nagZ Triggers Gonococcal Biofilm Disassembly

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Bacterial-bacterial interactions play a critical role in promoting biofilm formation. Here we show that NagZ, a protein associated with peptidoglycan recycling, has moonlighting activity that allows it to modulate biofilm accumulation by Neisseria gonorrhoeae. We characterize the biochemical properties of NagZ and demonstrate its ability to function as a dispersing agent for biofilms formed on abiotic surfaces. We extend these observations to cell culture and tissue explant models and show that in nagZ mutants, the biofilms formed in cell culture and on human tissues contain significantly more biomass than those formed by a wild-type strain. Our results demonstrate that an enzyme thought to be restricted to peptidoglycan recycling is able to disperse preformed biofilms.

Gonorrhea, caused by the exclusive human pathogen Neisseria gonorrhoeae (GC), is the second most common reportable bacterial disease in the US, affecting over 800,000 people every year1. While laboratory studies have contributed to a significant understanding of the mechanistic underpinnings of GC interactions with the host, during natural infection the organism probably exists on mucosal surfaces as a biofilm2. This observation leads to questions about potential differences in studies done with planktonic bacteria as compared to how the organism is growing in vivo. GC growth in biofilms also has implications for treatment, especially with the emergence of cefixime and ceftriaxone-resistant strains3,4.

GC can form a biofilm on glass, primary and transformed human cervical epithelial cells2. However, little is known about how gonococci form biofilms, the signals that regulate biofilm production or the mechanism by which gonococci disperse from the biofilm. DNA plays a critical role in organizing the neisserial biofilm matrix, with secretion of single-stranded DNA playing an important role in the initiation of gonococcal biofilms5. However, the source of this DNA is unclear as strains such as FA1090 and 1291 form biofilms, yet lack the type 4 secretion system associated with DNA secretion6. Addition of exogenous DNase or Spermine impairs biofilm formation and contributes to the disruption of the biofilm7,8. Since the human urogenital track is rich in polyamines, and these molecules can protect GC from host antimicrobials9, interactions with polyamines may be one mechanism by which GC leave a biofilm under physiological conditions. Studies on biofilms produced by other organisms have suggested several mechanisms of dispersal, including erosion10 and sloughing11. Erosion and sloughing can result from bacterial metabolism-associated processes, such as the secretion of glycosidases12 and nucleases13.

GC in biofilms appear to be intimately associated with each other through unknown mechanisms. Our previous studies demonstrated that N. gonorrhoeae MS11ΔOpa, a strain that lacks opacity protein expression, is impaired in its ability to form microcolonies14. Since Opa proteins can act as lectins and bind to LOS from neighboring cells15, we hypothesized that there must be a glycosidase that modulates LOS-Opa interactions to allow for separation individual cocci from a microcolony. We further predicted that this protein could regulate bacterial escape from a biofilm.

In this study, we characterized NagZ from N. gonorrhoeae and analyzed its role in biofilm development. We expressed nagZ in Escherichia coli, purified the protein and defined its biochemical properties. We show that a nagZ mutant produces a more robust biofilm that accumulates more biomass over time. We also show that exogenously added NagZ reduces the biofilm. Finally, we show that in the absence of NagZ, gonococci produce thicker biofilms on ex vivo-cultured human cervical tissue.

Results

NagZ is a non-essential β-N-acetylglucosaminidase. Using the CAZy database16, we determined that ORF0135 in N. gonorrhoeae FA1090 encoded a putative beta-hexosaminidase (EC 3.2.1.52) belonging to

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glycoside phosphorylase (GH3) superfamily. Glycosidases from GH3 are retaining enzymes and cleave their substrates in an acid/base-catalyzed two-step double-displacement mechanism involving a covalent glycosyl-enzyme intermediate in which a fully conserved aspartic acid functions as the catalytic nucleophile. Since ORF0135 is homologous to NagZ in a wide variety of gram-negative bacteria and contains the conserved aspartic acid (the homology to E. coli and Pseudomonas aeruginosa is shown in Supplemental Fig. 1) we propose renaming ORF0135 nagZ. This gene is present in all neisserial genomes currently available in NCBI, with a high degree of conservation (>97% identity) at the nucleotide level among GC strains (Figure S1). The gene synteny is identical in the pathogenic strains, but diverges significantly in commensal strains (data not shown).

