Investigating the role of pseudaminic acid on *Helicobacter pylori* adhesion to human cells

Student Researcher: Divya Bhargava
Advisor: Danielle Dube
Bowdoin College
Department of Chemistry

Abstract

Helicobacter pylori is a pathogenic bacterium whose antibiotic resistance is high and increasing (Kusters et al, 2006, Savoldi et al, 2018). Glycans and sugar modifications serve important roles in *H. pylori* pathogenicity and thus present compelling targets for novel treatments. Pseudaminic acid, a rare 9-carbon bacterial sugar, is a modification added onto *H. pylori* flagellar filament proteins FlaA and FlaB that is necessary for the bacterium's motility, a function critical to virulence (Schirm et al, 2003). As flagellar filament proteins are reported adhesins in other gram-negative bacteria, this study tests the hypothesis that genetic disruption of pseudaminic acid biosynthesis will impede bacterial adhesion to host cells, another function necessary for virulence (Haiko and Westerlund-Wikström, 2013). This hypothesis was tested by investigating the mammalian cell adhesion properties of two *H. pylori* strains mutated in parts of the pseudaminic acid biosynthesis pathway relative to wildtype *H. pylori*. To this effect, adenocarcinoma-derived gastric epithelial (AGS) cells were cultured with *H. pylori* strains, and bacterial adhesion to these host cells was assessed using flow cytometry and confocal microscopy. Our results suggest that pseudaminic acid modifications play an important role in *H. pylori* adhesion, as mutant strains showed diminished adhesion to AGS cells. Additionally, preliminary investigations into the role of pseudaminic acid in modulating host immune response to *H. pylori* indicate that pseudaminic acid may impact cytokine secretion by AGS cells as well.

Project Objectives

The primary objective of this study was to optimize a flow cytometry and confocal microscopy-based adhesion assay and then use the assay to investigate the role of pseudaminic acid on *H. pylori* adhesion to human cells. To achieve this goal, I tested the use of different methods of fluorescently labeling bacteria and different methods of releasing cells bound to the co-culture surface. Furthermore, I worked to develop a protocol that resulted in distinct and identifiable populations in flow cytometry plots to identify adherent bacteria in the samples analyzed. Once the flow cytometry-based assay was optimized, I used it to compare the adhesion of mutant strains of *H. pylori* to wildtype *H. pylori*, and gathered complementary evidence via confocal microscopy.

Methodology Used

Adenocarcinoma-derived gastric epithelial (AGS) cells (ATCC Number: CRL-1739) were used in *H. pylori* adhesion experiments. Prior to use, AGS cells were stored in liquid nitrogen for preservation and were thawed by warming in a 37°C water bath and seeded in T75 flasks in media which consisted of Ham's F12 Glutamax Nutrient Mix with 10% fetal bovine serum (FBS). Cells were incubated at 37 °C with 5% CO_2 and were passaged when they reached 80-90% confluency. Wildtype (WT) *H. pylori* strain G27 and *H. pylori* pseudaminic acid biosynthesis mutants ΔpseE and ΔpseB on the same isogenic background were used in these studies (Baltrus et al, 2009). The ΔpseE and ΔpseB mutant strains of *H. pylori* were created by Adedunmola Adewale through insertional inactivation of target genes via a chloramphenicol acetyltransferase cassette (Adewale, 2022).

To prepare for the adhesion assay, approximately 320,000 AGS cells and 640,000 AGS cells were seeded into wells of a 24-well tissue culture plate and 2-well chamber slide respectively and incubated overnight at 37 °C and 5% CO_2. Bacterial cells were harvested from horse blood agar plates and used to inoculate 3 mL broth cultures in Brucella broth, which contained an added 1 µL/mL of chloramphenicol for mutant strains harvested. These liquid cultures were incubated overnight at 37 °C and 14% CO_2 with shaking. To begin the co-culture the following morning, AGS cells were incubated for 2 hours in refreshed media which did not contain fetal bovine serum. During this time, overnight bacterial cultures were

washed in Brain Heart Infusion (BHI) broth and resuspended to a concentration of about $1.6*10^9$ cells/mL, or an OD_{600} of 1.6. Then they were treated with an anti-*H. pylori* fluorescently tagged antibody (Biotium BNC881335-100) and incubated for 30-60 minutes. After the 2-hour incubation of the AGS cells, the bacteria were washed and 20 µL of bacterial suspension was added to each well with AGS cells. This co-culture was incubated for 3-hours at 37°C and 14% CO_2.

During this 3-hour incubation samples of the pre co-culture bacterial suspension were prepared by combining 20 µL of bacterial suspension with 175 µL of phosphate-buffered saline (PBS) and 2 µL of a calibrated suspension of 6 µm polystyrene reference beads at a concentration of $1*10^8$ beads per mL used for quantification. These samples were analyzed using flow cytometry.

