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ROLE OF THE GENE HP0102 ENCODING A CONSERVED LPS GLYCOSYLTRANSFERASE IN THE PATHOGENESIS OF HELICOBACTER PYLORI

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ABSTRACT

Helicobacter pylori are a major human pathogen and are associated with chronic gastric inflammation, peptic ulcer disease and gastric cancer. Contact with host cells is recognized as a signal capable of triggering expression of bacterial genes important for host pathogen interaction. Adherence of H. pylori to the gastric epithelial cell lines AGS and MKN45 strongly upregulated expression of a gene HP0102 in the adhered bacteria as determined by qRT-PCR. In silico analysis suggested that HP0102 shows tremendous sequence conservation among different strains of H. pylori including several Indian clinical isolates and was predicted to encode for a glycosyltransferase enzyme. To elucidate the role of HP0102, a HP0102 knockout strain was constructed (ΔHP0102) and analyzed. The gene was found to be associated with two distinct phenotypes related to pathogenicity. In AGS cell-adhered H. pylori, it has a role in upregulation of cag A, a major virulence factor and consequent induction of the hummingbird phenotype in the infected AGS cells. HP0102 was also found to be involved in the glycosylation of bacterial lipopolysachharides (LPS) by glycostaining analysis. Bacterial LPS is a major virulence factor triggering the expression of cytokines via TLR 2 and TLR4 dependent signaling cascades. Results of a cytokine array using the cell culture supernatants of MKN45 cells (expressing both TLR2 and TLR4) infected with either H. pylori wild type or the ΔHP0102 strain suggested that the HP0102 mutant was impaired in inducing the expression of several cytokines including the proinflammatory cytokine IL-8. Further work is under progress to identify more specific functions of HP0102 and also identify other signaling networks that may be affected by LPS and its glycosylation state during the pathogenesis of H. pylori.

KEYWORDS: Helicobacter pylori Peptic ulcer disease, Gastric cancer and Cytokine array.

INTRODUCTION

Helicobacter pylori are a Gram-negative, microaerophilic bacterium which is specialized in colonization of the gastric mucosa.[1] Between 1979- 82, Australian pathologist, Robin Warren and Australian gastroenterologist, Barry Marshall, identified H. pylori and suggested a link to the development of stomach ulcers. H. pylori are slow growing microorganisms but act as a dominant pathogen that can cause peptic ulcers and gastritis that can lead to gastric cancer.[2] Human stomach acts as a reservoir providing most suitable environment for the growth of the H. pylori. Its name refers to both its spiral shape and the area of the lower stomach which it habitually colonizes between the stomach and small intestine.[3] The adaptation to such a harsh and acidic environment in human stomach reduces its competition with other bacterium and enhances its ability to cause chronic infection.[4] This makes H. pylori one of the most successful human bacterial parasites, which colonizes more than 50% of the human population.

The link between Helicobacter pylori and peptic ulcer, first recognized by Barry Marshall and Robin Warren in 1982, provided major insight into human gastric pathology. Colonization ranges from 50-100%, making Helicobacter pylori the most common infectious agent of humans in the world today.[4] Because of the prevalence and importance of Helicobacter pylori infection, understanding the mechanisms by which it colonizes the gastric mucosa and causes disease has received intense interest. Helicobacter pylori possess several putative colonization factors, including urease, various adhesins and flagellar motility, some of which have been shown to be necessary for gastric colonization. The most severe Helicobacter pylori mediated disease states are attributed to strains harbouring the cag pathogenicity island (cag PAI), a 40 kb DNA element. Analysis of the cag PAI sequence suggested that it encodes a putative secretion

apparatus with homology to type-IV secretion system, which are involved in the transfer of effector macromolecules into host cells.[5-6] In 1994, the National Institute of Health (USA) published an opinion stating that most recurrent duodenal and gastric ulcers were caused by H. pylori and recommended that antibiotics be included in the treatment regimen.[7-8] Warren and Marshall were awarded the Nobel Prize in Medicine in 2005 for their work on H. pylori.

MATERIALS AND METHODS

Media preparation

Brain Heart Infusion (BHI) Agar medium: All components were weighed by using a weighing balance and dissolved in 285ml of double distilled water in a 500ml conical flask and mixed gently. Then the flask was autoclaved at 121°C, 15lb/sq inch for 15 minutes. After autoclaving the flask was allowed to cool at rum temperature. Then 5% (15ml) horse serum (GIBCO) was added to the flask.15ml of media was poured on each sterile petri plates and gently swirled in a laminar air flow chamber. The plates were left aside over night for solidification and then stored at 4°C as shown in Table 1.[10]

Table 1: Brain Heart Infusion (BHI) Agar medium (300ml).

Components	Quantity
BHI powder	16.8g
Bacto agar	4.5g
Distilled water	Upto 285 ml

GC Blood Agar Medium: The above all components were weighed by using a weighing balance and dissolved in 170 ml of distilled water taken in a 250ml conical flask and mixed gently. Then the flask was autoclaved at 121°C, 15lb/sq inch for 15 minutes. After autoclaving 13.6ml defibrinated horse blood (Remel) was added to the flask and incubated at 80°C for 20 minutes and then allowed to cool at 50°C.After cooling 16ml horse serum (GIBCO) and 560µl isovitalex (BD BBL) was added to the flask and mixed properly. [20µg/ml Chloramphenicol was added to the flask when required.]Then 15ml of media was poured on each sterile petri plates and gently swirled. The plates were left aside over night for solidification and then stored at 4°C as shown in Table 2.[11]

Table 2: GC Blood Agar Medium (200ml).

Components	Quantity
GC agar base	4g
Bacto agar	480mg
Cysteine HCl	80mg
Distilled water	Upto 170ml

Brucella Agar: Above all the components were weighed by using a weighing machine and dissolved in 170ml of distilled water taken in a 250ml conical flask and mixed gently. Then flask was autoclaved at 121°C, 15lb/sq inch for 15 minutes. After autoclaving the flask was allowed to cool at 50°C.Then 5% (15ml) horse serum (GIBCO) was added to the flask.15ml of media was poured on each sterile petri plates and gently swirled. The plates were left aside over night for solidification and then stored at 4°C as shown in Table 3.[12]

Table 3: Brucella Agar (300ml).

Components	Quantity
Brucella Broth mixture	8.4g
Agar	4.5g (1.5%)
Cysteine HCl	120mg
Distilled water	Upto 285ml

Brucella soft agar plates: Brucella agar also prepared by following as mentioned Table 4 protocol and here only the amount of agar added was restricted to 0.3%. 5.194g of RPMI-1640 (GIBCO) and 1gm $NaHCO_3$ (.2%) were weighed and dissolved in 50ml (10%) heat inactivated Fetal Bovine Serum (FBS) [Pan Biotech] in a clean 500ml conical flask 5ml of Penstrep (5,000units/ml penicillin G sodium salt and 5000µg/ml streptomycin monosulfate) (GIBCO) was added to the flask. Then the final volume was made upto 500ml by adding required amount of distilled water. The components were mixed properly and filtered with 0.22µm filter units, collected in 50ml sterile falcon tubes and stored at 4°C.[13]

Table 4: RPMI-1640 Complete Medium (500ml).

