Exploring H. pylori: Virulence factors and growth optimisation

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To date, gastric cancer remains one of the most prevalent and life-threatening neoplasms worldwide (Sung et al., 2021), with one of its major causative factors being colonisation of the gastric epithelium by the microaerophilic, gram-negative, spiral-shaped bacterium Helicobacter pylori (H. pylori) (Marshall & Warren, 1984). Following an infection with H. pylori individuals may develop various conditions including gastritis, duodenal ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, gastric cancer and other extra gastric conditions (Malfertheiner et al., 2012, Fong., 2020). Presently, approximately half of the global population carries the often-lifelong burden of a H. pylori infection (Wroblewski et al., 2010). Despite its worldwide prevalence, the exact mechanism of action of H. pylori is still not completely understood.

The successful survival and colonisation of H. pylori is attributed to its diverse array of unique virulence factors. These virulence factors aid the bacterium in evading the human immune system and enable its resilience to the harsh acidic conditions of the stomach. To navigate the gastric environment and reach the host cell, the bacterium employs flagella for movement towards the gastric mucosa and adhesins to securely attach to gastric epithelial cells (Gu., 2017; Salama et al., 2013). During this process, H. pylori uses urease to modulate the acidic environment of the stomach, thereby elevating the pH in its immediate surroundings (Scott et al., 2002).

Upon attachment to gastric epithelial cells, H. pylori releases virulence factors that can modulate host cell signalling such as Cytotoxin-associated gene A (CagA). CagA is a protein encoded by a 40 kb pathogenicity island (PAI) in the bacterial genome and is delivered into host cells through a Type IV secretion system (T4SS) (Odenbreit et al., 2000). Once within the cell, CagA undergoes tyrosine phosphorylation mediated by Src and c-Abl kinases, triggering

a cascade of diverse aberrant signalling pathways (Yong et al., 2015). This cascade induces infected host cells to produce pro-inflammatory cytokines, recruit immune cells to the gastric epithelium, and initiate chronic inflammation. Subsequently, these events lead to profound alterations in cell polarity, morphology, and the initiation of oncogenic processes.

Recently, the identification of a novel H. pylori virulence factor known as Hp1012, has spurred research interest (Wessler & Posselt, 2023). This metalloprotease is presumed to play a crucial role in mediating the adhesion of H. pylori to host gastric epithelial cells. Existing ΔHp1012 mutants, such as p12 ΔHp1012 and N6 ΔHp1012, stand as invaluable tools for ongoing research investigating the function of Hp1012. However, it is noteworthy that the mutation of Hp1012 in both P12 and N6 H. pylori strains appears to adversely impact the bacterial growth rate, posing challenges in culturing these strains for further study.

The objective of this study was to identify the expression of virulence factors Hp1012 and Cag-A, in P12 WT as well as P12 ΔHp1012 infected AGS cells over varying durations. Additionally, we sought to investigate alterations in the growth curve of H. pylori WT and ΔHp1012 mutants derived from both P12 and N6 strains when subjected to a medium containing Brain Heart Infusion (BHI) and either β-Cyclodextrin (β-CD) or foetal calf serum (FCS). This comparative analysis aimed to identify potential improvements in using either β-CD or FCS in the growth medium when cultivating different strains and mutations of H. pylori. The overarching goal of these investigations is to enhance the cultivation conditions for H. pylori and gain a deeper understanding of the mode of action of the bacteria to enable more effective future research endeavours.

Materials and Methods

Infection Experiment

Bacterial culture:

H.pylori populations P12 wildtype and P12 ΔHp1012 were streaked onto wildtype plates from a cryo-stock and incubated under microaerophilic conditions in an anaerobic jar using CampyGen system (Oxoid) at 37 degrees.

Cell culture:

AGS cells were cultured in RPMI medium with 10% FCS and 1% L-glutamine, maintained at 5% CO2 and 37 degrees. The cells were then split every two days with a ratio of 1:6 and every three days with a ratio of 1:8. Cells were seeded on $10cm^2$ culture dishes at a volume of $1*10^6$ cells per dish. Approximately 16 hours prior to infections cells were serum starved through removal of FCS in the medium and incubated overnight.

Infection

AGS cells were infected with P12 wildtype at a multiplicity of infection (MOI) of 100 and harvested after 2, 4, and 6 hours. Additionally, a mock sample was created using PBS instead of bacteria and harvested after 6 hours. Subsequently, all harvested samples were preserved at -80 degrees until SDS-page run.

SDS page

Infection samples were measured using a Bradford assay to determine the protein concentration of the samples and ensure accurate and equal loading. Marker, mock, 2h, 4h and 6h samples loaded into SDS gel and run at 90V for 15 min and then 120V for 45 min.