The DNA encoding NagZ was cloned into the expression vector pET28a and the expressed protein purified. The molecular mass of NagZ as determined by SDS-PAGE (47 kDa) was consistent with the predicted mass for this protein (Supplemental Fig. 2). We determined the optimal enzymatic conditions for NagZ using p-Nitrophenyl N-acetyl-β-D-glucosaminide (pNP-GlcNAc) as a substrate. The optimal conditions for the assay were determined to be 37 °C, in KPO$_4$ buffer (200 to 400 mM) at a pH of 8.0 (Supplemental Fig. 2B,C). The enzyme retained 100% of activity after 48 hr at room temperature (data not shown). The kinetic parameters $K_m$ and $V_{max}$ against (pNP-GlcNAc) were 3.2 mM and 64 μmol min$^{-1}$ mg$^{-1}$ respectively, and these values are in the same order of magnitude as those for several other hexosaminidases. We calculated the specific activity of our purified protein as 1600 nmol min$^{-1}$ mg$^{-1}$. We screened for activity of this enzyme using p-Nitrophenyl conjugated to other sugar moieties (Supplemental Fig. 2D). Of the substrates that produced detectable cleavage products, NagZ had about 20% maximal activity on p-nitrophenyl-beta-D-N,N'-diacetyltolboise versus that measured with PNP-GlcNAc.

Because N. gonorrhoeae lacks the genes needed to encode a carbohydrate capsule, cell surface N-acetyl-glucosamine is only found associated with LOS. However, this would not preclude NagZ possessing the ability to cleave N-acetyl-glucosamine linked through other conformations. This led us to test Staphylococcus aureus, which produces an extracellular polysaccharide that is a 1-3-1-6 linked polymer, predominately composed of N-acetyl-glucosamine that is required for biofilm formation. When this polymer is degraded by treatment with dispersin B, the biofilm is disrupted. We tested to see if NagZ could disrupt a biofilm formed by S. aureus SH1000. NagZ treatment of a S. aureus biofilm had a negligible impact on its biofilm (Supplemental Fig. 2E). Conversely, treatment with Dispersin B significantly reduced the biomass of the S. aureus biofilm. Dispersin B treatment of gonococcal biofilms produced a negligible impact on its biofilm (data not shown). Given that prolonged treatment of a S. aureus biofilm with NagZ failed to have an impact on it, it is likely that NagZ lacks exo- and endo-1-3-1-6 N-acetyl-glucosaminidase activity.

Because NagZ is needed for the formation of monosaccharides from the released disaccharides during the cytosolic steps of the muropeptide-recycling pathway in E. coli, we hypothesized that the accumulation of peptidoglycan fragments that would occur in a nagZ mutant could affect gonococcal viability. We used a recombination strategy to delete this gene and verified that the deletion had been incorporated properly in the genome by DNA sequence analysis of the region containing the deletion. We measured the growth properties of the deletion mutant and the data indicate that in standard liquid growth media, there is no difference in growth between the parent and deletion strain (Supplemental Fig. 3A).

NagZ activity could be detected in the supernatant as the culture enters stationary phase (Supplemental Fig. 3A). We could not detect any NagZ activity in culture supernatants of Log-phase cells, but could readily detect its activity in the cell lysate. Bioinformatic programs to predict the cellular location of NagZ (Signal p4.024, PSORTb v3.025, PSLPred26 and MESSA27) suggested a cytosolic or periplasmic location for NagZ (data not shown). Using both bacterial lysates and broth supernatants isolated from overnight cultures from FA1090ΔnagZ, we were unable to detect beta-hexosaminidase activity, suggesting that NagZ is the only beta-hexosaminidase in N. gonorrhoeae FA1090.

