Role of hypothetical protein HP0897 in DNA replication of *Helicobacter pylori*

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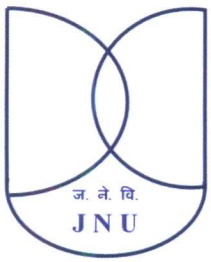

CERTIFICATE

The research work embodied in this thesis entitled **"Role of hypothetical protein HP0897 in DNA replication of *Helicobacter pylori*"** has been carried out by Mr. Ajay Kumar under my guidance at the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India. The work presented here is original and has not been submitted in part or full for any degree or diploma of any university/Institution elsewhere.

Date: 23.07.2018

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Dedicated to Humanity....

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Abbreviations

APS	Ammonium Per Sulfate
Amp	Ampicillin
ATP	Adenosine 5'-triphophate
bp	Base pair
BSA	Bovine Serum Albumin
CAM	Chloramphenicol
DTT	Di thiothreitol
dsDNA	double stranded DNA
EDTA	Ethylene Diamine Tetraacetic acid
IPTG	Isoproryl-ß-D-thio galactopyranoside
Kb	Kilo Base
kDa	kilo Dalton
M	Marker
^{o}C	Degree Centigrade
PAGE	Poly Acrylamide Gel Eletrphoresis
PBS	Phospahte Buffer Saline
PMSF	Phenyl Methyl Suphonyl Flouride
PCR	Polymerase Chain PCR
PEG	Polyethylene glycol
rpm	Revolution Per Minute
SDS	Sodium-Dodecyl Sulfate
ssDNA	Single Stranded DNA
TBE	Tris Borate EDTA
TEMED	N,N,N',N',Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) amino ethane
βME	β-Mercapto Ethanol
µl	Micro litre

Table of Content

Chapter 1

Review of Literature

Review of Literature

1.1. Introduction

For many years it was considered that peptic ulcer disease was due to stress condtions. It was hard to believe that a bacterium, *Helicobacter pylori,* can survive within acidic niche of the stomach. However, the discovery of *H. pylori* in gastric biopsies has changed our thinking about gastric diseases and their treatment.

Helicobacter pylori is classified into the epsilon subdivision under the Proteobacteria, order Campylobacterales, of Helicobacteraceae family. It is a gram-negative, slow growing, microaerophilic, flagellated and motile bacterium (figure 1.1). It exists in three shapes spiral or curved shape, rod shape and spherical shape (figure 1.2). *H. pylori* colonize the gastric epithelium of stomach and its favorable niche is pylorus region of the stomach hence its name *H. pylori. H. pylori* infected person exhibit gastric inflammation along with gastritis and peptic ulcers. 80-90% of patients infected with *H. pylori* develop gastric ulcers and are at risk of developing stomach cancer and MALT lymphoma (Allen, 1999). About half of the World and more than 80 percent population of India is infected with *H. pylori (Thirumurthi and Graham, 2012). H. pylori* was first isolated and cultured by Barry Marshall and Robin Warren in 1982. The *Helicobacter* genus was created in 1989 (Owen, 1998). Several strains of *H. pylori* have been sequenced after that.

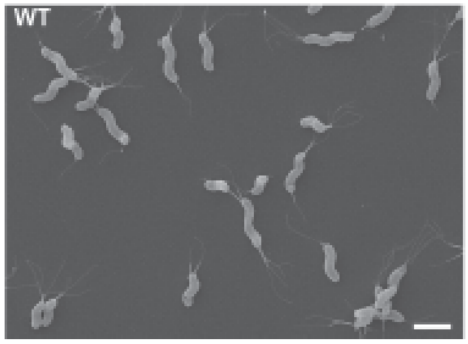


Figure1.1 Scanning Electron Microscope image of *H. pylori*. Scale bar is 2 µm (Adoptedfrom Sycuro, *et al.*, 2013)

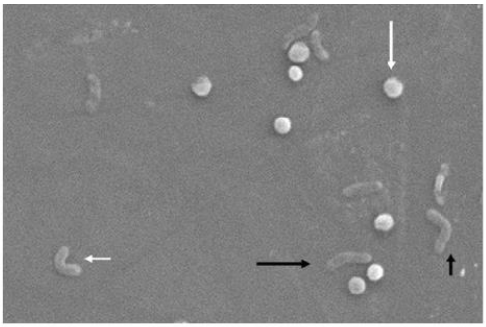


Figure 1.2 Scanning electron microscope image of Coccoid and spiral *H. pylori.* Coccoid (large white arrow), U(small white arrow), V (small black arrow) and spiral (large black arrow) forms. Image adopted from Azevedo NF, *et al.*, 2007

1.2. Epidemiology of *H. pylori* in the world

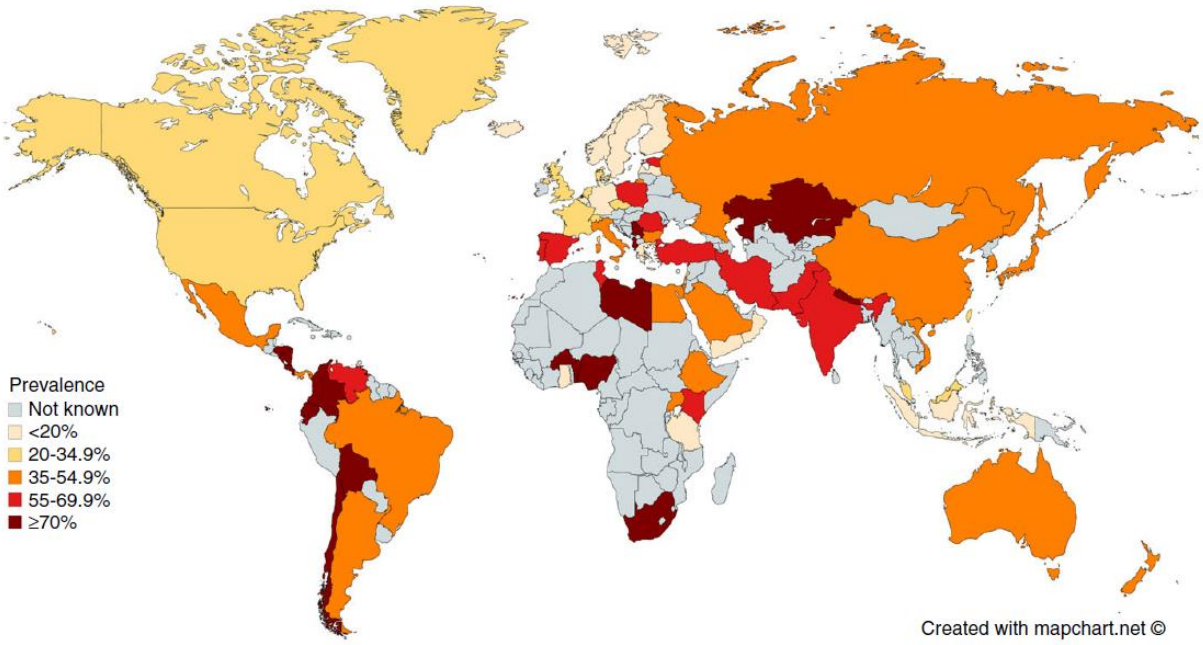


Figure 1.3: Graphical representation of the predominance of *Helicobacter pylori* infection across the world. (Zamani M, *et al.*, 2018)

H. pylori infections are very less prevalent in developed countries than developing countries. The infection rates of *H. pylori* are highest in Latin America and the Caribbean and lowest in Northern America and Oceania. There is fairly good homogeneity of *H. pylori* infection in regions of North America, and Latin America/Caribbean, but huge variations are observed in country-based occurrence among Asian countries (Zamani et al., 2018). These variations are observed in unequal prevalence rates from Kazakhstan (79.5%) and Indonesia (10.0%). Similarly, different prevalence rates can be seen within the countries of Europe, with the highest rate from Serbia (88.3%) and lowest rate from Belgium (11.0%). The highest HP infected from Nigeria, Portugal, Estonia, Kazakhstan and Pakistan and on the contrary countries like Switzerland, Denmark, New Zealand, Australia and Sweden poses the lowest HP infected population. (Hooi et al., 2017).