After the 3 hour co-culture period was complete, chamber slides were prepared for analysis by aspirating culture media. 20 µL of Fluoromount (Sigma Aldrich F4680) with 1 µg/mL of DAPI was placed on the slides and used to add a coverslip. Slides were analyzed on a Leica 6B confocal microscope in LAS X software. The co-cultures in the 24-well plate were used for flow cytometry analysis. Co-culture supernatant from these wells was removed, centrifuged at 15,000 rpm to remove any cells, and stored at -80°C for later experimentation. Then remaining media was aspirated and the co-cultures were incubated in 0.1 mL of 0.25% trypsin-EDTA for 5-10 minutes. Trypsin was quenched with 0.9 mL of tissue culture media, and samples were mixed gently to break up any clumps of cells. Then 500 µL of media containing adherent cells was combined with 5 µL of reference beads and samples were analyzed using flow cytometry.

Cytokine production by AGS cells in response to bacterial challenge was tested using an enzyme-linked immunosorbent assay (ELISA). A Human CXCL-8 DuoSet Enzyme Linked Immunoassay (ELISA) kit (R&D Systems, Minneapolis, MN) was used to detect relative concentrations of CXCL-8 in supernatant harvested from the *H. pylori* and AGS cell co-cultures. ELISA assays were conducted according to manufacturer instructions. Briefly, a 96-well plate was coated with Capture Antibody the night prior to the experiment, and the next day block buffer, samples and CXCL-8 standards, a biotinylated goat anti-human CXCL-8 Detection Antibody, streptavidin conjugated to horseradish peroxidase, and a solution made of equal parts H_2O_2 and tetramethylbenzidine were added step-wise with wash steps in between. Then, colorimetric analysis was performed using a SpectroStar Nano microplate reader (BMG Labtech, Ortenberg, Germany) at 450 nm wavelength. Cytokine concentration data was derived from an 8-point human CXCL-8 standard curve.

Results Obtained

A distinct *H. pylori* population was observed in the flow cytometry plots of plate-bound cells. This population was hypothesized to represent *H. pylori* that had adhered to gastric epithelial cells because all non-adherent *H. pylori* had been aspirated and washed away. Analysis of all populations present on these plate-bound flow cytometry plots demonstrated a trend that there were fewer mutant, plate-bound *H. pylori* than wildtype, plate-bound *H. pylori*. When the counts of plate-bound *H. pylori* were standardized based on the pre co-culture counts of bacteria, which represent the amounts of bacteria added to the co-culture, a significant difference in these counts was seen between wildtype and mutant strains of *H. pylori*. This difference was replicable, although there was variation between experiments on whether mutant adhesion was low enough that it was comparable to the negative control or lower than that of wildtype *H. pylori* but still significantly higher than the negative control.

Data from confocal microscopy also suggested that there was a limited amount of adhesion of *H. pylori* pseudaminic acid biosynthesis mutants compared to wild-type *H. pylori*. The nuclei of AGS cells appeared blue in all samples, with a small amount of green signal as well due to bleedover in the detection of the two wavelengths of fluorescence. However, samples with wildtype *H. pylori* included also had much brighter and smaller, more concentrated spots of green signal around the periphery of the AGS cells, which were hypothesized to represent bacteria being labeled by the fluorescently tagged antibody. Samples with mutant strains of *H. pylori* largely lacked these smaller brighter spots.

When testing the supernatant of these co-cultures for cytokine production by AGS cells in response to bacterial challenge, some variability was observed. The first time the ELISA was performed to test for CXCL-8 production, only AGS cells that had been incubated with wildtype *H. pylori* had CXCL-8 present in their conditioned media. However, the second time the ELISA was performed, both wildtype and ΔpseE *H. pylori*-induced CXCL-8 production. In fact, CXCL-8 production in response to challenge with ΔpseE was significantly higher than CXCL-8 production in response to challenge with wildtype *H. pylori*.

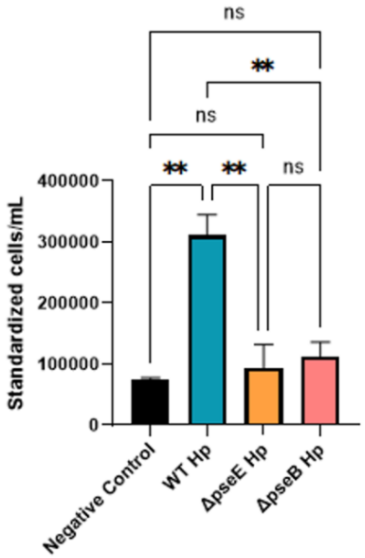
Significance and Interpretation of Results

The fact that counts of plate-bound *H. pylori* pseudaminic acid biosynthesis mutants were significantly lower than those of wildtype *H. pylori* indicates that these mutants are demonstrating diminished adhesion capabilities. This data is further supported by the complementary microscopy data which showed the presence of adhered *H. pylori* only in the wild-type samples. This suggests that pseudaminic acid modifications play an important role in adhesion of *H. pylori* and that they may have potential as a target for novel therapeutic strategies.