Components	Quantity
RPMI-1640	5.194g
$NaHCO_3$	1g
Fetal Bovine Serum(FBS)	50ml (10%)
Penstrep	5ml
Distilled water	Upto 500ml

Maintenance of Helicobacter pylori culture: A heavy loopful of H. pylori culture was scrapped out from 48 hour old GC blood agar plate and streaked onto a fresh GC agar plate with a sterile inoculating loop for mass culturing of the bacteria. The plate was then incubated at 37°C under microaerophilic conditions (10% CO_2) for 48 hours and subcultured every 2 days.[14]

Maintenance of AGS cell line: A confluent T-25 tissue cultue flask was taken and the media was discarded completely. The flask was washed twice with 3ml 1X PBS.1ml Trypsin-EDTA(0.5%) was added and incubated at 37°C.3ml of complete RPMI-1640 medium was added and the bottom of the flask was washed gently. The media was collected in a 15ml tube and centrifuged at 1200rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1ml of RPMI-1640 complete media (including 10% FBS and Pencillin + streptomicin).[15]

Adherence of H. pylori with AGS cell line: A heavy loopful of bacterial culture was scraped out from a 48

hour cultured GC plate and suspended in 1ml filtered PBS in a 1.5ml microcentrifuge tube. The tube was centrifuged at 4000 rpm for 5mins at room temperature. The pellet was washed with 1X PBS and the pellet was re-suspended in RPMI-1640 incomplete medium (without serum and antibiotics).The Optical Density of the cells were measured at 600nm by UV spectrophotometer and the volume for 1 O.D cells were calculated (1 O.D=5×10^8 cells). The required amount of cells (5×10^8) was added to the AGS monolayer at multiplicity of infection (MOI) of 50 in 0.5ml of RPMI media. The T-25 flask was incubated at 37°C under microaerophilic condition as described above. The cultured AGS cell line in T-25 flask was observed under phase contrast microscope (Zeiss) for confluency. The experimental flask was washed with 3ml of filtered 1X PBS and then PBS was pipette out completely.4ml of incomplete media was added in the experimental and control flask(without the cell line).5×10^8 cells(H. pylori) added in both experimental and control flask.500µl of FBS(Pan Biotech) was added to both the experimental and control flask. The flasks were incubated under microaerophilic condition for 2 hours. After incubation 10µl of supernatant was taken from both the flasks for CFU (Colony Forming Unit) assay. This was considered as CFU taken before wash. The control flask was kept for RNA isolation.[16]

Isolation of adhered bacteria flask: The media was fully decanted from the flask and washed thrice with 3ml 1X PBS. Again 1ml of 1X PBS was added and 10 µl was taken from it and mixed with 990 µl 1XPBS for CFU assay. This was considered as CFU taken after last wash. The remaining PBS was decanted and 1ml of 0.25% Triton X 100 was added and incubated for 2 minutes at room temperature. The lysed mixture was taken in an eppendorf tube and vortexed vigourously for 1-2 minutes and then centrifuged at 8000rpm for 2 minutes. Triton X 100(which had no effect on the viability of H. pylori) was decanted and 1ml of PBS was added and vortexed vigorously.10 µl of the above mixture was taken for CFU assay (this was considered as CFU taken for adhered bacterial cells) and the remaining solution was kept for RNA isolation. 0.5µl seeded in one T25 flask so that the cells were expected to get fully confluent after 48 hours of incubation at 37°C in a 10% CO_2 incubator.[17]

RNA isolation from H. pylori cells by Trizol method: A loopful of H. pylori cells were scraped out from a 48 hour old cultured GC blood agar plate and suspended in 1ml of filtered 1X PBS in a 1.5ml eppendorf tube. The tube was centrifuged at 8000rpm for 2minutes at room temperature. Supernatant was discarded and pellet was resuspended in 1ml Trizol. The samples were incubated and vortexed vigorously for 15 minutes in ice to permit the complete lysis of bacterial cells.200µl of Chloroform per ml of Trizol was added, shaken vigorously by hand and vortexed for 15 minutes and intermittently kept in ice. Then the samples were incubated in ice for 15 minutes, then at room temperature for 2-3 minutes. Then the samples were centrifuged at 12000g for 15 minutes at 4°C.400µl of the aqueous phase was transferred into a fresh tube.400µl of Isopropanol was added and mixed by inversion for precipitating the RNA from the aqueous phase. The sample was vortexed and incubated at room temperature for 10minutes.The samples were centrifuged at 12000g for 10 minutes at 4°C.The supernatant was discarded and the pellet was washed in 1ml of 75% ethanol and stored at -70°C overnight. RNA pellet in 1ml of 75% ethanol was mixed by vortexing and centrifuged by 7500g for 10 minutes at 4°C.The RNA pellet was dried briefly for 5-10minutes and dissolved in 10µl DEPC (Diethyl pyrocarbonate) water then vortexed for 10 minutes. Then heated at 65-70°C for 5-10 minutes Vortexed for 2minutes and kept in ice from the total content 2µl of the sample was taken and mixed with 998µl of DEPC water. The absorbance (O.D) of the solution was taken at 260nm.[18]

Isolation of chromosomal DNA by Sodium Perchlorate ($NaClO_4$) Method: A loopful of H. pylori culture was scrapped out from the 48 hour old GC blood agar plate and suspended in 1ml of 1X filtered PBS. The sample was then centrifuged at 6000rpm for 3mins.The pellet was dissolved in 100µl of Sodium chloride Tris EDTA (STE) (0.1M) 0.5% SDS from 10% SDS stock and 5µl of Proteinase K (50µg/ml) was added to the sample. The sample was incubated at 37°C for overnight. Next day the samples were brought at room temperature and again 5µl 10% SDS was added. Equal volume of Tris saturated phenol (120µl) was added and kept on ice for 10 minutes. The sample was centrifuged at 6000rpm for 5-10 minutes and the aqueous supernatant was taken in a fresh eppendorf tube. Then equal volume of Phenol chloroform (1:1) was added and kept on ice for 10 minutes. Again it was centrifuged at 6000rpm for 10 minutes and the aqueous supernatant was taken in a fresh eppendorf tube.1M sodium perchlorate ($NaClO_4$) was added and then equal volume of chloroform was added to the sample and then kept on ice for 10 minutes. The sample was centrifuged at 6000rpm for 10 minutes and the supernatant was taken in a fresh eppendorf tube.4µl R Nase (composition: 10mg RNase,15mM NaCl,5mM Tris pH8,volume adjusted to 1ml) was added to the sample and incubated at 37°C for 30 minutes. Then equal volume of chloroform was added to the sample and kept on ice for 10 minutes. The sample was centrifuged at 6000rpm for 10minutes. This step was again repeated. The supernatant was taken in a fresh eppendorf tube and 1ml of cold ethanol was added to the sample. The sample was then centrifuged at 13000rpm for 15 minutes. The pellet was dissolved in 200µf distilled water and stored at 4°C.[19]

Isolation of plasmid DNA (pRY109) from Escherichia coli: 1.5ml overnight culture was taken in a eppendorf tube centrifuged at 8000rpm for 2minutes.The supernatant was discarded and another 1.5ml overnight culture was added to the pellet. Centrifuged at 8000 rpm for 2minutes and supernatant was discarded. Pellet was

resuspended in 100µl solution and kept in ice for 5minutes.200µl freshly prepared solution-II [composition: 4M NaOH and 10% SDS] was then added and kept in ice for 5minutes. 150 µl solution-III [composition: 5M CH_3COOK (pH4.8) and glacial acetic acid] was then added and kept on ice for 20 minutes. The mixture was then centrifuged at 10,000rpm for 10 minutes. The supernatant was transferred to a fresh eppendrof tube and equal volume of phenol-chloroform was added to the tube mixed well and kept in ice for 3 minutes. The mixture was centrifuged at 10,000rpm for 10 minutes. Aqueous layer was transferred in a fresh eppendorf tube and equal volume of chloroform was added to the tube mixed well and kept on ice for 3 minutes. Then the mixture was centrifuged at 10,000 rpm for 10 minutes twice. Aqueous layer was transferred in a fresh eppendorf tube.0.3M NaCl was added to each eppendorf tube. Equal volume of isopropanol was added mixed well and kept at room temperature for 1 hour. Centrifuged at 13,000rpm for 20 minutes Supernatant was discarded and the pellet was resuspended in 1ml of 80% ethanol and kept in ice for 20 minutes. Centrifuged at 13,000 rpm for 20 minutes Supernatant was discarded and the pellet was allowed to dry at room temperature until it became transparent.4 µl RNase (50-100 µg/ml) was added to the pellet and incubated at 37°C for 30 minutes. The pellet was then dissolved in 10-20 µl of millipore water or in TE buffer.[20]