The variation in infection rates within these continents can be attributed to various reasons as a lifestyle, environmental factors, socioeconomic status, education levels and dietary factors.

In 1994, The International Agency of Cancer, upon various demographical studies worldwide, classified *H. pylori* as a Class I carcinogen for gastric cancer. Since then the bacterium is considered to be one of the causative factors of gastric cancer.

1.3. Reservoir

Earlier it was believed that *H. pylori* could exist only inside the host and it cannot survive in extragastric environment outside its host. However several new studies reported the presence of *H. pylori* in fresh water, rivers and sewage water (Lu et al., 2002, Hulten et al., 1998, Mazari-Hiriart et al., 2001a, Mazari-Hiriart et al., 2001b, Enroth and Engstrand, 1995). The bacteria possess quorum-sensing genes like *luxS* (Cole et al., 2004) and two-component system ARS (Servetas et al., 2016) which have been shown to be involved in formation of biofilm. Biofilm formation in water or vegetables may help bacteria to survive in extragastric environment for longer period and play an important role in transmission of these bacteria. Biofilm formation on gastric mucosa may be one possible explanation for eradication therapy failure.

1.4. Transmission

The mechanism of transmission of bacteria is still elusive as there is no direct evidence how this bacteria is transmitted from infected host to an uninfected one. Three routes of infection of these bacteria can be postulated. The primary transmission mechanism of of *H. pylori* is considered to be fecal-oral route as evidenced by the existence of bacteria in feces (Li et al., 1996) and sewage water (Nayak and Rose, 2007) using PCR and culture techniques. The second transmission route of *H. pylori* infection is thought to be oral-oral route because *H. pylori* is found in dental plaque and saliva (Nguyen et al., 1995). *H. pylori* infection is more common in communities where infants and children are fed on food pre-masticated by the mother (Yucel, 2014). Another route of infection can be nosocomial spread of this bacterium. *H. pylori* can be transmitted by endoscopes contaminated with *H. pylori*. It occurs when there is manual endoscope washing (Langenberg et al., 1990). The transmission frequency of nsoscomial spread is measured to be 4 in 1000 endoscopies (Tytgat, 1995).

1.5. Genome

Helicobacter pylori has a circular chromosome of size around 1.7 Mbp and 39% GC content. The genome of H. *pylori* contains 1590 predicted coding sequences with a well-defined system for motility, for DNA modification and restriction, and also for scavenging iron. (Tomb et al., 1997). Out of 1590 coding sequences, about 1,091 identified by database match and 499 coding sequences are not found in the database. *H. pylori* genome encodes 36 tRNA species which are identified by tRNA scan-SE. These are organised in 7 groups and 12 single genes. Ribosomal RNA includes 23S-5S RNA and 16S RNA along with an orphan 5S RNA gene (Tomb et al., 1997). *H. pylori* chromosome has the bipartite origin of replication (*oriC*) where it is divided into two sub origins *oriC1* and *oriC2* which are separated by *dnaA* gene in between them (Donczew et al., 2012). DnaA boxes are present in both *oriC1* and *oriC2;* however, DNA unwinding element is present near to *oriC2. H. pylori* chromosome possess most of the genes required for DNA replication and cell division. However, many genes which otherwise essential for these fundamental processes in *E. coli* are missing in *H. pylori*. For example, orthologues of DnaC, ftsQ, ftsN and many other proteins are not present (Doig et al., 1999). The components of DNA repair and recombination system has been annotated in *H. pylori* including nucleotide excision repair, base excision repair, , *uvrABCD, recBCD* and *ruvABC. H. pylori* has *recA, recO, recR* and *recJ*, but lacks *recF and recQ. H. pylori* lacks mismatch repair system. *H. pylori* strains show diversity in both nucleotide sequence and gene content. Natural transformation and horizontal gene transfer increase chances of homologous recombination and facilitate its genome to diversify (Dorer et al., 2011). *H. pylori* genome also posses virulence genes that are responsible for the disease. The major virulence genes include genes encoding type IV secretion system, *cagA and vacA.* These virulence genes are injected directly into host cell via type IV secretion system. CagA protein upon injection in host epithelial cells phosphorylates many proteins and leads to alterations in host cell metabolism. VacA protein triggers the formation of acidic vacuoles in host epithelial cells and its presence is associated with tissue damage and the disease (Atherton et al., 1995). As *H. pylori* grow in an acidic environment of the stomach, it has evolved genes to tolerate highly acidic conditions. Its genome encodes urease enzyme that converts urea into ammonia and CO_2. Other mechanism for acid tolerance may involve its ability to establish a positive inside membrane potential (Matin et al., 1996) and release of factors that inhibit acid

production by the parietal cells (Labigne and de Reuse, 1996). *H. pylori* possess various outer membrane proteins (OMPs) for adherence to gastric epithelial cells. Important OMPs are BabA, SabA, AlpA and AlpB (Tomb et al., 1997).

1.6. Pathophysiology

H. pylori can be transmitted from person to person as there are reports of its occurrence in the environment in various niches. As discussed above, *H. pylori* can be transmitted through three routes. Oral-fecal, oral-oral and nosocomial spread are three suggested routes. By any of the three routes, the bacterium can enter the mouth of a host and travels down the esophagus where the pH is neutral. As the bacterium enters the stomach, the pH drops down to ~2. Most of the microorganisms cannot tolerate this low pH and are killed. But *H. pylori* survive this low pH because of adaptive colonization factors developed during the course of evolution. The various colonization factors developed by *H. pylori* include urease enzyme, flagella for motility, chemotaxis, helical shape and outer membrane proteins (Gu, 2017).