**NagZ acts on peptidoglycan.** As gonococci grow they release the peptidoglycan fragments removed from the saccus. However, they release a significant portion of these fragments into the medium, and characterization of these fragments produces a reproducible chromatographic profile. We tested NagZ for its ability to digest metabolically-labeled peptidoglycan fragments. Using a boiled preparation of NagZ as a negative control, we generated the expected profile (Fig. 1). The data in Fig. 1A show that the addition of NagZ altered the profile of peptidoglycan fragments, where the peptidoglycan monomer peak shifted to later fractions (smaller size) from the sizing columns, consistent with the removal of N-acetylglucosamine from the peptidoglycan monomers. Similarly, the free disaccharide peak was eliminated and a peak appeared for free 1,6-anhydro-N-acetylmuramic acid. No significant changes were seen to the peaks for peptidoglycan dimers and tetrasaccharide-peptide, suggesting that these molecules are not substrates of NagZ. These data are consistent with the known activity of NagZ in other bacteria, acting to remove N-acetylglucosamine from peptidoglycan monomers and free disaccharide.

**NagZ does not act upon LOS.** Because NagZ was able to cleave substrates containing N-acetylglucosamine, and neisserial LOS contains N-acetylglucosamine in a variety of conformations, we assayed NagZ for its ability to act upon neisserial LOS. Purified LOS from N. gonorrhoeae F62 was digested with NagZ and its mobility compared with undigested LOS. Because F62 expresses several different isoforms of LOS containing terminal and internal N-acetylglucosamine, and NagZ was unable to act on any of these components, whether at a terminal or internal location (Fig. 1B), we concluded that NagZ lacks 1-3 exoglycosidase activity and α1-2 endoglycosidase activity. We also performed these assays by adding NagZ to intact cells, and analyzing the LOS after treatment. The data obtained was the same as that obtained using purified LOS, indicating that the enzymatic activity is not linked to the presence of a specific LOS conformation (Data not shown).
The nagZ deletion strain forms a thicker biofilm. Gonococcal strains growing in biofilms are under significant metabolic stress. As such, we would expect that the extracellular milieu of a biofilm would contain cytosolic enzymes because stressed cells are dying and dying cells are lysing. We hypothesized that a nagZ mutation could alter the structure of a gonococcal biofilm. The wild type and ΔnagZ strains of N. gonorrhoeae FA1090 were compared for their ability to form a biofilm under static and dynamic conditions. Dynamic biofilms were developed at the liquid air interface on the inner surface of culture tubes. After 24 hr, the bacterial culture was aspirated and the biofilm dried. The data (Fig. 2, panel A) is a visual representation of the biofilm that was formed on a culture tube. The data in Fig. 2, panel B is a quantification of the biomass of this biofilm, using a crystal violet staining procedure. Statistical analysis using Student’s t-test showed a significant increase in biomass of the knockout strain over the wild type strain (n = 6, p < 0.001) of approximately 4 fold. A similar phenotype was observed when biofilms were made under static conditions (Fig. 2C). This biofilm enhancement was lost when the mutant was complemented (Fig. 2C). We performed confocal microscopy on static biofilms grown for 36 hr, using Hoechst stain to visualize the biofilm. The mutant strain shows a denser and taller biofilm (~4 fold), consistent with quantification of biomass using crystal violet (Fig. 2D). We compared the ability of FA1090 and FA1090ΔnagZ to form biofilms over time. The biomass associated with FA1090ΔnagZ continued to accumulate over 3 days, while the biomass of a biofilm produced by FA1090 peaked around 48 hr (Fig. 2E).

Effect of NagZ on gonococcal biofilm formation. We analyzed biofilm evolution over time using scanning electron microscopy. After 12 hrs, the biofilms produced by FA1090 and FA1090ΔnagZ looked different. The biofilm produced by FA1090 appeared patchy with numerous nucleation sites while the biofilm produced by FA1090ΔnagZ appeared diffuse across the entire field of view with no obvious nucleation sites (Fig. 3A). These biofilms are dramatically different in their appearance at 12 hrs. FA1090ΔnagZ biofilms showed an increase in cell density over time, while the FA1090 biofilm density seemed to peak at 48 hr. This difference cannot be explained by differences in the growth rate as both strains give the same growth profile (see Supplemental Fig. 3A) or due to the presence of other extracellular components as the overall biomass of the mutant biofilm is greater.