Western blotting

Gel removed and assembled with membrane between Whatman filter paper to transfer proteins onto nitrocellulose membrane via western blotting. Transfer run for 90 min at 0.14 A. Then membranes were blocked (3% BSA blocking solution) and incubated in primary antibody overnight. After incubation the membrane was incubated once more with secondary antibody for 1.5 hours. Western blot quantified using Bio-Rad ChemiDoc™ MP Imaging System.

Stripping

Membrane was stripped using stripping buffer and β-Mercaptoethanol to allow detection with different primary and secondary antibodies. The membrane was stripped twice to allow detection of three proteins (CagA, P-CagA and GAPDH).

Primary Antibody	Dilution	Secondary Antibody	Dilution
4G10	1:2500 in 3% BSA	Anti-mouse-HRP	1:15000 in Rotiblock
CagA1B2	1:2500 in 3% BSA	Anti-rabbit- HRP	1:15000 in Rotiblock
GAPDH	1:5000 in Rotiblock	Anti-rabbit- HRP	1:15000 in Rotiblock

Table 1: Primary and matching secondary antibodies used to detect CagA, P-CagA and GAPDH in western blot. Phosphorylated CagA in P12 infected AGS cells detected with 4G10 primary antibody and Anti-mouse-HRP. Unphosphorylated CagA detected with CagA1B2 and Anti-rabbit-HRP. GAPDH positive control detected with GAPDH antibody and Anti-rabbit-HRP.

Bacterial growth curve

Bacterial culture:

Agar plates cultivated with P12 wildtype, P12 Δ Hp1012, N6 wildtype and N6 Δ Hp1012 from cryo-stock.

BHI medium:

BHI medium prepared with 1% β-CD and filtered to sterilise. Bacteria were collected and dissolved in BHI medium, and OD measured. Then measured OD was used to calculate volume of bacterial substrate that needed to be added to Erlenmeyer flask and filled to 20mL with BHI medium to obtain an OD of 0.1. Erlenmeyer flasks with bacteria were incubated overnight at 37 degrees to allow bacteria to acclimate to fluid culture.

Microplate reader:

Following incubation, the OD of the fluid bacterial culture was measured and diluted to OD 0.1 with either BHI medium + 10% FCS or BHI medium + 1% β-CD. Samples (P12 WT + β-CD, P12 WT + FCS, N6 WT + β-CD, N6 + FCS) were loaded into an 86-well plate and incubated for 43.5 hours in a ClarioStar microplate reader to measure the absorbance (OD600) of the samples every 30 min.

Results

Infection Experiment

Figure 1 depicts the expression patterns of CagA, Phosphorylated CagA (P-CagA), and GAPDH in AGS cells treated with P12 WT H. pylori for varying durations (2, 4, and 6 hours). Lane one represents a mock sample of AGS cells treated with PBS and harvested after six hours that reveals no discernible bands for CagA or P-CagA expression, except for a 120kDa band too small to indicate CagA. This additional banding at approximately 120kDa remains present in all samples. In lane two, AGS cells treated with P12 WT for 2 hours exhibit robust bands for both CagA and P-CagA expression. Lane three displays AGS cells infected for 4 hours, showing reduced expression of both P-CagA and CagA compared to the two-hour sample. In lane four, AGS cells after 6 hours of infection exhibit the lowest levels of phosphorylated and unphosphorylated CagA. Overall, AGS cells infected with WT H. pylori consistently demonstrate CagA and P-CagA expression, with the strongest banding observed at 2 hours. GAPDH served as a positive pipetting control and was present in all samples with weaker banding in the 2- and 6-hour samples.