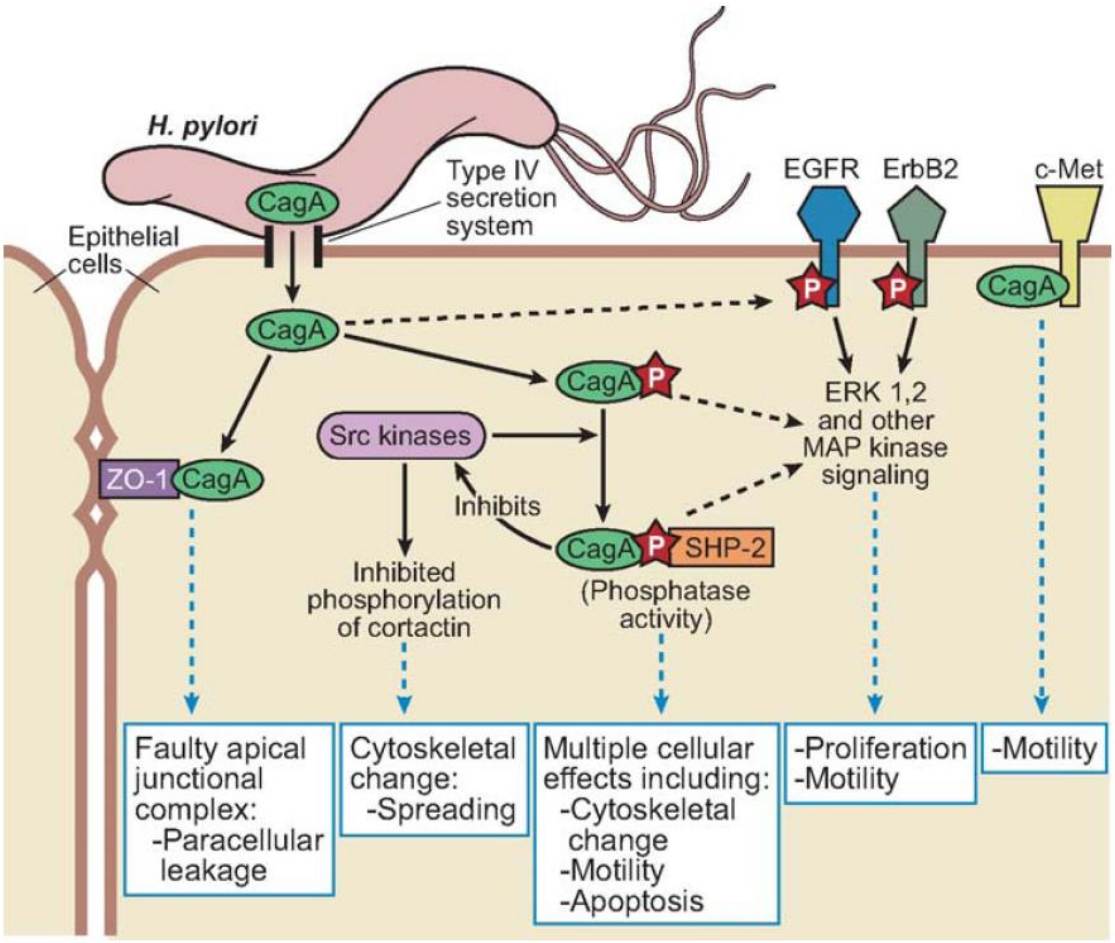


Figure 1.4 CagA modulating the various cellular signaling pathways. CagA protein is injected into the host cell and phosphorylated by Src kinases and leads to various cellular outcomes by modulating host cell signaling pathways. (Image adopted from Atherton JC, 2006).

H. pylori possess a bunch of 6-8 flagella at one pole of the cell. The helical shape of the bacteria and flagella provide bacteria with mechanical advantage to pierce through the viscous mucous membrane on the stomach lining and move through the viscous layer with the help of flagella (Martinez et al., 2016). The flagella of *H. pylori* possess a sheath around it and a bulb-like ending at the terminus of each flagellum (Gu, 2017). The bacterium neutralizes its immediate surrounding by urease activity. *H. pylori* urease enzyme hydrolyzes the urea into CO_2 and NH_3 that buffers the acidic pH around it (Roesler et al., 2014). The bacterium proliferates in the stomach by binary fission and can be passed through the digestive system and then the cycle will be completed. The *H. pylori* colonization leads to gastritis and peptic ulcer disease (Shiotani and Graham, 2002). During *H. pylori* infection, G

cells of the antrum are activated to secrete gastrin hormone (Blaser and Atherton, 2004). The gastrin hormone stimulates parietal cells in the stomach which secrete more acid into the stomach lumen. Increased acid levels in the stomach lumen cause damage to the duodenum and this lead to the ulcer development (Schubert and Peura, 2008). The patients infected with *H. pylori* can be symptomatic or asymptomatic depending upon the type of *H. pylori* strain they are harboring. There are three types of *H. pylori* strains: Type I, intermediate and Type II (Enroth et al., 2000).

These strains can have different effects on host depending on the type of virulence factors they posses. Type I and Type II strains are categorized on the basis of cag-Patogenicity Island (cag PAI). cag- PAI is a 40 kb chromosomal region that encodes genes for type IV secretion system and *cagA* gene. Type IV secretion system assembles into the membrane and forms a needle-like an apparatus that directly injects various virulence factors into the host cells (Backert et al., 2015). The major virulence factors injected into host cells are CagA (cytotoxin-associated gene A) and VacA (vacuolating cytotoxin).

CagA protein gets phosphorylated at tyrosine residue of its EPIYA motif upon entering into the host cell. Phosphorylated as well as unphosphorylated CagA interacts with various proteins of signaling pathways of the host cell (fig. 1.4 and 1.5). About 20 proteins of host cells are reported to be interacting with CagA (Yamaoka, 2010). The Src homology-2 domain-containing phosphatase 2 (SHP2) of host cell signaling one of the most studied factors that interacts with EPIYA motif of CagA.

This interaction of CagA with signaling pathways leads to changes in cell physiology and ultimately carcinogenesis (Higashi et al., 2002, Yamaoka, 2010). CagA protein is also associated with the induction of proinflammatory cytokine IL-8 which is a potent neutrophil-activating chemokine (Censini et al., 1996, Yamaoka et al., 2001). The other virulence factor VacA induces vacuolation, membrane channel formation, apoptosis, interaction with cell membrane receptors and induction of a proinflammatory response (Atherton, 2006, Cover and Blanke, 2005, Kusters et al., 2006).