We hypothesized that the differences seen in the biofilms made by FA1090 and FA1090ΔnagZ were due to the localized presence of NagZ in the extracellular milieu. For this to happen, we hypothesized that NagZ could gain access to the extracellular milieu through autolysis. We analyzed the impact of exogenously added NagZ on biofilms produced by FA1090ΔnagZ. Static biofilms were formed and after treatment with NagZ protein, the biofilms were visualized using SEM. NagZ treatment reduced the thickness and density of the biofilm formed by FA1090ΔnagZ (Fig. 3B). The reduction in biomass was quantified, and the data indicate more than 50% of the biofilm was removed with the addition of exogenously added NagZ (Fig. 3C). Addition of NagZ to the bacterial suspension at the beginning of biofilm formation also resulted in a similar reduction in biofilm formation after 24 hr (data not shown). After NagZ treatment, strands of nucleic acid were still apparent in the biofilm (Fig. 3B); treatment with DNase not only removed the extracellular DNA but also degraded the biofilm (data not shown). The data in Fig. 3D show the presence of extracellular DNA in biofilms produced by both strains.
The lack of nagZ does not alter blebbing. *N. gonorrhoeae* generates outer membrane vesicles and alterations in vesicle formation have been observed in bacteria with defects in peptidoglycan metabolism. Since NagZ is involved in peptidoglycan recycling, it is possible that NagZ could affect biofilm formation indirectly by increasing the release of outer membrane vesicles. Using high resolution SEM to visualize the biofilm architecture and the ultrastructure of bacteria within the biofilm, we determined the number and size of blebs made by FA1090 and FA1090ΔnagZ. We found that there was no difference in bleb size or distribution between the two strains, ruling out a membrane blebbing defect as the cause for difference in biofilm formation (See supplemental Fig. 4).
Viability of cells within a biofilm. SEM images showed what appears to be DNA entangling the bacterial aggregates in a random fashion. Since FA1090 lacks the gonococcal genetic island implicated in DNA secretion\(^3\), the source of DNA could be through bacterial lysis or leakage of DNA from dead cells. The data in Fig. 4A show the double-stained (Hoechst and propidium iodide (PI)) biofilms visualized by confocal microscopy (Hoechst stains both dead and live bacteria whereas PI only permeates the membranes of dead bacteria). Figure 4B shows the quantification of these images by comparing the mean fluorescence intensity ratio (FIR) of Hoechst staining to PI staining. No significant difference in the proportion of live to dead bacteria was observed, although the amount of dead bacteria increased over time as seen by an increase of fluorescence intensity of PI. By 72 hrs, both biofilms contain a significant portion of dead cells.

Effect of NagZ expression on Biofilm formation on human cells. We determined the ability of FA1090 and FA1090\(\Delta\)nagZ to form a biofilm on polarized T84 cells. Biofilms formed on the apical surface were visualized by SEM (Fig. 5A). The surface area covered by the biofilms was quantified using ImageJ and the percent of the area covered by biofilm determined. The data (Fig. 5B) show that the mutant strain covered significantly more surface area than the wild type strain, consistent with experiments performed on glass coverslips. Although we could not quantify the total biomass of these two biofilms, it appeared that the FA1090\(\Delta\)nagZ biofilm was taller than the FA1090 biofilm.

To establish the clinical relevance of NagZ in biofilm formation, we obtained cervical tissues from women undergoing uterine surgeries and sourced by the National Disease Research Interchange (NDRI). Tissue samples were infected with \(\sim 10^9\) bacteria per tissue sample, and after 48 hr incubation, the presence of GC on the sample visualized by confocal microscopy. The Map tiling function was used to take images of small sections of the tissue.
as a Z-stack, and all the images were tiled using automated software to create a Z-stack of the entire cervix specimen. The stacks were Z-projected as shown in Fig. 6A. The relative number of cervical cells in a given sample area was defined based on the fluorescence intensity of the Hoescht stain (blue), which stains nuclei. The overall shape of the explant was shown by actin staining (green). The relative number of GC of in a sampled area was determined based on the red fluorescence, which was the result of immunostaining GC. The biofilm quantity was defined as the ratio of red fluorescence intensity to blue fluorescence intensity. This was then normalized to 100 (Fig. 6C). This data indicates that the \( \text{nagZ} \) strain produced a biofilm with four times the number of GC compared to wildtype strain, which is in agreement with experiments performed on abiotic surfaces.