PCR-Polymerase chain reaction

PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies.[21]

Quantitative Real-Time PCR (qRT-PCR): The purity of the RNA sample was checked by spectrophotometer. 2 µg of RNA isolated from H. pylori cells incubated under different conditions using Trizol reagent (Invitrogen) was taken for the quantitative real time analysis. Samples were treated with RNase free DNase I (1U/ µg, Roche) in the presence of an RNase inhibitor (RNasin, Invitrogen). Samples were then incubated for 40 minutes at room temperature. 2 µl (25mM) EDTA was added to the samples. Samples were incubated in a water bath at 75°C for 10 minutes. After 10 minutes samples were taken out and immediately kept in ice for 'heat chill' shock. Quantitative real time PCR (qRT-PCR) was performed using SYBR green one step RT-PCR kit (Takara) in an iCycler IQ5, Real-time PCR detection system (Roche).[22] The wells of the plate were loaded with the above reagents and with RNA and primers of choice, covered with a piece of transparent sealing tape and the plate was then roatated for 5 minutes the plate was then placed on the Roche Real-time PCR detection system after setting the following thermal cycling parameter. A melting curve was also generated to verify that a single product was amplified. From threshold cycle (C_T) values; the relative levels of expression of the genes under different conditions were calculated using the $2^{-\Delta\Delta CT}$ method using 16SrRNA as control.

Construction of Chloramphenicol acetyl transferase (cat) cassette for vector free allelic replacement mutagenesis: The vector-free allelic-replacement mutagenesis strategy used to generate HP0102 deficient mutant. Chloramphenicol resistance (Cm^r) cassette cat originating from was amplified with the primers cat F and cat R (listed in Table-2) and the plasmid pRY109 was used as the template. H. pylori SS1 genomic DNA was isolated as previously described. Four pairs of gene specific primers designated HP0102 P1/HP0102 P2 (for HP0102) and HP0102 P3/HP0102 P4 (for HP0102) were used to PCR amplify the up- and downstream regions, respectively, of each target gene in order to produce fragments of approximately 500 base pairs (bp) flanking the region to be deleted. The P2 and P3 primers contained 5' overhangs complementary to the cat gene sequence. Amplicons generated with each primer pair (Left flank, Right flank, and cat) were amplified in a second PCR with P1 and P4 primers to generate the long PCR product (mutation construct or allelic replacement cassette). This product was used for natural transformation of H. pylori, followed by selection on the appropriate antibiotic chloramphenicol in this case.[23]

PCR reaction mixture: In each case primers P1 and P2 refers to primers for amplification of left flanking sequence of HP0102 P1 and HP0102 P2 for amplification of left flanking sequence of HP0102. In each case primers P3 and P4 refers to primers for amplification of left flanking sequence of HP0102 P3 and HP0102 P4 for amplification of left flanking sequence of HP0102.The reaction mixtures were placed in a thermal cycler and was run according to the following thermal cycling parameters as shown in Table 5.[24]

Table 5: PCR reaction mixture.

Components	Left flank	Chloramphenicol acetyl transferase (cat)	Right flank
Template	100ng (H. pylori G27 genomic DNA)	100ng (pRY 109)	100ng (H. pylori G27 genomic DNA)
10X Taq buffer (Thermo scientific)	5µl	5µl	5µl
10mM dNTPS	1µl	1µl	1µl
Taq polymerase(5U/µl) (Thermo scientific)	0.1µl	0.1µl	0.1µl
Forward primer (10pm)	1µl (P1)	1µl (cat F)	1µl (P3)
Reverse primer (10pm)	1µl (P2)	1µl (cat R)	1µl (P4)
MilliQ water	Made upto 50 µl	Made upto 50 µl	Made upto 50 µl

Purification of the PCR products using kit (QIAGEN): The products obtained from the above PCR reaction were subjected to purification using QIAGEN PCR purification kit. 5 volumes Buffer PB was added to 1 volume of the PCR product mixture. A QIAquick column was placed in a provided 2 ml collection tube. To bind DNA, the sample was applied on to the QIAquick column, allowed to stand for 5 minutes and centrifuged at 13,000rpm for 30–60 seconds. The flow-through was discarded and the QIAquick column was placed back in the same tube. To wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged at 13,000 rpm for 60 seconds The flow-through was discarded and the QIAquick column was placed back in the same tube. The QIAquick column was centrifuged once more at 13,000 rpm for 60 seconds in the provided 2 ml collection tube to remove residual wash buffer. Then each QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl milli Q water (pH 7.0–8.5) was added to the center of the QIAquick membrane,was allowed to stand for 5minutes and the column was centrifuged at 13,000rpm for 60 seconds.[25]

Visualization of the PCR product by Agarose gel electrophoresis: 10µl of the purified PCR product was mixed with 5µl 6X gel loading dye and was run on a 2% agarose gel along with a 100bp ladder and then the bands were visualized under gel doc (Biorad).[26]

Splicing by Overlap Extension (SOE) PCR: The corresponding flanking sequences of HP0102 was taken and mixed with the amplified cat gene in a 1:1:1 proportion. All the reagents except the primers P1 and P4 (corresponding to HP0102) were added and the reaction mixtures were placed in a thermal cycler after setting the following thermal cycling parameter. After 6 cycles the thermal cycling was paused and primers P1 and P4 were added. Then again thermal cycling was continued abiding the following protocol. Thermal cycling was paused and primers P1 and P4 were added. Then again thermal cycling was continued abiding the following protocol.[27]

Visualization of the PCR product by Agarose gel electrophoresis: 10µl of the purified PCR product was mixed with 5µl 5X gel loading dye and was run on a 1.5% agarose gel along with a 1kb ladder and then the bands were visualized under gel doc (Biorad).[28]

Transformation of Helicobacter pylori cells with the mutant constructs

DAY-1: A heavy loopful of H. pylori wild type cells were scraped out from a GC blood agar plate and washed with 1X PBS. Then it was divided equally in two 1.5ml microcentrifuge tubes and centrifuged at 8000rpm for 2 minutes. The supernatant was discarded and the pellet in each tube was resuspended in 100µl of BHI broth. 100µl of the each of the mutant constructs (for ΔHP0102) prepared by SOE PCR and was added in one microcentrifuge tube containing the resuspended culture. The entire mixture from microcentrifuge tube was dropped on the centre of GC blood agar plate and was then incubated for 24 hrs under microaerophilic conditions for uptake of DNA by bacterial cells.[29]

DAY-2: After 24hrs of incubation the, bacterial cultures were streaked onto chloramphenicol (cam) GC blood agar plate to obtain isolated colonies. Then the plates were incubated for 48hours at 37°C under microaerophilic condition.[29]

DAY-4: After 48 hours of incubation 10 single colonies from mutant was picked with sterile toothpicks and then streaked onto chloramphenicol (20µg/ml) GC blood agar plate. Then the plate was incubated for 48hrs at 37°C under microaerophilic condition.[29]