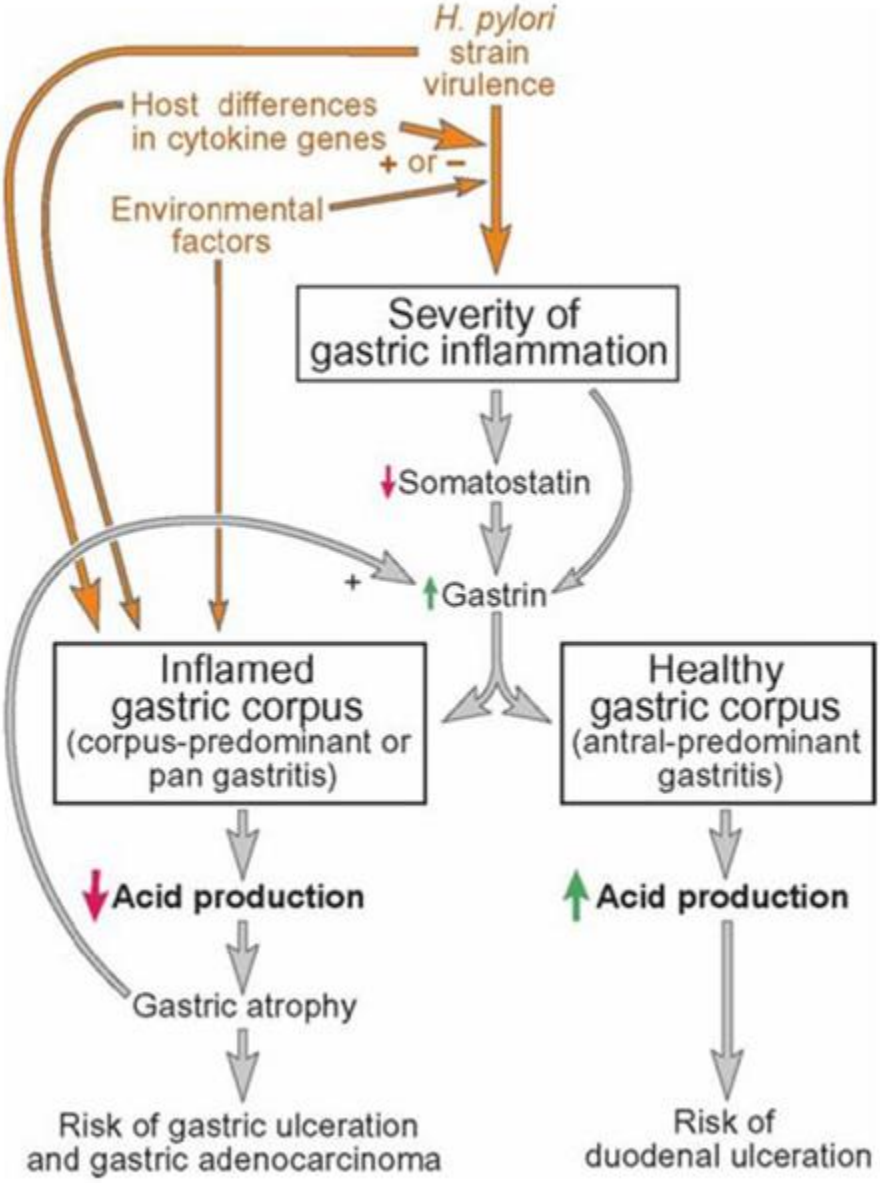


Figure 1.5 External factors (orange color) modulating *H. pylori* induced disease (in boxes). Type of *H. pylori* strain, host immune system and environmental factors may regulate severity of disease.

The *H. pylori* develop cancer of the stomach by two pathways. One pathway involves inflammation of the cells by the indirect action of *H. pylori* and the other pathway involves modification of host proteins and high rates of mutations by the direct action of the CagA protein on host cells. These two pathways work in a co-operative manner to develop gastric cancer. The indirect pathway involves the induction of innate and adaptive immunity. CD4T cells are activated in *H. pylori* infected person which release interferon gamma and

interleukin-4 (IL-4). Interferon gamma is the major cause of gastritis. In the direct pathway, CagA interacts with several host pathways and leads to changes in cell morphology (Humming bird phenotype). Phosphorylated CagA leads to activation of β-catenin signaling by disrupting E cadherin and β-catenin complex. CagA protein also disrupts tight junctions and cell polarity. All these cumulative changes lead to the development of gastric cancer (Saadat et al., 2007, Franco et al., 2005).

1.7. Diagnosis and Treatment

Various detection methods of *H. pylori* infection are currently available, but the selection of method depends on the age of the individual, cost, advantage and disadvantage of each method and accessibility. Diagnostic tests before the treatment of the infection involve invasive and noninvasive methods:

1) Invasive Methods: Histology, culture from biopsy samples, Polymerase chain reaction, rapid urease test of biopsies.

2) Noninvasive methods: Serology includes enzyme immunoassay (EIA).

Tests used to confirm eradication of *H. pylori* include urea breath test and stool antigen test (Garza-Gonzalez et al., 2014).

The infection of *H. pylori* is treated within one week "triple therapy". The triple therapy includes one proton pump inhibitor (omeprazole) and two other antibiotics (clarithromycin and amoxicillin) (Malfertheiner et al., 2017). The two antibiotics are included to prevent bacteria to develop resistance against one particular antibiotic. Proton pump inhibitor prevents excess acid secretion and helps to heal stomach lining.

1.8. DNA replication and Cell division can be effective drug targets

Development of antibiotic resistance is a major challenge to combat infectious disease. Being present in half of the World population, antibiotic resistance to *H. pylori* is a major threat to human health. Therefore, it is critical to develop new drug targets against *H. pylori*. DNA replication and cell division are two important processes for survival and proliferation of the bacteria in the host. Targeting these processes can effectively control the disease burden.

Replication proteins are good targets of antibiotic designing. Many inhibitors of replication have been used to cure infections due to pathogenic bacteria. For example, quinolones specifically targeting the gyrase enzyme involved in replication. Many quinolones are available that inhibit gyrase action and stall DNA replication (Liou et al., 2011). Moreover, *H. pylori* have many unique features regarding DNA replication and cell division. These unique features can be considered while selecting a drug against *H. pylori.* Such type of drug will specifically target *H. pylori.*

1.9. Bacterial cell cycle

As DNA controls the genetic makeup of the organism, its duplication and faithful transfer to progeny is necessary for maintenance and perpetuation of organisms which is accomplished by DNA replication process. The duplication of genetic material by replication and its equal

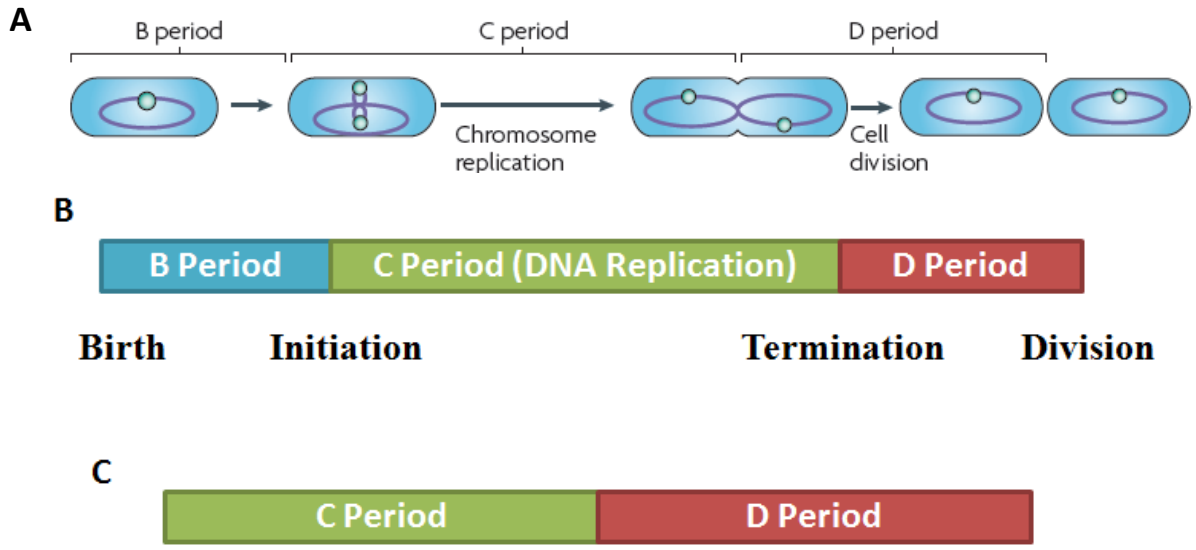


Figure 1.6 Prokaryotic cell cycle. (A) The bacterial cell cycle comprises B, C and D period. (Image adopted from Wang JD, 2009) (B) And (C) Bacterial cell cycle under slow and rapid growth conditions respectively.

distribution to progeny cells through cell division comprise complete cell cycle. The cell cycle in eukaryotes is well defined by various steps. In bacteria, the cell cycle is continuous with no defined steps. However, for the sake of simplicity, the bacterial cell cycle can be divided into three periods named B, C and D period (Wang and Levin, 2009) (fig. 1.6.A and B). B period starts from the cell birth until DNA replication begins. C period is defined by

the synthesis of DNA. It extends from initiation of replication to termination of DNA replication. D period is characterized by the cell division. It starts after the completion of replication and ends with the division of mother cell into two daughter cells. Under fast growth conditions the cell cycle consists of C and D periods only (fig. 1.6.C). Under fast growth conditions, a new round of replication starts before the completion of the previous one. This phenomenon is known as multifork replication (Wang and Levin, 2009). Bacteria adapt multifork replication to coordinate the timing of DNA replication with the cell cycle (fig. 1.7).