**Discussion**

While *N. gonorrhoeae* can form biofilms *in vivo* and *in vitro*, the mechanism by which they are formed and the processes that mediate their dispersal remain unclear. We show that when GC lack the ability to make NagZ, they produce more robust biofilms that continue to accumulate over time (Fig. 2) and that exogenously added NagZ reduces these biofilms (Fig. 3). Because NagZ plays a role in peptidoglycan (PG) turnover in other organisms, NagZ can be considered another example of a moonlighting enzyme.

Electron microscopic studies of cervical biopsy specimens from patients with culture-proven *N. gonorrhoeae* infection have revealed evidence of biofilms; these biofilms appeared to be only a few bacteria thick. It is unclear why these biofilms do not take on the qualities of those seen on abiotic surfaces or on tissue culture cells. Because...
∆nagZ biofilms are significantly thicker on tissue culture cells and cervical explants than FA1090, we suggest that the release of NagZ during autolysis is limiting the accumulation of biomass. It has been hypothesized that GC biofilms produced during colonization of the cervix results in asymptomatic carriage. How *N. gonorrhoeae* suppresses development of an adaptive immune response during natural infection in the presence immunostimulatory peptidoglycan fragments is unclear. We suggest that removal of N-acetylglucosamine from toxic peptidoglycan monomers renders them immunosilent, and that the released N-acetylglucosamine suppresses neutrophil functions and proinflammatory cytokine induction. Hence, the liberation of N-acetylglucosamine from peptidoglycan fragments by NagZ can serve to suppress the immunological signaling when the gonococcus colonizes cervical mucosa.

**Experimental Procedures**

**Bacterial strains and plasmids.** *N. gonorrhoeae* were grown in standard gonococcal medium (Difco, MI), designated GCP if used as phosphate-buffered broth, and designated GCCK if used with agar, supplemented with 1% Kellogg's supplement, at 37°C and 5% CO2. Strain F62Δ8-1 has been previously described and is a strain that is genetically deleted for lgtA. All GC strains were phenotypically non-piliated and Opa negative. *E. coli* BL21(DE3) and *E. coli* DH5α MCR (Life Technologies, MD) were grown in L broth (LB) at 37°C or 30°C.

Antibiotics included in media were used at the following final concentrations (μg ml⁻¹): ampicillin 100, kanamycin 50. Plasmid pUC19 (New England Biolabs) and pET28a(+) (Novagen) were used to make deletions in various *N. gonorrhoeae* strains and to express NagZ protein, respectively. *S. aureus* SH1000 strain was obtained from Dr. Jeffery Kaplan, George Washington University.

**Chemicals and Reagents.** All reagents were purchased from Sigma-Aldrich, unless stated otherwise. Polymerase chain reactions were performed using Pfu polymerase (Thermo Scientific Molecular Biology, Pittsburgh, PA) or GoTaq polymerase (Promega, Madison, WI), and carried out according to the manufacturer's instructions. All restriction enzymes were sourced from New England Biolabs (Beverly, MA). A sample of Dispersin B was obtained from Dr. Jeffery Kaplan, George Washington University.

**Construction of mutants.** A list of all primers used is provided in Table 1. A DNA fragment carrying the nagZ gene of *N. gonorrhoeae* FA1090 along with ~850 bp of right and left flanking DNA sequences was amplified using primers hexoF and hexoR. The resulting amplicon (~2700 bp) was cloned into the pUC19 EcoRI and HindIII sites and transformed into *E. coli* DH5α MCR, resulting in the formation of pUC19:nagZ. One of the isolated clones was used for further experiments. To disrupt nagZ in the gonococcus, a Kanamycin (Kan) resistance gene was excised from pK1844 with SmaI, the DNA fragment purified from agarose gel and inserted into the EcoRV site of pUC19::nagZ. A plasmid from a presumptive *E. coli* clone carrying pUC19::nagZ::Kan was isolated and the construct was verified by DNA sequencing (Macrogen, USA). This plasmid DNA was cleaved with EcoRV and ligated with double-strand oligo DNA prepared from DUSF and DUSR, which contain two EcoRV sites and *N. gonorrhoeae* DNA uptake sequence, after cleaving with EcoRV. Kanamycin resistant transformants were
The column was washed with 100 ml of buffer B containing 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole and 10% (v/v) glycerol and the proteins eluted with a 6 ml of buffer B containing increased concentration of imidazole (20 mM, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM). N-acetyl-β-D-hexaminidase eluted at 150 mM–200 mM. The fractions containing the purified proteins were pooled and dialyzed against buffer B and 1 M KPO₄ buffer pH 8.0 (final concentration of 200 mM), 50 μl of water and 2 μg of enzyme (final concentration of 1.3 U/ml). Enzyme activity was assayed using various p-nitrooligosaccharides as substrate.