Microscopic analysis of AGS cell lines following its adherence with H. pylori wild type and H. pylori ΔHP0102 mutant cells: A heavy loopful of bacterial cultures(wild type, ΔHP0102)were scraped out from 48

hour cultured GC plates and suspended in 1ml filtered PBS in a 1.5ml microfuge tubes. The tubes were centrifuged at 4000 rpm for 5mins at room temperature. The pellets were washed with 1X PBS and the pellet were resuspended in RPMI-1640 incomplete medium.(without serum and antibiotics).The O.D of the cells were measured at 600nm by UV spectrophotometer and the volume for 1 O.D cells were calculated (1 O.D=5×10^8 cells).The required amount of cells (5×10^8) was added to the AGS cell line monoloayer of 50% confluency grown in T-25 flasks at multiplicity of infection(MOI) of 50 in 0.5ml of RPMI media. One of the flasks was incubated with 5x108 wild type cells, one with 5x108 ΔHP0102 mutants and the third one with 5x108 mutants. Another T-25 flask was kept as control in which no bacterial cells were added. The flasks were then incubated under microaerophilic conditions (10% CO2).After every one hour following infection the flasks were checked under the microscope for visible morphological changes in the AGS cell line. Images of the infected AGS cell line were taken after 5 hours following infection.[30]

AGS cell line fixation for microscopy: 5ml of cell fixation solution (PBS-4.25ml, 40% Formalin-250µl, 25% Glutaraldehyde-500µl) was prepared for cell fixation. The tissue culture flask was taken the media fully decanted from it and then washed with 1X PBS twice.1ml of fixative solution was then added to the flask and allowed to stand for atleast 10 minutes. Cell fixative solution was fully decanted from the flask and washed with 1X PBS twice. 2ml of 1X PBS was added to the flask to prevent the cells from drying. Finally images were taken under phase contrast microscope as necessary.[31]

Isolation of H. pylori Lipopolysaccharides (LPS): H. pylori strains grown in GC agar plates for 48 hours were harvested (1 O.D), resuspended in 200 µl of 1X SDS-buffer (2% β-mercaptoethanol, 2% SDS and 10% glycerol in 0.1M Tris-HCl, pH 6.8, pinch of bromophenol blue), boiled for 15 minutes, followed by incubation at room temperature for 15 minutes. Next, 5 µl of both D NaseI (10mg/ml) and R Nase A (10mg/ml) were added and incubated at 37°C for 30 minutes, followed by addition of 10 µl of Proteinase K (10mg/ml) and incubation at 60°C for 3 hours. To each sample 200 µl of ice cold Tris saturated phenol was next added and incubated at 65°C for 15 minutes with occasional vortexing. The samples were next mixed with 1 ml of diethyl ether at room temperature and centrifuged at 20,600 ×g for 10 minutes. The bottom blue layer was carefully extracted and 15 µl of the sample was separated on a 12% SDS-polyacrylamide gel and visualized either by silver staining (Pierce) or by using the Pro-Q Emerald 300 Lipopolysaccharide gel staining Kit (Molecular Probes) as per manufacturer's instructions.[32]

RESULTS AND DISCUSSION

Identification of genes of H. pylori which are regulated by attachment to gastric epithelial cells and understanding their role in virulence: Contact with host cells is recognized as a signal capable of triggering expression of bacterial genes important for host pathogen interaction. Previously, it has been demonstrated that the expression of H. pylori virulence genes, cag A is strongly induced in bacteria following adherence to epithelial cell lines. In an endeavor to identify factors necessary for host cell contact dependent virulence gene expression, an adherence assay was utilized where H. pylori can adhere to gastric epithelial cells and express its virulence properties under laboratory conditions.

Adherence of H. pylori to the gastric epithelial cell line AGS: Different strains of H. pylori have been reported to efficiently adhere to AGS cells in-vitro. To test whether, H. pylori strain SS1 can also efficiently adhere to AGS cells an adherence assay was performed (Fig.1.A) H. pylori grown to the mid logarithmic phase of growth were incubated with AGS cell line for different time intervals and the number of AGS-adhered bacteria was determined by CFU assay on BHI agar plates. Results of the CFU analysis suggested that the number of adhered bacteria increased gradually up to 12 hours but did not increase any further when examined up to 24 hours (Fig.1.B).

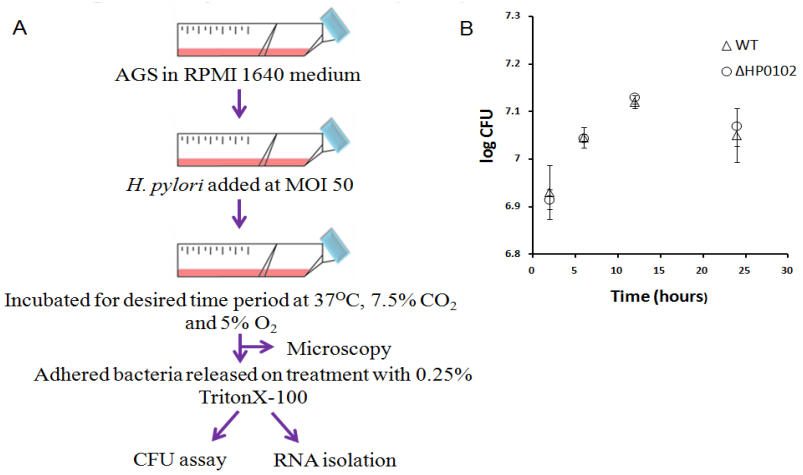


Figure 1: Adherence of H. pylori strain SS1 to the gastric epithelial cell line AGS. (A) Schematic description of adherence assay. (B) H. pylori SS1 WT and ΔHP0102 strains were added to semi confluent AGS monolayers at MOI 50 and CFU of the adhered bacteria was estimated at different time intervals.

Previous studies have identified genes that are essential for colonization of H. pylori in a mice model. The functional roles of only a sub-set of genes identified in these studies are known, while most of the genes lack functional annotation. Moreover, a definite correlation could not be established between any of these genes and regulation of virulence gene expression. HP0102 has been identified as factor essential for colonization of H. pylori. Expression of HP0102 following host cell contact and the role of this gene in H. pylori virulence has been investigated in this study and the results are described in the following sections.

Expression of the HP0102 gene is upregulated in H. pylori following adherence to AGS cells: Semi-confluent monolayers of AGS cells were infected with H. pylori, and expression of the HP0102 gene was examined in the adhered bacteria by qRT-PCR at different time points after adherence (Fig.2). HP0102 expression was also examined in unadhered bacteria grown under identical conditions without cell line or isolated from the supernatant of H. pylori infected AGS monolayer. Significant increase in HP0102 expression was consistently observed in AGS adhered H. pylori as compared to the unadhered controls (Fig.2). No difference in HP0102 expression was observed between unadhered bacteria grown without cell line and those isolated from the supernatant of infected AGS monolayers (Fig.2), suggesting that HP0102 induction required direct contact of the bacteria with the AGS cells and was not due to a component secreted by the cell line.

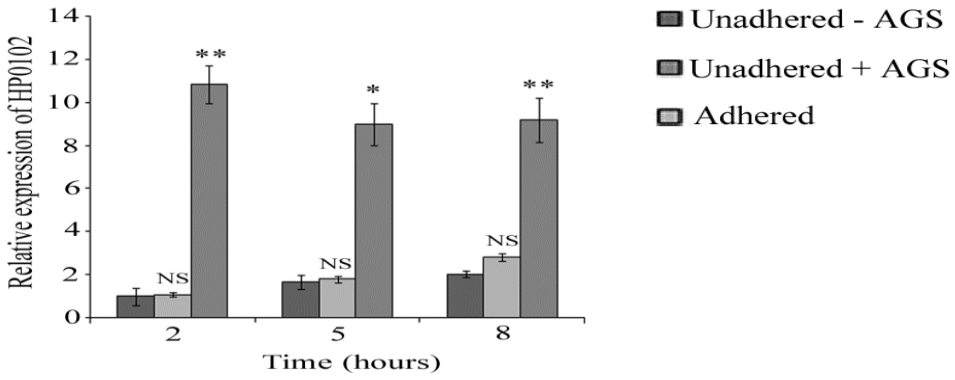


Figure 2: Expression of HP0102 in AGS-adhered and unadhered H. pylori strains Expression of HP0102 at different time points was estimated in unadhered and AGS-adhered H. pylori by qRT-PCR.