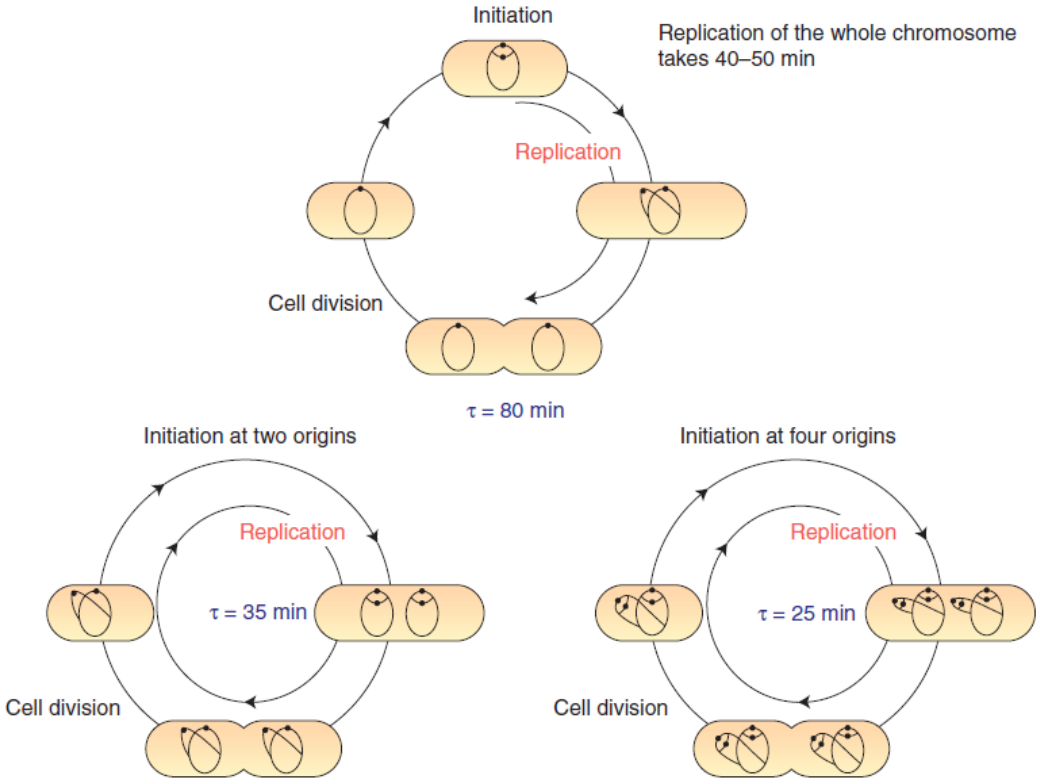


Figure 1.7 Schematic diagram showing replication and cell cycle under slow growing and fast growing conditions. Under slow-growth conditions, cell division starts when DNA replication is completed (Cell cycle 80 minutes). In fast-growing bacteria (35 or 25 minutes), replication initiation occurs at each *oriC* in the partially duplicated chromosome and cell division occurs before the previous round of replication is completed. Multifork replication in fast-growing bacteria helps to coordinate the timing of replication with cell division. (Image adopted from Skarstad K, 2013)

1.10. Chromosomal DNA replication

DNA replication is conserved among all forms of life froms from viruses to humans. Bacterial DNA replication starts through a single chromosomal origin and occurs just the once per cell cycle (Boye et al., 2000). If the onset of replication is not coordinated with cell cycle cell growth is severely affected by the change in gene copy number, DNA damage, and instability of genetic material (Arias and Walter, 2007, Simmons et al., 2004). DNA replication principally divided into three stages: (1) initiation which involves origin firing by dedicated AAA+ family of proteins, (2) Elongation which extends new DNA strands by using parental strands as template (3) termination of DNA replication and decatenation of duplicated chromosomes. This work in this thesis is mainly related to replication initiation proteins of *H. pylori.*

1.10.1. Initiation of DNA replication

Initiation of replication occurs through the single origin of replication in the chromosome in bacteria as compared to eukaryotes where replication starts at multiple origins. Origins are recognized by dedicated AAA+ initiator proteins that can be a homomeric polymer as DnaA initiator in bacteria or heteromeric polymer as in eukaryotes is ORC (Origin Recognition Complex). Bacterial replication initiation involves four steps: 1) *ori*C recognition by initiator factors (Skarstad et al., 1993) 2) melting of *oriC* (Bramhill and Kornberg, 1988) 3) loading of two copies of replicative helicases on to single-stranded DNA (Fang et al., 1999) 4) recruitment of replisome primase, polymerase and clamp loader that proceed directionally opposite from the origin (Funnell et al., 1987).

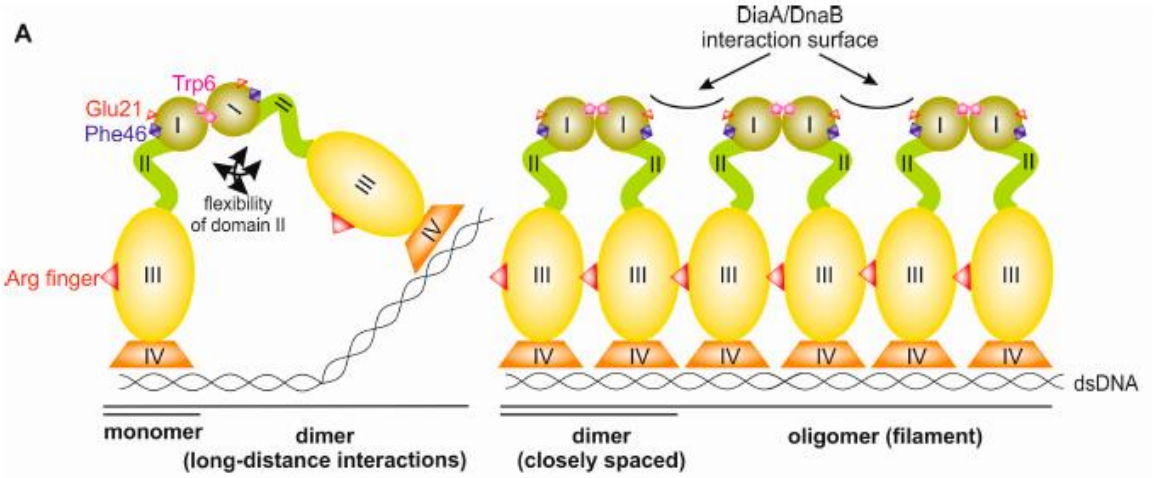
DnaA initiator protein recognizes and binds to DnaA boxes (9 mer repeats) within the *oriC.*ATP bound DnaA is active in binding to DNA and assembles into spiral form at *oriC* to form nucleoprotein complex. The *oriC* also contains sites for IHF, fis and HU. These factors assist the initiation process along with DnaA (Kasho et al., 2014).