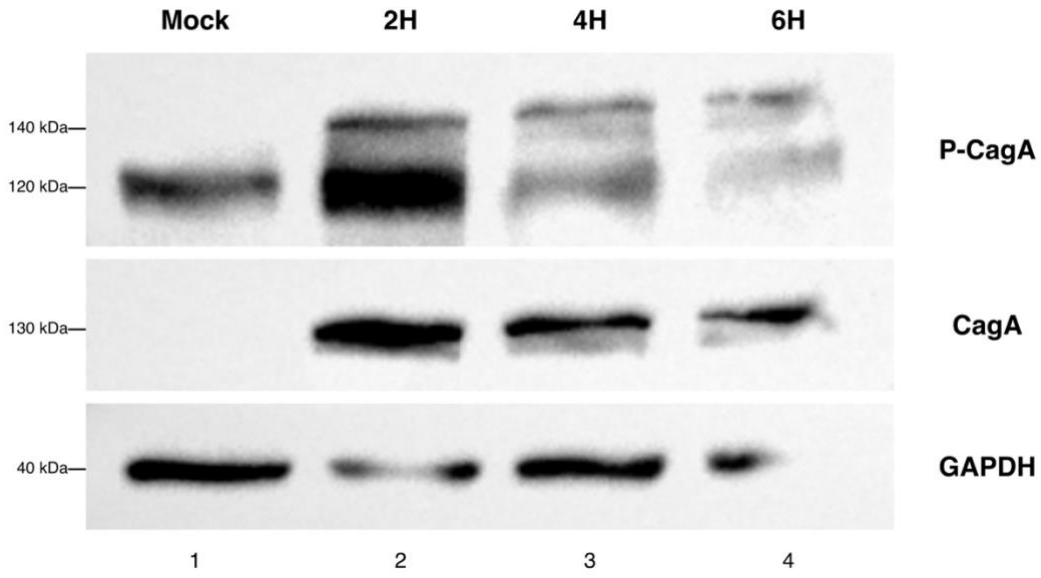

Figure 1: Western blot indicating expression of Cag A, Phosphorylated Tryrosine (P-CagA) and GAPDH in AGS cells infected with P12 WT. AGS cells infected with P12 wildtype for two, four and six hours. Cells were cultured in RPMI. P-Tyr (4G10) detects phosphorylated CagA. GAPDH acted as pipetting control.

Bacterial growth curve

The growth curves of Helicobacter pylori strains N6 and P12 were analysed over a 43-hour period in two distinct culture media: BHI supplemented with β-CD and BHI supplemented with FCS (Figure 2). Comparing P12's growth in the two media types, it is evident that P12 exhibited faster growth in the BHI + FCS medium than in the BHI + beta-cyclodextrin medium. The log phase was more sustained and reached a higher cell density when FCS was present, suggesting that P12 thrives more efficiently in the presence of FCS. Similarly, N6 displayed enhanced growth in the BHI + FCS medium compared to the BHI + beta-cyclodextrin medium. The lag phase was shorter, and the exponential phase was more robust in the presence of FCS. Interestingly, N6 cultured in beta-cyclodextrin seemed to experience a decline in cell numbers after 36 hours, indicating a potential adverse effect on its viability in this specific medium.