Results represent the average of at least three independent experiments. Transcript levels are expressed relative to that in unadhered WT strain at 2 hours arbitrarily taken as 1.Error bars represent SD. The statistical significance (P values) of the differences in gene expression between AGS adhered and unadhered H. pylori strains are indicated. * $P \leq 0.05$; ** $P \leq 0.01$; NS, Not significant.

Prevalence and conservation of HP0102 gene in H. pylori strains: To detect the prevalence of the HP0102 gene in H. pylori strains, the gene sequence of strain 26695 was used as a query to search for homologous

sequences in H. pylori genomes (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene was present in all the 69 H. pylori strains whose genome sequences are available and a high degree of sequence conservation of the gene (more than 93%) was observed among the different strains. Since it is not known if any of the 69 strains were Indian isolates, 15 H. pylori clinical strains isolated from India (Table 6) were examined for the presence of the HP0102 gene. PCR using primers flanking the HP0102 ORF as well as gene internal primers clearly indicated the presence of HP0102 in all isolates (data not shown). Furthermore, sequencing of the PCR products revealed a high degree of conservation of the gene sequence. These results indicated that the HP0102 gene is almost ubiquitously present and highly conserved in H. pylori strains.

Table 6: HP0102 sequence conservation analysis in H. pylori Indian clinical isolates as well pylori strains.

H. pylori strain	Isolated from patients with		cag PAI*	HP0102 sequence conservation[a] (%)	HP0102 gene sequence accession #
	DU*	NUD*			
I-121	+	-	+	96	KT932667
135 (1)	+	-	+	96	KT932666
I-30	+	-	+	96	KT884537
I-90	+	-	+	96	KT884534
I-230	+	-	+	96	KT884538
San52	-	+	+	96	KT884542
San77	-	+	+	96	KT884535
San10	-	+	+	95	KT884539
San64	-	+	+	95	KT884540
162	+	-	+	95	KT884533
Osc51	-	+	+	95	KT884545
I-10	+	-	+	94	KT884541
Osc30	-	+	+	94	KT884543
152 1(A)	+	-	+	94	KT884536
155 1(A)	+	-	+	94	KT884544

Sequence conservation was analyzed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) considering the HP0102 gene sequence of H. pylori strain 26695 as reference. Interestingly, NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis also suggested that HP0102 protein shares homology with glycosyl transferase enzymes that are found in various other bacterial species and contains a conserved glycosyl transferase (GT-2) domain (Fig.3). Further analysis suggested that HP0102 shares similarity with members of protein super-families which are involved in the glycosylation of bacterial Lipopolysaccharide (LPS). Next, to investigate the role of HP0102, a ΔHP0102 deletion mutant was constructed and analyzed.

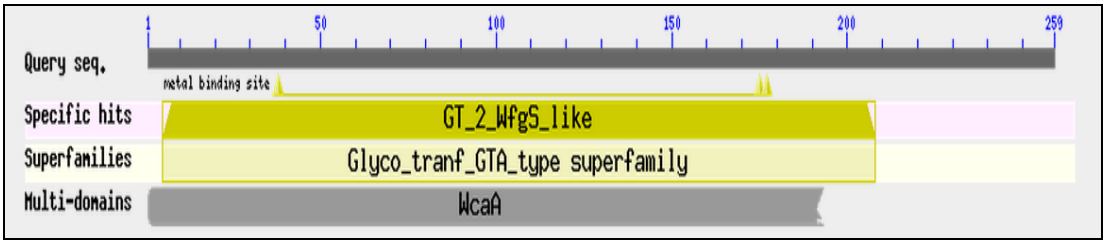


Figure 3: Prediction of a putative glycosyl transferse domain in HP0102 protein by NCBI BLAST.

Construction of ΔHP0102 mutant by non vectorial allelic replacement method: The left flanks and right flanks of the genes to be mutated i.e.HP0102 was amplified and the products was observed in an agarose gel. The flanks had sizes around 500bps and distinct band of the expected size was observed in respective lanes denoting that the flanks were properly amplified. The cat gene was also amplified from the plasmid pRY109. A product of 738bp (between the 700 and 800 bp of the marker ladder) suggested that the cat gene was properly amplified. The left and right flank regions of the gene repG was amplified as positive controls as shown in Figure 4.

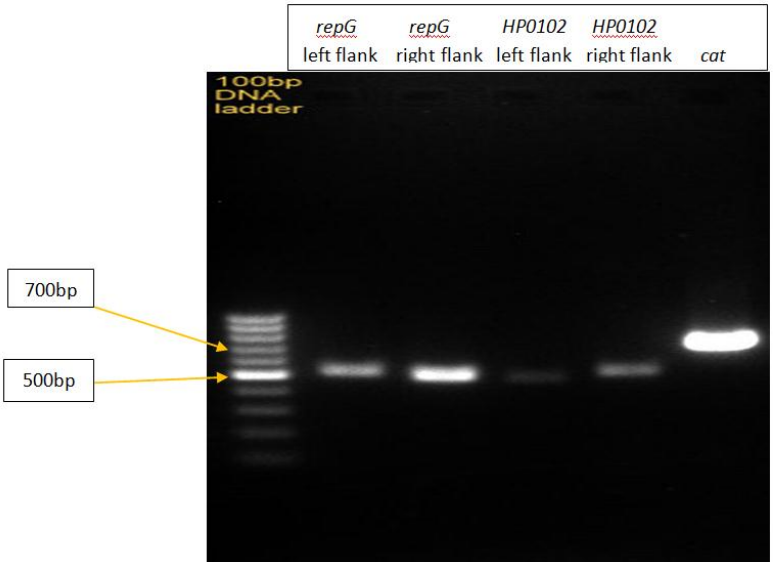


Figure 4 Showing the amplified left and right flanks of repG (positive control) HP0102 and also the amplified cat gene.

Result of mutation cassette construction by SOE PCR: The chloramphenicol resistant cassette (cat gene) fused to the left and right flanks of the gene HP0102 was constructed by SOE PCR and was run on a 1.5% Agarose gel with a 1kb ladder as the marker. Presence of a prominent band between 1.5kb and 2kb in respective lanes signified that the SOE PCR was successful. Thus the obtained product was used for transformation. The construct of cat flanked by the flanking sequence of cat was denoted as cat mutation construct and the construct of cat flanked by the flanking sequences of HP0102 was denoted as ΔHP0102 mutant construct. The exact size of ΔHP0102 mutation construct was 1.76kb as shown in Figure 5.