1.10.2. DnaA initiator

DnaA proteins consist of four functional domains. N-terminal domain I is responsible for intermolecular interactions between DnaA monomers and with other interacting partners that

modulate the activity of DnaA. Domain II acts as a linker region. Domain III is responsible for oligomerization and filament formation that unwinds DNA at DNA unwinding element (DUE). Domain IV interacts with double-stranded DNA. The domain structure of DnaA and a comparison of its four domains in different bacteria including *H. pylori* are shown in figure 1.8.

A



B

	Domain I	Domain II	Domain III	Domain IV
General information	~10 kDa; structure similar to KH domains; sequence poorly conserved	2 kDa-28 kDa; unorganized structure; the least conserved between species	~25 kDa; includes AAA+ motif; binds and stabilizes ssDNA via ISM motif	~13 kDa; includes HTH motif; binds dsDNA
Interactions				
Escherichia coli	DnaA domain I, DnaB, HU, Dps, DiaA, L2, YfdR, β-clamp-Hda	not known	DnaA domain III, β-clamp-Hda	DnaA boxes, β-clamp-Hda
Bacillus subtilis	SirA	not known	DnaA domain III. SojA, YabA, DnaD	DnaA boxes
Helicobacter pylori	HobA	not known	not known	
Streptomyces coelicolor	N-terminus of DnaA	not known	not known	

Figure 1.8 Schematic diagrams of domains of bacterial DnaA initiator protein. (A) Domains of DnaA and their functions in bubble formation at origin of replication. B) Comparison of functional domains of DnaA among different bacterial species including *H. pylori*. (Figure adopted from Anna Zawilak-Pawlik, 2017)

1.10.3. Origin of Chromosome Replication

Most of the bacteria possess single origin of replication (*oriC*) per chromosome where bidirectional replication starts. *OriC* consists of 9-mer and 13-mer repeat sequences which are recognized by DnaA protein (Fig 1.8.A). DnaA binds to DnaA boxes with different

affinities. Three sites of *oriC* (R1, R4, and R2) have high to medium affinity for both DnaA-ATP and DnaA-ADP (Fuller et al., 1984). Only DnaA-ATP binds at lower affinity sites of *oriC* (R3, R5/M, I1, I2, I3, C1, C2, C3, τ1, and τ2) (McGarry et al., 2004, Frimodt-Moller et al., 2015). *H. pylori* is unique as it is the first gram negative bacteria where *oriC* is divided into two sub origins named oriC1 and oriC2 which are separated by DnaA gene in between them. HpDnaA binds to both *oriCs,* but HpDnaA dependent melting of DNA occurs at DUE near to *oriC2* (fig. 1.9.b). HpDnaA binds to *oriC2* in topology specific manner. Only supercoiled *oriC2* binds HpDnaA. Binding of DnaA to *oriC1* takes place in a sequence specific manner (Donczew et al., 2012).

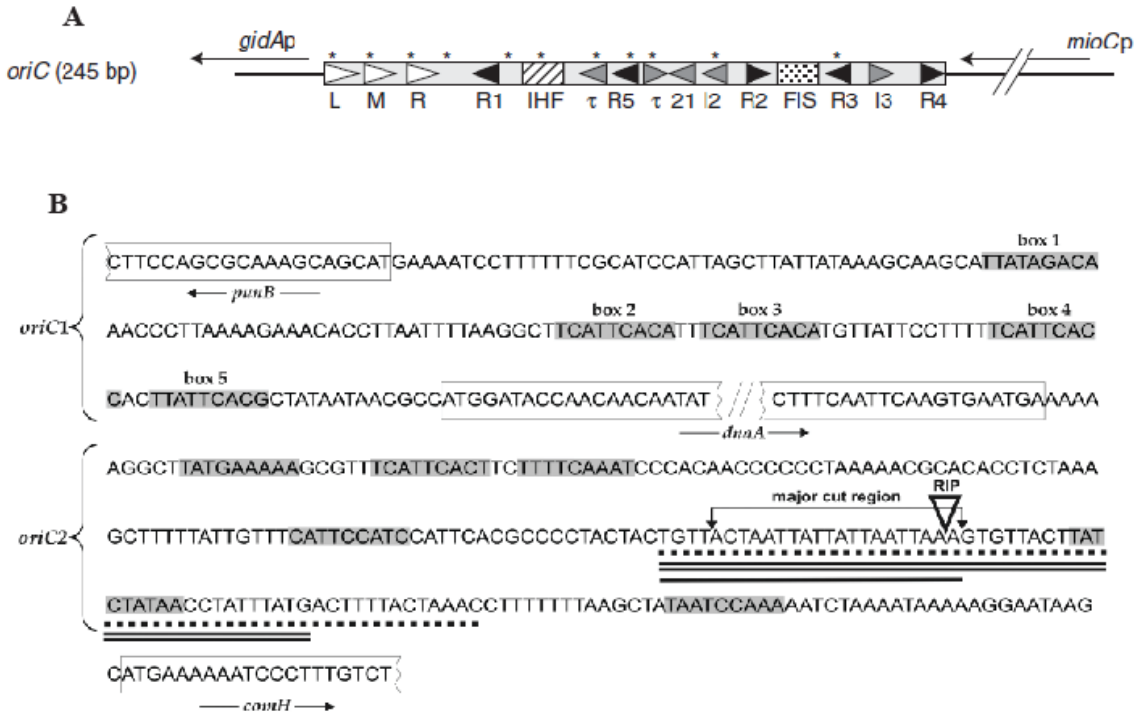


Figure 1.9 Schematic representation of *oriC* region in bacteria. (A) Basic organization of *oriC* in *E. coli*. High to medium affinity sites (R1, R4 and R2) for DnaA-ATP and DnaA-ADP. Lower affinity sites (R3, R5/M, I1 to I3, τ1 and τ2 are indicated that only binds DnaA-ATP. Fis (dotted box) and IHF (box filled with lines) sites are shown. L, M and R represent AT-rich region. (B) Organization of Bipartite *oriC* of *H. pylori* is shown.

1.10.4. Helicases

Genetic information is stored in duplex DNA of the genome. To read and use this information for various cellular processes, double stranded DNA has to be transiently

separated into single strands. Helicases are enzymes that separate double stranded DNA into single strands in ATP dependent manner. There are DNA and RNA helicases. DNA helicases are involved in DNA replication where they initiate DNA replication by unwinding DNA at positions on chromosome called origin sequence (*ori*C). DNA helicase continues to unwind DNA and forms a structure called replication fork. Helicases use ATP to provide energy to break hydrogen bonds between nucleotide base pairs in double stranded DNA. Apart from DNA replication other helicases also play role in DNA repair and homologous DNA recombination. RNA helicases bind and remodel RNA or RNA protein complexes in ATP dependent manner. RNA helicases are involved in shaping the form of RNA during transcription, splicing, and translation.