Furthermore, the initial ELISA data suggest that pseudaminic acid may play a role in modulating host immune response in response to *H. pylori* infection. The fact that the AGS cells challenged with ΔpseB *H. pylori* did not produce CXCL-8, a pro-inflammatory cytokine, suggests that lack of a functional pseudaminic acid biosynthesis pathway impacts host ability to respond to and perhaps recognize these bacteria. However, the variability demonstrated in ΔpseE *H. pylori's* ability to induce production of CXCL-8 indicates that further replicates of this experiment are needed to establish conclusions about these trends. Yet the preliminary trend shown in this work has been previously observed. Cytokine production in response to certain glycosylation mutants of *H. pylori* has been shown to be variable and has indicated that phase variability may play a role. To continue this project, further replicates of the assay testing for CXCL-8 production should be performed and production of other cytokines should be assayed for as well. The ability of *H. pylori* glycans, like structures modified with pseudaminic acid, to modulate immune response provides another reason why these molecules are such compelling targets for new treatments.

Figures/Charts

A) Standardized counts of plate-bound *H. pylori*



B) 40x confocal microscope images showing green signal from anti-*H. pylori* fluorescently tagged antibody

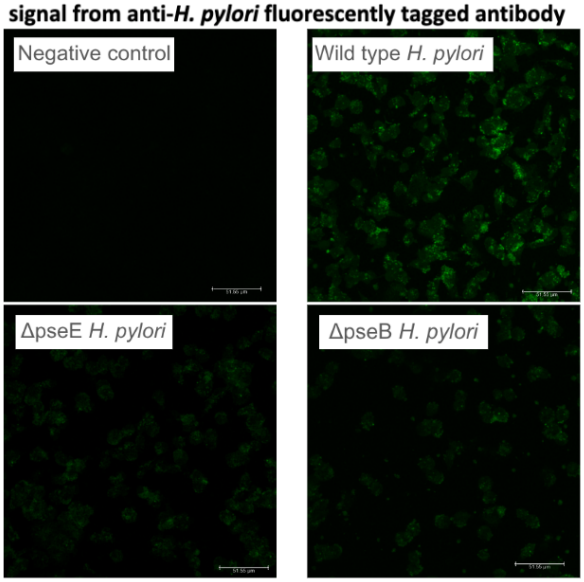


Figure 1. A) Standardized counts of plate-bound *H. pylori* from preliminary adhesion assay. B) 40x confocal microscope images from Alexa 488 channel showing green signal from anti-*H. pylori* fluorescently tagged antibody. Standardized counts were determined by dividing counts of plate-bound *H. pylori* by the coefficient of the pre co-culture value. Standardized counts of plate-bound *H. pylori* show diminished adhesion in pseudaminic acid biosynthesis mutant strains.

Acknowledgments and References

I would like to thank my mentor Professor Danielle Dube for allowing me to work in her research laboratory and to learn from her wealth of knowledge. Next, I would like to thank my lab manager Karen Moulton for teaching me laboratory techniques and for providing me with invaluable support. I would also like to thank my current and past labmates, in particular, Will Surks ’26 for his patience, kindness, and willingness to teach me the complexities of our project. Finally, I would like to thank Lisa Ledwidge for training me on Bowdoin’s confocal microscope and the Maine Space Grant Consortium for funding my work this summer.

Adewale, A. P. Identification of Genes Involved in *Helicobacter Pylori* Glycolipid and Glycoprotein Biosynthesis. Honors Proj. Bowdoin Coll. 2022.

Baltrus, D. A., Amieva, M. R., Covacci, A., Lowe, T. M., Merrell, D. S., Ottemann, K. M., Stein, M., Salama, N. R., & Guillemin, K. (2009). The complete genome sequence of *Helicobacter pylori* strain g27. Journal of Bacteriology, 191(1), 447–448.

Haiko, J., & Westerlund-Wikström, B. (2013). The role of the bacterial flagellum in adhesion and virulence. *Biology*, *2*(4), 1242–1267.

Kusters, J. G., van Vliet, A. H. M., & Kuipers, E. J. (2006). Pathogenesis of *Helicobacter pylori* infection. *Clinical Microbiology Reviews*, *19*(3), 449–490.

Savoldi, A., Carrara, E., Graham, D. Y., Conti, M., & Tacconelli, E. (2018). Prevalence of antibiotic resistance in *Helicobacter pylori*: A systematic review and meta-analysis in world health organization regions. *Gastroenterology*, *155*(5), 1372-1382.e17.

Schirm, M., Soo, E. C., Aubry, A. J., Austin, J., Thibault, P., & Logan, S. M. (2003). Structural, genetic and functional characterization of the flagellin glycosylation process in *Helicobacter pylori*. *Molecular microbiology*, *48*(6), 1579-1592.