| Primer Name | DNA sequence |
|-------------|--------------|
| hexoF       | GGCAATTCAACGCCGATCTCGATAC |
| hexoR       | GCAAGCTTTTTCCGGCATGATCTAAC |
| DUSF        | ATGGCGATATCCGGCTCTGAAAGATTCTGGATATCGAT |
| DUSR        | ATCGATGATATCCGGCTCTGAAAGATTCTGGATATCGAT |
| Ngo0135F    | CTAGTACGTAGCAGGGCTCCCATATTC |
| Ngo0135R    | CGCTCGAGTAAAGGCCTTCCGACCTTTTA |

Table 1. List of Primers.

selected, the plasmid DNA isolated and checked for the presence of the uptake sequence. Plasmid DNA from such clones was used for transformation of *N. gonorrhoeae* strains by liquid transformation method⁴⁵ and selected on GCK plates containing kanamycin (50 μg/ml). *nagZ* was deleted by a spot transformation technique⁴⁶ in *N. gonorrhoeae* FA1090. The *nagZ* complemented strain was constructed using a PCR amplicon generated with primers hexoF and hexoR to transform the deleted strain, using a spot transformation technique⁴⁵.

Expression and purification of NagZ protein. DNA fragment encoding *nagZ* isolated from *N. gonorrhoeae* FA1090 was amplified using primers Ngo0135F and Ngo0135R and the resulting amplicon (1300 bp) was cloned into pET28a(+) using Nhel and XhoI, resulting in the formation of plasmids pET28a(+)::nagZ. To purify NagZ, a single colony generated by transformation of *E. coli* BL21(DE3) with pET28a(+)::nagZ was used to inoculate 100 ml of LB broth with kanamycin. Cultures were incubated at 37 °C, and when the OD₆₀₀ of the culture reached 0.6, IPTG was added to a final concentration of 1 mM. Incubation was continued at 30 °C for an additional 12 hr. Cultures were centrifuged and bacterial pellets were resuspended in 10 ml buffer A containing 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 20 mM imidazole, 10 mM -mercaptoethanol, 0.1% (v/v) Tween 20, 100 μM PMSF. After sonication, the cellular debris was removed by centrifugation at 40 000 g for 1 hr and then the supernatant applied to a 3 ml Ni-NTA agarose column previously equilibrated with 100 ml of above buffer. The column was washed with 100 ml of buffer B containing 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole and 10% (v/v) glycerol and the proteins eluted with a 6 ml of buffer B containing increased concentration of imidazole (20 mM, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM). N-acetyl-β-D-hexaminidase eluted at 150 mM–200 mM. The fractions containing the purified proteins were pooled and dialyzed against buffer B and reapplied to a Ni-NTA agarose column. The purity of the enzyme was determined by running an aliquot on a 15% SDS-PAGE. PageRuler™ prestained protein ladder (170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa) (Fermentas) was used as protein molecular weight marker.

LOS purification and analysis. LOS was purified from broth-grown cells by hot phenol-water method⁴⁷. LOS was then concentrated by lyophilization and extraction with hot phenol-water continued until the preparation obtained minimal absorbance at 200 nm. LOS was analyzed on a 16.5% Tris-Tricine gel at a fixed current of 380 nM. After 30 min incubation at 37 °C, the reaction was stopped by addition of 50 μl of 200 mM glycine NaOH buffer pH 10. Water (860 μl) was then added and the release of p-nitrophenol from the substrate was monitored at 405 nm. One unit (U) of enzyme was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from pNP-GlcNAc per min at 37 °C.

