concentration of bound sialic acid in culture supernatants from that of the uninoculated medium control. Sialic acid consumption was calculated by subtracting the concentration of total sialic acid in each culture supernatant from that of the uninoculated medium control.

Detection of nanH genes in G. vaginalis clinical isolates

Although many of the strains in our collection have draft genome sequences available, these sequences are often comprised of many contigs and thus remain incomplete. Therefore, PCR was used as a more stringent test of the presence or absence of a gene rather than relying on potentially incomplete draft genome sequences. In our analysis, we only included *G. vaginalis* strains that were available for culture, sialidase activity assay, and PCR confirmation of *nanH1*, *nanH2*, and *nanH3*. *G. vaginalis* strains were grown anaerobically on NYCIII or HBT agar until colonies reached ~1 mm in diameter (generally 36 to 48 h). Agar plates were then removed from the anaerobic chamber, and colony PCR was performed with Ex Taq polymerase (Clontech) and the following primer pairs (Table 1): G. vag sia universal F3/G. vag sia universal R1 for *nanH1*, G. vag nanH2 qPCR F/G. vag nanH2 qPCR R for *nanH2*, and G. vag nanH3 qPCR F/G. vag nanH3 qPCR R for *nanH3*. Annealing temperatures were 54 °C for *nanH2* reactions and 51 °C for *nanH1* and *nanH3* reactions. Extension time was 30 s for all three PCR assays. Expected amplicon sizes were 636 bp for *nanH1*, 348 bp for *nanH2*, and 322 bp for *nanH3*.

PCR amplification of nanH2 and nanH3 in human vaginal specimens

This study used samples from the Contraceptive CHOICE project at Washington University. CHOICE received IRB approval at the Washington University School of Medicine, and all participants gave their written informed consent at enrollment and their permission to use vaginal specimens for future studies. This subproject was also IRB-approved (ID 201108155). Both the CHOICE study and this substudy were conducted according to the principles expressed in the Declaration of Helsinki. The human vaginal specimens used in this study were from a previously published subset of CHOICE participants for whom Nugent scores were

published previously and additional vaginal material was available (56).

Genomic DNA was isolated from vaginal swabs eluted in 0.1 M sodium acetate (pH 5.5). Insoluble material was pelleted by centrifugation and processed using the Wizard Genomic DNA Purification Kit (Promega). Amplification of *nanH2* and *nanH3* was performed in 96-well PCR plates (Phenix Research) with Intact Genomics Taq polymerase (catalog no. 3249) and the primer pairs G. vag nanH2 MP F1/G. vag nanH2 MP R1 or G. vag nanH3 qPCR F/G. vag nanH3 qPCR R. Genomic DNA was diluted 10-fold into a PCR plate. 2 μl of each diluted DNA sample was then transferred to a fresh PCR plate on ice, and 18 μl of ice-cold PCR master mix was added to each well. All primers were used at a final concentration of 200 nM. Genomic DNA isolated from GED7760B (*nanH2* only) or JCP8066 (*nanH3* only) served as positive controls. The annealing temperature was 51 °C for both primer sets, and the extension time was 30 s. Amplification was performed for 35 cycles. The expected amplicon sizes were 460 bp for *nanH2* and 322 bp for *nanH3*.

PCR products were separated on 1% agarose gels and visualized under UV light with ethidium bromide staining. Bands at the expected sizes were categorized by an observer blinded to the Nugent status of the samples. Very faint bands were categorized as negative because in all cases we observed, the replicate reaction did not yield a visible band. After all *nanH2* and *nanH3* reactions were separately categorized as positive or negative, we created two summary categories: either *nanH2* or *nanH3* and neither *nanH2* nor *nanH3*. We then performed the analyses described in Fig. 7. The Wilson–Brown method was used to compute confidence intervals. One sample gave discordant results between the two replicate reactions performed and could not be categorized based on the above summary variables; this (Nugent intermediate) sample was excluded from the final analysis.

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