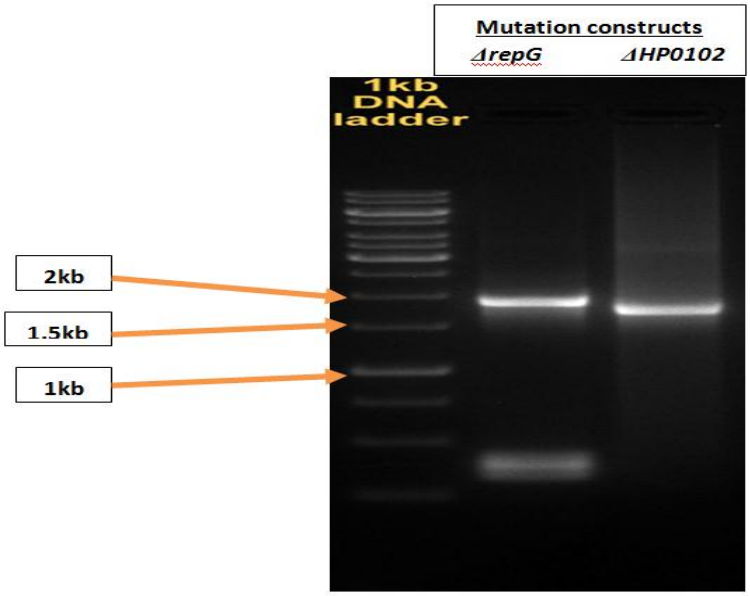


Figure 5: Gel picture showing the mutation constructs, the third lane from the left contains the constructs containing the chloramphenicol resistant cassette with flanks of HP0102 gene. Mutation constructs for the gene repG was generated as a positive control of SOE PCR.

Confirmation of mutant by PCR: To confirm the successful construction of mutant in HP0102 the DNA isolated from the wild type and mutant (ΔHP0102) were subjected to PCR and analyzed by agarose gel electrophoresis.(Fig.10) PCR products with primers P1-P4 shows a shift in the HP0102 mutant (ΔHP0102) with respect to the wild type H. pylori.(Lane 2-3). Presence of cat gene in the mutant and its absence in the wild type strain (lane 4-5) and presence of HP0102 in the wild type and its absence in the mutant (lane 6-7) proved that the cat cassette successfully replaced the HP0102 gene. Successful amplification of cagA for both the wild type and mutant (ΔHP0102) acted as the positive control lane (lane 8-9) as shown in Figure 6.

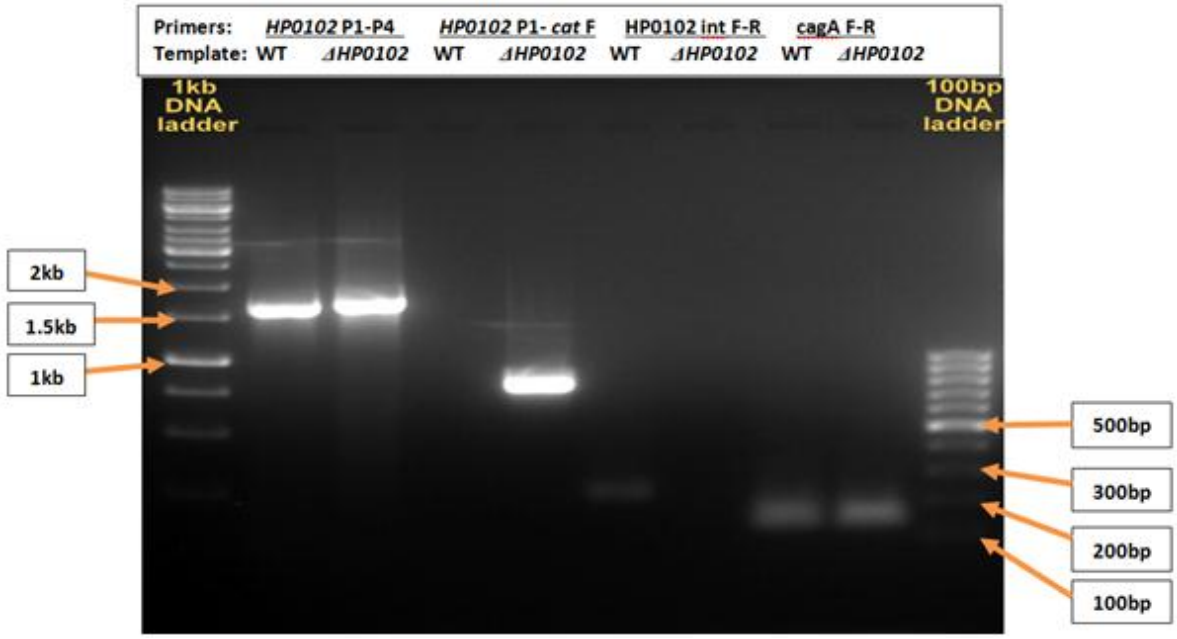


Figure 6: Agarose gel electrophoresis of the PCR samples for mutant confirmation. Lane2-Wildtype genome amplified with primers HP0102 P1 and HP0102 P4. Lane3-genome of ΔHP0102 mutant amplified with primers HP0102 P1 and HP0102 P4. Lane 4-5 wild type and ΔHP0102 mutant amplified with primers cat internal F and HP0102 P1. Lane 6-7 wild type and ΔHP0102 mutant amplified with the primers of HP0102 F and HP0102 R. Lane 8-9 wild type and ΔHP0102 mutant amplified with the primers of cag A (cag A F and cag A R).

Following mutant construction the relative growth rates of H. pylori WT and ΔHP0102 mutant was estimated by CFU analysis. Results of the CFU analysis suggested that both the WT and ΔHP0102 strains demonstrated similar growth kinetics and further suggested that HP0102 was not essential for growth of H. pylori under the growth conditions used in our laboratory as shown in Figure.

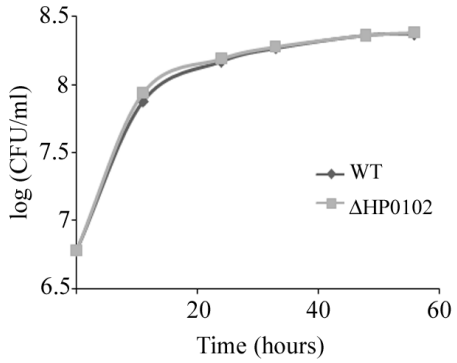


Figure 7: Growth kinetics of HP0102 wild type and mutant.

HP0102 is required for the glycosylation of H. pylori LPS: LPS isolated from both the H. pylori SS1 WT and ΔHP0102 strains were separated by SDS-PAGE and visualized either by silver staining or by using a glycolipid staining kit. A similar pattern of bands corresponding to LPS could be observed by silver staining for both the strains (Fig.8A). Upon staining with the glycolipid staining kit, bands corresponding to the glycosylated LPS could be observed in the WT sample. However, no visible bands could be observed for LPS isolated from ΔHP0102 strain when stained using the glycolipid staining kit (Fig.8B), suggesting a role of HP0102 in LPS glycosylation.

Bacterial LPS is known to be involved in the process of adhesion to host cells in the early stages of infection. Taken together with the fact that the gene HP0102 is also strongly upregulated in AGS-adhered H. pylori, the possibility that the gene might have a role in pathogenesis was next investigated.

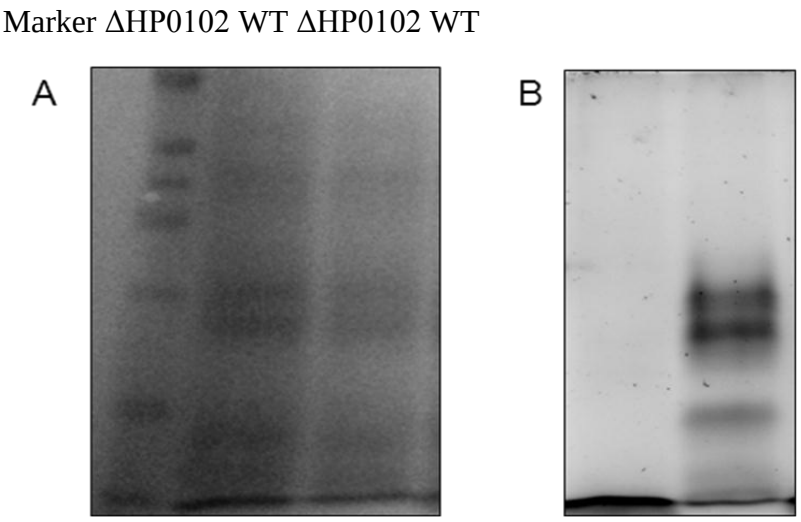


Figure 8 HP0102 is required for the glycosylation of H. pylori LPS. Analysis of H. pylori LPS isolated from SS1 WT and ΔHP0102 by (A) Silver staining and (B) Glycolipid staining.