Hexosaminidase assay. Enzyme activity was assayed using various p-nitrooligosaccharides as substrate. The standard reaction mixture consisted of 20 μl of substrate solution at a final concentration of 1.2 mM, 20 μl of 1 M KPO₄ buffer pH 8.0 (final concentration of 200 mM), 50 μl of water and 2 μg of enzyme (final concentration of 380 nM). After 30 min incubation at 37 °C, the reaction was stopped by addition of 50 μl of 200 mM glycine NaOH buffer pH 10. Water (860 μl) was then added and the release of p-nitrophenol from the substrate was monitored at 405 nm. One unit (U) of enzyme was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from pNP-GlcNAc per min at 37 °C.

Biofilm formation. To prepare biofilms, bacteria from overnight cultures grown on GCK agar plates were suspended in GCP with sodium bicarbonate and Kellogg’s supplement to a final concentration of 10⁶ bacteria per ml. Dynamic biofilms were grown in new heat-sterilized glass tubes by adding 1 mL of the bacterial suspension per tube, and incubating in a rotary shaker for 48 hr. Static biofilms were obtained by making a suspension of bacteria to a Klett of 100 in GCP and adding an aliquot to culture flasks using published methods⁴⁷. The biofilms were quantified by staining with 1% w/v crystal violet. The crystal violet stained biofilm was eluted out by dissolving in 30% acetic acid and the absorbance read at 590 nm in a spectrophotometer.

The same protocol was used to study biofilm formation on polarized epithelial cells. T84 epithelial cells were seeded at 10⁴ per transwell insert in 24 well plates and grown for 10 days. The cells were considered polarized once the transepithelial electrical resistance (TEER) levels reach above 1500 Ω.cm². Bacteria was added on the apical surface and biofilm formed for different time points. For scanning electron microscopy, the transwell membrane was fixed in glutaraldehyde and the membrane cut and removed from the insert before staining.

Confocal Microscopy analysis. Static biofilms were grown in 35 mm glass bottom microwell dish with No. 15 coverglass (MatTek, MA) for 48 hr. Biofilms were washed with PBS three times and stained with Hoechst stain for 15 min fixed with 4% PFA for 20 min visualized with Leica TCS SP5 X confocal microscope (Leica Microsystems, IL, USA).

Scanning electron microscopy. Static biofilms were grown for 36 hr over a glass coverslip placed inside a 24-well cell culture plate. Briefly, the coverslips were gently washed with PBS, and fixed with 2% glutaraldehyde.

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Scanning electron microscopy. Static biofilms were grown for 36 hr over a glass coverslip placed inside a 24-well cell culture plate. Briefly, the coverslips were gently washed with PBS, and fixed with 2% glutaraldehyde.
for 60 min at RT and then overnight at 4°C. The coverslips were fixed the next day using 1% osmium tetroxide, dehydrated by a series of washes with increasing concentrations of ethanol, dried by critical point drying method, and finally coated with gold-palladium alloy. Samples were visualized with Amray 1820D microscope (20 kV) and Hitachi S4700 microscope (5 kV).

**Cervical infections.** Women undergoing uterine surgeries provided informed consent for the use of cervical tissue samples, which were obtained from NDRI (National Disease Research Interchange). The University of Maryland Institutional Review Board approved of the experiments and methods, which were carried out in accordance with the approved guidelines. This endocervical tissue was received within 24 h post-surgery. Samples were cut into ~2.5 cm (L) × 0.6 cm (W) × 0.3 cm (H) pieces, incubated in CMRL-1066 (GIBCO) plus antibiotics for 24 h, and switched to antibiotic-free media for 24 h. Figure 6 the tissue was infected with approximately 10⁹ bacteria per tissue sample. After a 48 hr incubation period, the tissues he tissue was fixed with paraformaldehyde, cryopreserved in gelatin, cryosectioned, and immunostained. Antibody used to label GC was as previously described. Images were acquired by confocal microscopy using a map tiling function was used to take images of small sections of the tissue as a Z-stack, and all the images were tiled using automated software to create a Z-stack of the entire sample.

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Author Contributions
The original study was conceptualized by D.C.S. and S.V.B. The experimental design was formulated by D.C.S., S.V.B. J.D.L. and J.P.D. The data was collected by S.V.B., A.P. and J.D.L. and interpreted by all authors. The manuscript was prepared by S.V.B. and D.C.S., with all other authors contributing to the editing of the final manuscript.

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