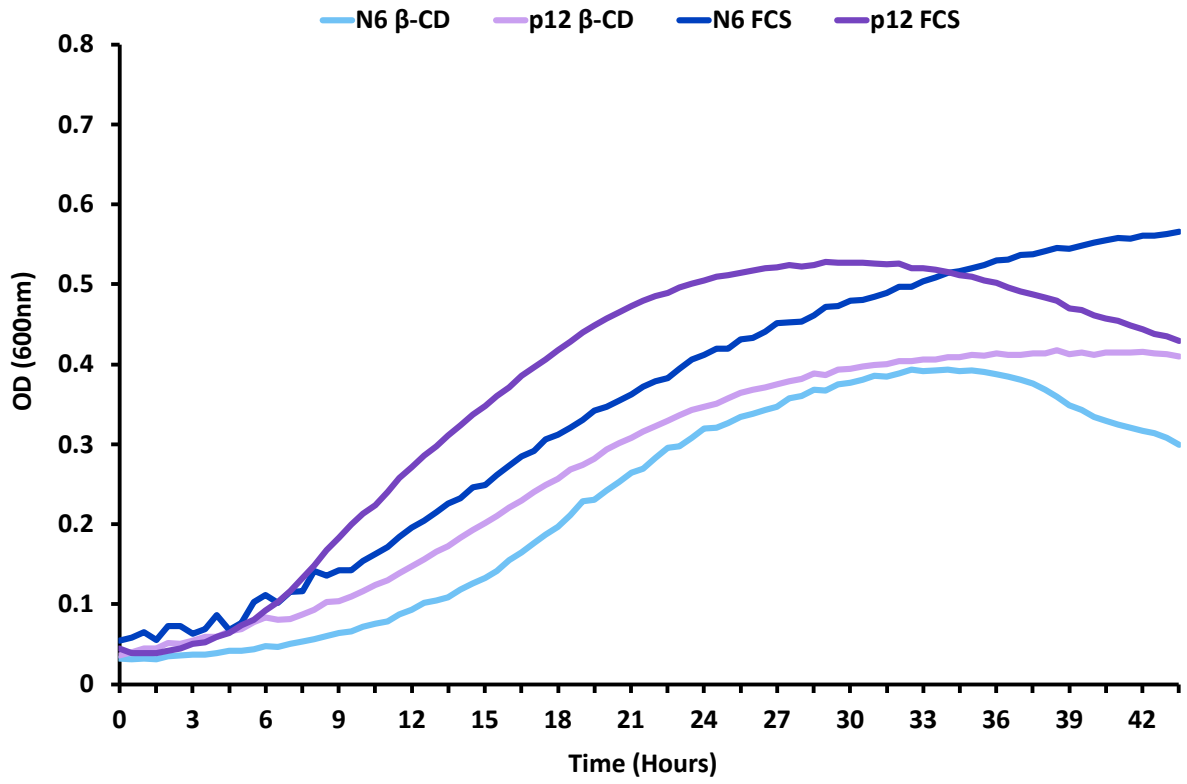

Figure 2: Growth curve indicating N6 and p12 strain growth over 43-hour period in BHI medium with addition of β-CD or FCS. Absorbance (OD 600) measurements taken every 30min with ClariStar microplate reader. BHI medium enriched with either 1% β-CD or 10% FCS. Blank subtracted before plotting.

Discussion

The primary objective of this study was to assess the presence of virulence factors Hp1012 and CagA in P12 WT and P12 ΔHp1012 infected AGS cells over a 6-hour period. Simultaneously, we aimed to establish a growth curve for H. pylori WT and ΔHp1012 mutants derived from both P12 and N6 strains cultured in Brain Heart Infusion (BHI) medium supplemented with either β-Cyclodextrin (β-CD) or fetal calf serum (FCS). The comprehensive investigation aimed to deepen our understanding of H. pylori's actions during AGS cell infection and refine the growth conditions for H. pylori WT and ΔHp1012 mutants.

To investigate the temporal dynamics of CagA in H.pylori infected host cells, AGS cells were infected with P12 WT and harvested after two, four, and six hours (Figure 1). As anticipated, cells treated with PBS instead of P12 WT exhibited no expression of CagA in either its phosphorylated or unphosphorylated state. This is because CagA is produced by H.pylori itself and is not synthesized by AGS cells, additionally CagA can only be phosphorylated when it is injected (via T4SS) into the cytoplasm of a host cell as bacteria do not have the ability to phosphorylate tyrosine (Backert et al., 2001). Therefore, it makes sense that P12 WT-infected samples (Figure 1) show the presence of both phosphorylated and unphosphorylated H. pylori. Additionally, all samples indicated extra banding at 120kDa (Figure 1) which is not consistent with the size of P-CagA, this extended banding is most likely a result of the 4G10 antibody detecting other phosphorylated tryrosine residues that are not related to CagA. Interestingly, the highest CagA expression was observed at the two-hour time point, a deviation from the expected trend based on previous studies in which CagA expression should remain relatively constant and P-CagA should increase over time (Krisch et al., 2016).

This inconsistency may stem from a potential loading error or complications during membrane stripping. Thus, further investigations are needed to determine if there is any true differences in the expression of CagA and P-CagA in H.pylori infected cells over time.

Unfortunately, unforeseen contamination resulted in the loss of AGS cells before infection with P12 ΔHp1012, limiting our ability to analyze Hp1012 expression in these mutant strains. Emphasizing the importance of careful experimental procedures and the necessity for repeated experiments to ensure reliable results.

In assessing the most favorable conditions for H. pylori growth, a growth curve spanning 43 hours compared N6 and P12 WT strains in BHI medium supplemented with either FCS or β-CD. While the initial goal was to optimize the growth conditions for ΔHp1012 strains, these mutants proved challenging to cultivate in liquid medium, necessitating their exclusion from the study. Instead, the study was continued using only N6 and P12 WT bacteria. The resulting growth curve (Figure 2) indicates that P12 WT in BHI + FCS medium offered the most reliable bacterial culture for rapid growth. Conversely, if sustained proliferation is required over an extended period, N6 + FCS may be preferable, as it exhibited no signs of reaching the logarithmic decline (death phase) after 43 hours, unlike P12 WT in BHI + FCS. Notably, both P12 and N6 strains cultured in BHI + FCS showed more exponential growth during the log phase compared to those in BHI + β-CD, highlighting FCS as a more suitable medium for the growth of N6 and P12 H. pylori strains.

In conclusion, our study confirms the expression and phosphorylation of CagA in all H. pylori-infected cells. However, further investigations are needed to analyze potential differences in signal strength with an increase in the duration of AGS infection. Additionally, FCS emerges as a preferable supplement to BHI medium for cultivating N6 and P12 WT H. pylori strains. Notably, N6 in BHI + FCS demonstrated prolonged viability compared to P12 WT. Future research should prioritize the proper execution of experiments involving ΔHp1012 strains to provide comprehensive insights into the intricacies of H. pylori pathogenicity.

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