1.10.5. Basic structural and functional features of helicases.

DnaB helicase forms a hexameric ring structure with each subunit around 52 kDa. The hexameric ring arranged into a doughnut shape with an internal diameter of 40 Å (San Martin et al., 1995). DnaB hexameric ring can adopt C6 or C3 rotational (Yang et al., 2002) symmetry. Helicase functions include ATP binding and hydrolysis by Walker A and Walker B motifs, DNA binding activity, helicase activity and oligomerization activity. There are four super families of helicases based on their amino acid sequence motifs (Gorbalenya et al., 1988). DnaB replicative helicase belongs to superfamily IV. These helicases are found in bacteria and bacteriophage systems (Hall and Matson, 1999).

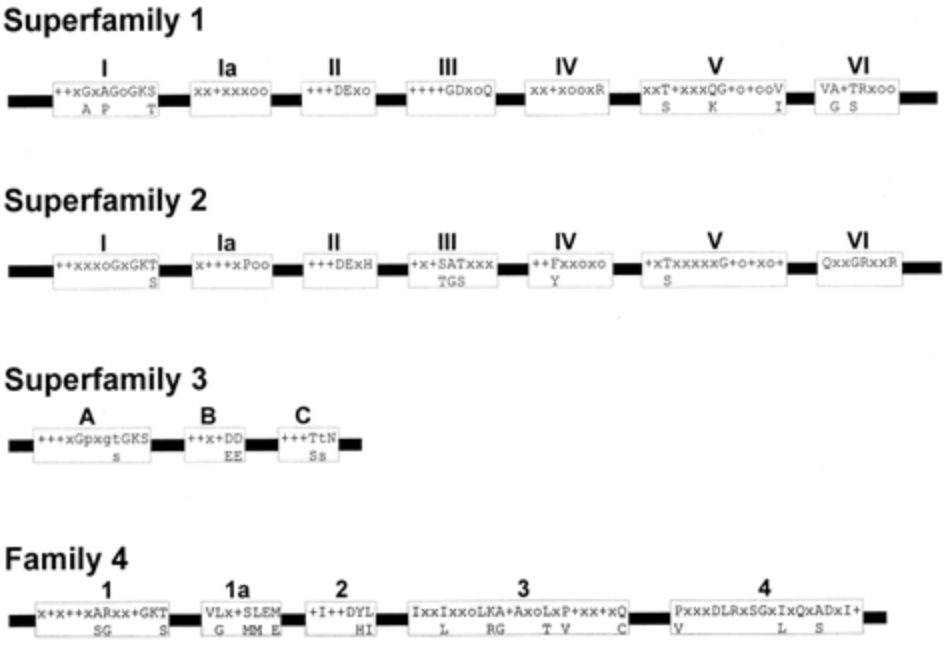


Figure 1.10 Schematic diagrams of different superfamilies of helicases and their motifs. Open boxes represent the conserved motifs and consensus amino acid sequence are represented by letter code inside the box. (+) sign represents a hydrophobic residue, o represents a hydrophilic residues and x can be hydrophobic or hydrophilic. (Adopted from hall and Matson, 1999).

1.10.6. Helicase loaders

Origin melting at *oriC* by the action of DnaA protein is followed by loading of DnaB onto the single-stranded DNA. Helicase loading is achieved by specific protein/s which assist helicase loading onto DNA. These helicase loader proteins are conserved among different bacteria with few exceptions like *Helicobacter pylori* and *Mycobacterium tuberculosis*. The helicase loaders belong to AAA+ superfamily of proteins and have conserved structural domains. The DnaC possesses ATP binding and weak ATPase activity (Davey et al., 2002). ATP bound DnaC forms a stable complex with DnaB and load DnaB onto DNA. After loading of DnaB, hydrolysis of bound ATP releases DnaC (Davey et al., 2002). In gram-positive bacteria, the helicase DnaC is loaded on to DNA with the help of helicase loader DnaI and two accessory proteins DnaB and DnaD (Bruand et al., 2001). Recently, in *H. pylori* Hp0897 with unique sequence has been shown to play a role of helicase loading and may be a possible homolog of helicase loader in these pathogenic bacteria.

1.10.7. Mechanism of helicase loading

The loading of hexameric helicase rings onto origin of chromosome marks the first step in the replisome assembly. Bacteria can possess one or more dedicated helicase loader proteins to load two helicases at the newly melted duplex DNA at *oriC*. DnaB forms a Doughnut shaped hexameric ring of identical subunits which is arranged into two tiers. N-terminal domains (NTD) of six monomers of DnaB form one tier and C-terminal domains form second tier (Bailey et al., 2007). Adjacent NTDs of DnaB form trimer of dimers configuration making a collar that binds to single-stranded DNA (Lo et al., 2009). CTD tier also binds to DNA during translocation of helicase along the DNA strand (Nitharwal et al., 2012). DnaB encircles lagging DNA strand and moves in 5' to 3' direction. In this process, the ATPase domain of HpDnaB faces duplex DNA in the fork (Jezewska et al., 1998, Kaplan, 2000, LeBowitz and McMacken, 1986). The unwinding of duplex DNA is considered to be due to a steric mechanism that excludes the leading strand from the inner channel of DnaB (Hacker and Johnson, 1997, Kaplan, 2000). In *E. coli* DnaB is loaded onto DUE by the joint action of DnaA and DnaC (Fang et al., 1999, Davey et al., 2002, Marszalek and Kaguni, 1994). To load DnaB hexameric ring onto ssDNA, DnaC interacts with DnaB in the specific ratio which is 6:6 for DnaB●DnaC complex in *E. coli*. For DnaB●DnaC complex formation C terminus of DnaB interacts with N terminus of DnaC (Chodavarapu et al., 2016).

