Investigating the role of glycan biosynthesis in *Helicobacter pylori* adhesion to human cells

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Abstract

The pathogenic and carcinogenic bacterium *Helicobacter pylori* (*H. pylori*) is accumulating resistance to antibiotics (Kusters et al. 2006, Savoldi et al. 2018). Many of its interactions with the host proceed through glycan-based mechanisms. Bacterial glycans are structurally diverse and vary across different species and serotypes of bacteria. These characteristics make *H. pylori* glycan components compelling pathogen-specific targets for novel treatment methods, which are direly needed. *H. pylori* expresses a range of glycans on its surface, including complex O-linked glycoproteins, pseudaminic acid-modified glycoproteins, and lipopolysaccharides (LPS) (Champasa et al. 2013, Schirm et al. 2003, Teng et al. 2022). This study tests the hypothesis that these glycan structures play a role in *H. pylori* adhesion to host cells, a function critical for pathogenicity. To test this hypothesis, human adenocarcinoma-derived gastric epithelial cells (AGS cells) were challenged with glycan biosynthesis mutant and wild-type *H. pylori*. The relative counts of bacteria adhered to AGS cells after a co-culture were assessed via flow cytometry and confocal microscopy. Findings indicate that pseudaminic acid and some O-linked glycoprotein biosynthesis mutants show diminished adhesion to host cells, while in general, LPS biosynthesis mutants do not. This work provides a foundation for further exploration of *H. pylori* glycans as targets for novel antibiotics.

Project Objectives

The primary objective of this study was to assess the role of biosynthesis of several glycan structures, including complex O-linked glycoproteins, glycoproteins containing pseudaminic acid as a single monosaccharide modification, and lipopolysaccharides, in *H. pylori* adhesion to human gastric epithelial cells. This work will evaluate glycans as new molecular targets in the treatment of *H. pylori* infections. To achieve this goal, a flow cytometry and confocal microscopy-based adhesion assay protocol was used to compare the adhesion of mutant strains to wild-type *H. pylori*.

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, thawed by warming in a 37°C water bath, and seeded in T75 flasks in media that contained Ham's F12 Glutamax Nutrient Mix with 10% fetal bovine serum (FBS). The cells were incubated at 37°C with 5% CO_2 and were passaged every 3 days or when they reached 80-90% confluency. Wildtype (WT) *H. pylori* strain G27 and *H. pylori* glycan biosynthesis mutants ΔpseE, ΔpseB, Δwzk, ΔwaaL, Δ579, Δ580, and Δ1179 on the same isogenic background were used in these studies (Baltrus et al, 2009). Pseudaminic acid mutant strains ΔpseE and ΔpseB, glycoprotein mutant strains Δ579, Δ580, and Δ1179, and LPS mutant strains Δwzk and ΔwaaL were created and characterized by Dube Lab members Andrew Mulholland '21, Adedunmola Adewale '22, and Karen Moulton via insertional inactivation with a chloramphenicol acetyltransferase cassette (Moulton et al. 2020, Mulholland 2021, 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 a 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. 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 that 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 bacterial suspension (20 µL) 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 bacterial suspension (10 µL) with phosphate-buffered saline (PBS, 85 µL) and a calibrated suspension of micrometer-scale polystyrene reference beads (5 µL) at a stock concentration of $2.5*10^6$ beads per mL. These samples were analyzed using flow cytometry. Data for 10,000 events was gathered.

After the 3-hour co-culture period was complete, chamber slides were prepared for analysis by aspirating culture media. Fluoromount (Sigma Aldrich F4680, 20 µL) with DAPI (1 µg/mL) was used to mount 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 -20°C for later experimentation. Then, remaining media was aspirated, and the co-cultures were incubated in 0.25% trypsin-EDTA (0.1 mL) for 5-10 minutes. Trypsin was quenched with tissue culture media (0.9 mL), and samples were mixed gently to break up any clumps of cells. Last, media containing adherent cells (500 µL) was combined with reference beads (5 µL), and samples were analyzed using flow cytometry.