Role of HP0102 in induction of the hummingbird phenotype in AGS cells: H. pylori SS1 WT and SS1 ΔHP0102 strains were added to AGS monolayers at MOI 50 and the number of adhered bacteria was enumerated at different times after adherence (Fig.9). No difference in the number of AGS-adhered bacteria was observed between the WT and ΔHP0102 strains upto 24 hours examined, indicating that HP0102 might have no role in the adherence of H. pylori to gastric cells. However, a remarkable difference in morphology was observed between AGS cells infected with WT or ΔHP0102 strain. When infected with the H. pylori SS1 WT strain, AGS cells exhibited the characteristic elongation and scattering, known as the hummingbird phenotype. However, much lower scattering and elongation was observed in AGS cells after adherence of the ΔHP0102 strain Since the hummingbird phenotype is associated with production and subsequent translocation of CagA from adhered H. pylori to the gastric cells, cagA expression was next examined in AGS-adhered SS1 WT and ΔHP0102 cells as shown in Figure 9.

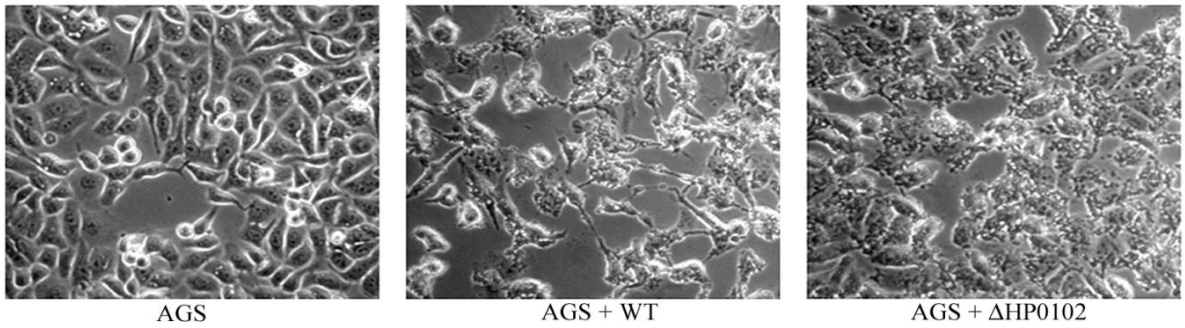


Figure 9: Morphology of AGS cells following adherence of H. pylori strains. AGS monolayers were incubated with H. pylori SS1 (WT) and ΔHP0102 for 5 hours, washed to remove unadhered bacteria and observed on an inverted microscope in phase contrast at 20 × magnification The number of elongated cells were counted and expressed as percent of total cells in each sample. Bar diagram represents the means ± SD of 3 experiments. The statistical significance of the differences (P values) in number of elongated cells between WT and the mutant strain is indicated. * P ≤ 0.05; *** P ≤ 0.001.

Role of HP0102 in cagA induction in AGS-adhered H. pylori: Expression of the cag A gene was observed in WT and ΔHP0102 strains following adherence to AGS cells and compared to that in unadhered bacteria. As reported earlier (Raghwan et al., 2013), about 4 fold upregulation of cag A expression was consistently observed in the WT strain within 2 hours of adherence (Fig.10). However, consistent with the lack of hummingbird formation practically cagA expression was not observed in AGS-adhered ΔHP0102 strain upto 8 hours examination period (Fig.10). In the complement ΔHP0102/HP0102 strain, cag A expression was similar to the WT strain (Fig.10). The difference was not observed in cag A expression of unadhered bacteria between WT and ΔHP0102 strains.

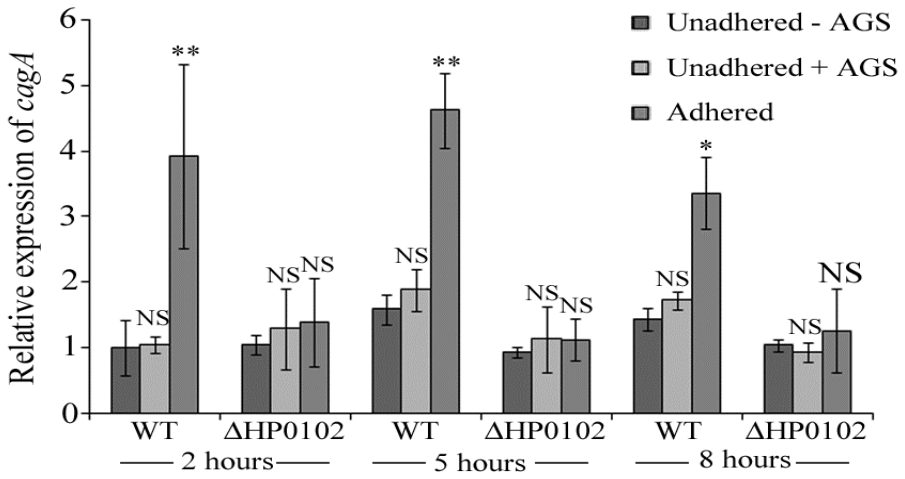


Figure10: Expression of cagA in AGS-adhered and unadhered H. pylori strains. Expression of cagA at different time points was estimated in unadhered and AGS-adhered WT and ΔHP0102 mutant by qRT-PCR. Results represent the average of at least three independent experiments. Transcript levels are expressed relative to that in unadhered WT strain arbitrarily taken as 1.Error bars represent SD. The statistical significance (P values) of the differences in gene expression between AGS adhered and unadhered H. pylori strains are indicated. * P ≤ 0.05; ** P ≤ 0.01; NS, Not significant.

Next, Western blot analysis determined that the levels of Cag A protein also increased in AGS adhered WT bacteria but not in the mutants of HP0102 (Fig.11). Hence, significantely Cag A phosphorylation was observed in WT infected AGS cells. While, only minor amounts were detected in AGS cells infected with ΔHP0102. These results suggested that the ΔHP0102 mutant was specifically defective in induction of cag A following adherence to AGS cells, indicating that HP0102 might have a role in host cell contact dependent upregulation of cag A expression in H. pylori.

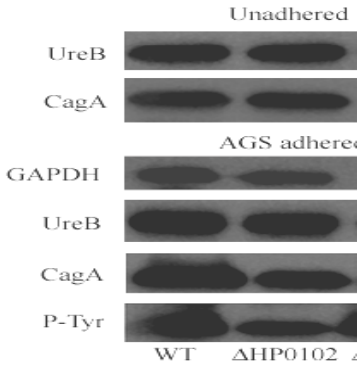


Figure 11: Western blot analysis. CagA was estimated in unadhered H. pylori and H. pylori infected AGS cultures. UreB was used as internal control. Phosphorylated CagA was estimated in H. pylori infected AGS cultures using GAPDH as internal control.

Expression of HP0102 and cagA Is Upregulated in H. pylori Following Adherence to MKN-45 Cells: H. pylori LPS has been reported to induce a cytokine response in host cell upon infection via the receptors TLR2 and TLR4. Since AGS do not express these receptors, we next examined whether HP0102 has a role in **cagA** upregulation during **H. pylori** infection of MKN-45 cells which is a gastric epithelial cell line expressing TLR2 and TLR4. Upon adherence of **H. pylori** WT to MKN-45 cells, HP0102 and **cagA** were both upregulated, but the upregulation was not observed upon infection with the ΔHP0102 strain (Figure 12), similar to the results obtained with the AGS cell line.