Results Obtained

A distinct *H. pylori* population present in the flow cytometry plots of plate-bound cells was observed. 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 for ΔpseE, ΔpseB, Δ579, and Δ1179 mutant strains, there were significantly fewer adherent bacteria than were observed for the wild-type when these counts were standardized to the pre co-culture counts of bacteria, which represent the amounts of bacteria added to the co-culture (Figure 1A, 1C). Standardized counts of adherent Δ580 and ΔwaaL *H. pylori* were similar to wild-type counts, while interestingly, standardized counts of adherent Δwzk were significantly higher than wild-type counts (Figure 1B, 1C).

Data from confocal microscopy also suggested that there was a limited amount of adhesion of *H. pylori* pseudaminic acid biosynthesis mutants and two out of three O-linked glycoprotein biosynthesis mutants (Δ579 and Δ1179) compared to wild-type *H. pylori* (Figure 2). The nuclei of AGS cells appeared blue in all samples, with a small amount of green signal as well due to bleed over in the detection of the two wavelengths of fluorescence. However, samples with wild-type *H. pylori* also included much brighter and smaller, more concentrated spots of green signal around the periphery of the AGS cells, which were hypothesized to represent bacteria labeled by the fluorescently tagged antibody. Samples with ΔpseE, ΔpseB, Δ579, and Δ1179 mutant strains of *H. pylori* largely lacked these smaller, brighter spots.

Significance and Interpretation of Results

The fact that standardized counts of select glycan biosynthesis mutant strains of *H. pylori* were significantly lower than those of wild-type *H. pylori* indicates that these mutants demonstrate diminished adhesion capabilities. These data are further supported by the complementary microscopy data, which showed a significant presence of adherent *H. pylori* only in the wild-type, Δwzk, ΔwaaL, and Δ580 samples. This suggests that pseudaminic acid modifications play an important role in adhesion of *H. pylori*, as do parts of complex O-linked glycoproteins, which are the parts encoded by the Δ579 and Δ1179 enzymes. The fact that these structures appear to play a role in adhesion suggests that they may have potential as targets for novel therapeutic strategies.

Figures/Charts

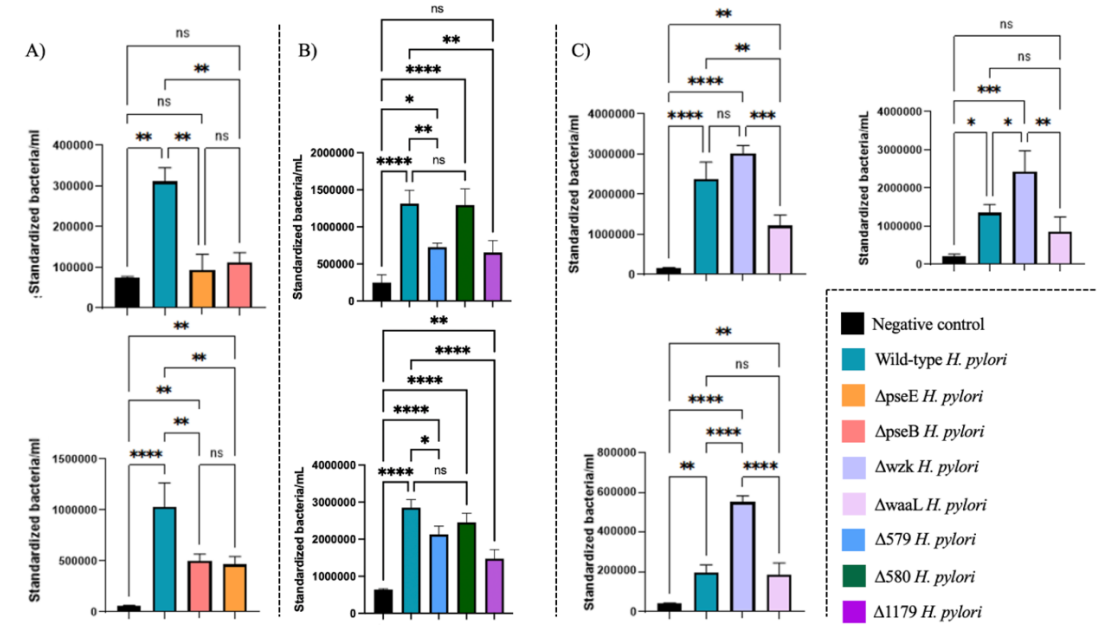

Figure 1. Standardized counts of *H. pylori* from flow cytometry plots. Counts were standardized to pre co-culture amounts of bacteria added. Figure sections represent replicate experiments. A) ΔpseE and ΔpseB *H. pylori* demonstrated consistently lowered standardized counts of bacteria compared to the wild-type. B) Δ579 and Δ1179 *H. pylori* demonstrated consistently lowered counts compared to wild-type while Δ580 *H. pylori* counts were similar to wild-type in both replicate experiments. C) Variability was observed in counts of Δwzk and ΔwaaL *H. pylori* compared to wild-type, though in general, mutations in LPS biosynthesis do no appear to adversely impact bacterial adhesion.

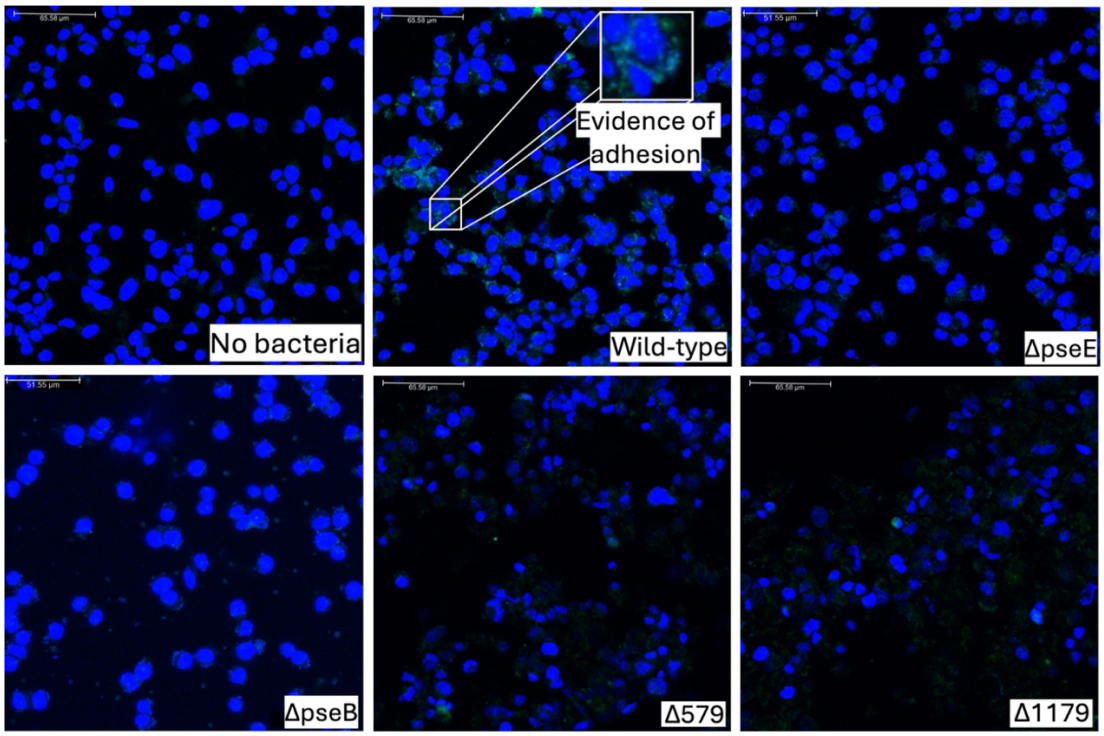

Figure 2. Confocal microscopy images of co-cultures of AGS cells and various strains of *H. pylori*. AGS cells are stained with DAPI (blue) and *H. pylori* are labeled with a fluorescently tagged antibody (green). Mutant co-cultures shown are ones which exhibited minimal adhesion compared to wild-type.

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