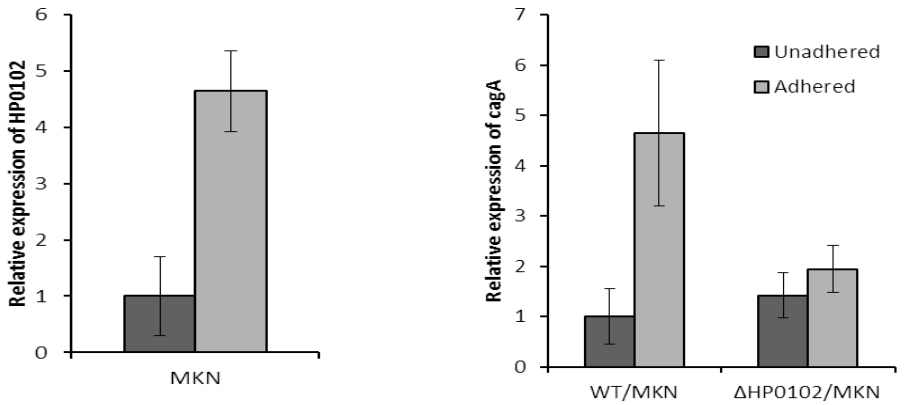
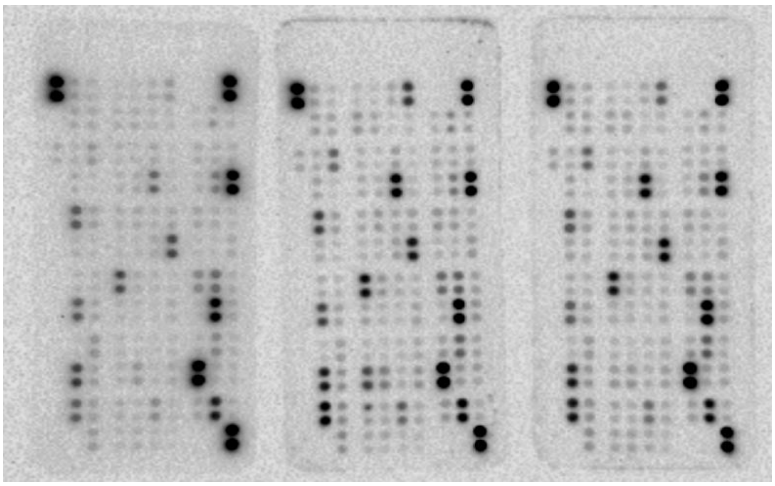


Figure 12: Induction of HP0102 and cagA upon infection of MKN-45 cells. Left panel: RNA was extracted from Helicobacter pylori SS1 2 hours after adherence to MKN-45 cells (adhered) or from H. pylori incubated under identical conditions without cell line (unadhered), and HP0102 expression was estimated by quantitative reverse transcription polymerase chain reaction analysis. Right panel: Expression of CagA was estimated in AGS-adhered or unadhered H. pylori SS1 wild type (WT) and ΔHP0102. Relative expression levels were calculated as fold induction of gene expression in adhered bacteria, considering the expression in unadhered bacteria arbitrarily as 1. Bar diagram represents the means ±SD.

Role of HP0102 in production of cytokines after adherence to MKN-45 cells: Next, a cytokine array was performed using the cell culture supernatants of MKN-45 cell from uninfected and cultures infected with H. pylori wild type and ΔHP0102 mutant (Fig.13). Absence of the gene HP0102 led to impaired production of several cytokines including CCL20, CXCL1, IL8, CD147, CCL3/4, CXCL10 and others in MKN-45 cell. The mean pixel density of some selected cytokines released from MKN-45 cells has been shown in Figure 14. The results indicate that HP0102 seems to have a significant role in inducing the inflammatory response in host cells upon infection with H. pylori.



Uninfected WT ΔHP0102

Figure 13: Cytokine Array profile of MKN-45 cells infected with H. pylori WT and ΔHP0102 strains.

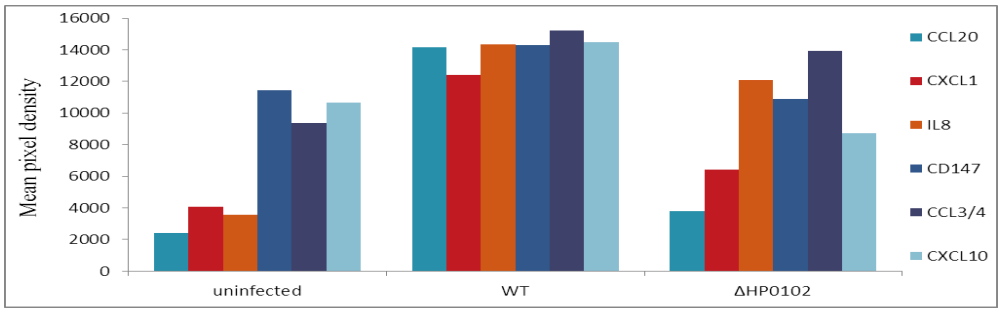


Figure 14: Graph of cytokine Array analysis showing the release of cytokines before and after infection with H. pylori WT and ΔHP0102 strains.

CONCLUSION

The ubiquitous presence and high degree of conservation of the HP0102 gene in all the 84 H. pylori strains including 15 Indian isolates examined, as well as upregulation of expression of the gene following adherence to gastric cells suggested that the gene might have an important function in pathogenicity. Indeed, it has been demonstrated in this study that HP0102 has a role in two different processes related to pathogenicity, a) induction of the hummingbird phenotype in AGS cells following H. pylori adherence and b) LPS glycosylation.

Initial analysis by qRT-PCR revealed that the mRNA levels of HP0102 increases after adherence to AGS and MKN-45 cells, further suggesting that HP0102 gene product must have some role after it infects AGS cells. Next, for understanding the role of HP0102, a deletion mutant was constructed by non vectorial allelic replacement method in H. pylori strain SS1. The growth kinetics and the rate of adherence to AGS cells of the HP0102 mutants was similar to that of the wild type. However, it was also seen that the severity of infection in AGS cells (demonstrated by the typical humming bird phenotype) was much less when AGS cells were infected with ΔHP0102 mutants as compared to the wild type strain. Unlike wild type H. pylori, expression of the gene cagA, responsible for this phenotype was not found to increase in HP0102 mutants following host cell contact. Further investigation is required to understand how HP0102 influences cagA expression upon host cell contact and whether the results obtained in a cell culture model also hold true in vivo.

HP0102 was also found to be involved in the glycosylation of bacterial lipopolysachharides (LPS) by glycostaining analysis. Bacterial LPS is a major virulence factor triggering the expression of cytokines via TLR 2 and TLR4 dependent signaling cascades. Absence of the gene HP0102 led to impaired production of several cytokines including CCL20, CXCL1, IL8, CD147, CCL3/4, CXCL10, etc in MKN-45 cells. The results indicate that HP0102 seems to have a significant role in inducing the inflammatory response in host cells upon infection with H. pylori. Further work is under progress to identify more specific functions of HP0102 and also identify other signaling networks that may be affected by LPS and its glycosylation state during the pathogenesis of H. pylori. Overall, HP0102 appears to be an important factor involved in the regulation of LPS structure and the induction of cagA expression (a major determinant of gastric cancer) upon host cell contact. Since, cagA is the major toxin responsible for inducing gastric cancer, decrease in levels of cagA in the absence of HP0102 gene product might be of importance in developing therapeutic approaches to cure infection or cancer caused by H. pylori.

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