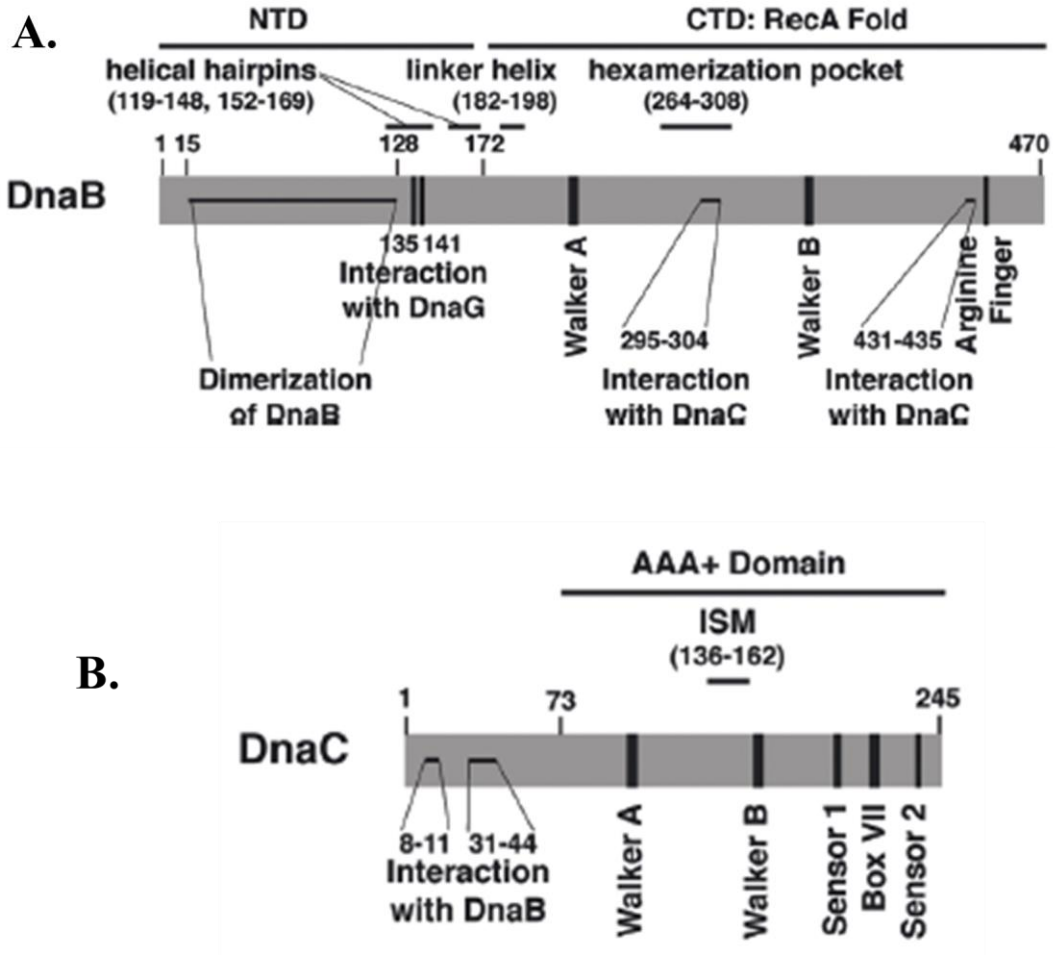


Figure 1.11 Schematic diagram of the domain structure of DnaB and DnaC showing sites of interaction of two proteins. (A) In DnaB NTD and CTD domains are separated by a linker region. Small NTD region showing dimerization domain, residues 135 to 141 involved in interaction with primase. Large CTD contains Walker A and Walker B motifs and residues for interaction with DnaC. (B) Domains of DnaC showing interaction site for DnaB, ISM motif, Walker A and Walker B motifs. (Adopted from Sundari Chodavarapu, 2015).

The recent studies have explained the long-lasting enigma of loading of closed DnaB hexameric ring onto single-stranded DNA. These reports suggest that DnaC traps DnaB in open ring confirmation. The first step of loading mechanism involves the binding of N-terminal of DnaC to RecA fold of DnaB. This interaction induces some conformational changes that generate a restraint within the NTD collar of DnaB. This restraint allow the interface between DnaB monomers to open. The ATP stimulates AAA+ domains of neighboring DnaC monomers to form oligomers of DnaC protomers which stabilize DnaB

hexamer in an open ring form. This open lock washer conformation allows DnaB to interact sufficiently with DNA and encircle single-stranded DNA. The interaction of DnaB with other factors such as primase results in conformational changes and/or ATP hydrolysis results in the release of DnaC from the helicase complex. The release of DnaC renders DnaB active in ATPase and helicase function (Arias-Palomo et al., 2013).

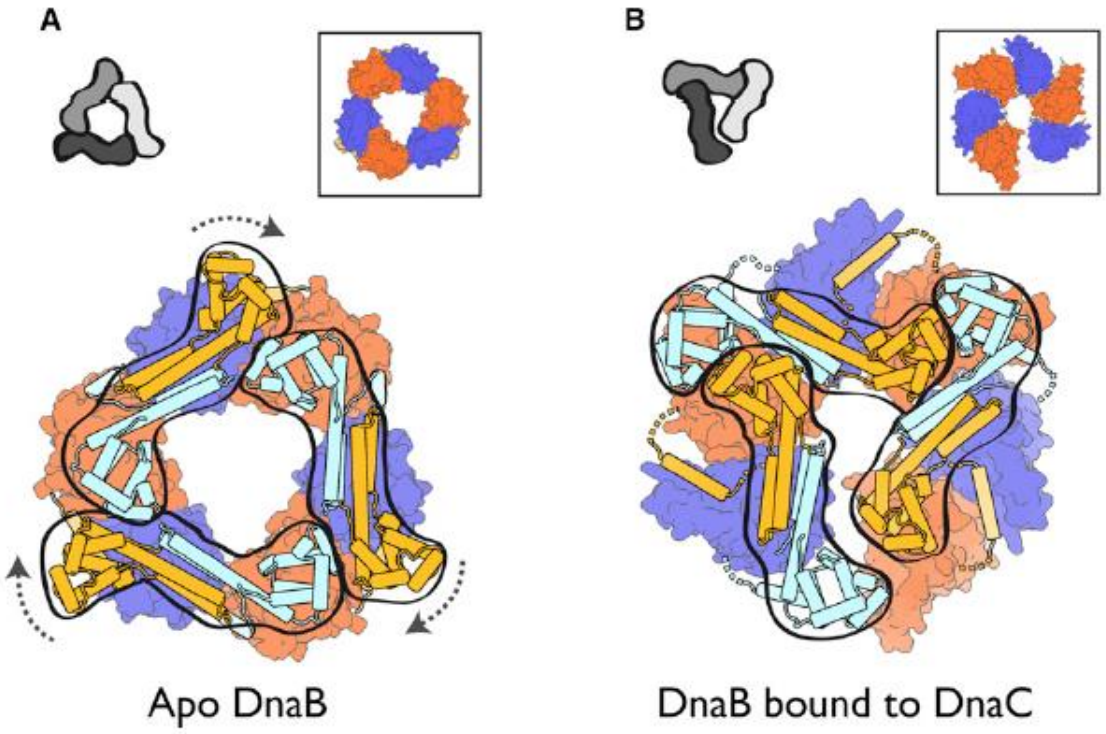


Figure 1.12 Remodeling NTD of DnaB by the interaction of DnaC which leads to DnaB ring opening. (A) NTDs of DnaB in the absence of ATP formed wide and closed triangular collar. DnaB recA domains are displayed as surfaces whereas NTDs and linker regions showed a light blue and orange cylinders. (B) NTDs of DnaB within DnaBC complex undergoes conformational changes from closed ring state, forming new arrangements between dimers. DnaB RecA fold forms a cracked spiral (boxed inset). DnaC is omitted for clarity. (Image adopted from Arias Palomo, E., 2013)

1.10.8. How helicase is loaded in bacteria that lacks helicase loader

There are many bacteria which lacks clear homolog of helicase loader. For example, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Pseudomonas aerugenosa*, *Gammaproteobacteria* contain no gene homologous to helicase loader. In the absence of

helicase loader how these bacteria are surviving is a long lasting question. Either these bacteria have helicases which do not require the assistance of helicase loaders and may be using some other mechanism for loading or these bacteria have helicase loaders with completely diverged sequences. Recent findings from our lab have shown that *H. pylori* DnaB interacts with a hypothetical ORF0897 and it is a promising candidate for helicase loader homolog as it functionally interacts with DnaB and modulates its functions. Other reports have suggested that Rv004 plays a role in DNA replication by interacting with DNA and DnaB and affects DnaB-DnaA complex formation. Rv004 belongs to *DciA* family which is helicase operators. So these studies reveal that helicase loader is not required in all bacterial species. DnaA may mediate helicase loading to some extent. Therefore, further research is required to find out how initiation factors coordinate to assist helicase loading and what are the various mechanisms of helicase loading exist among diverse species.

1.11. Status of helicase and helicase loader in *Helicobacter pylori*

The replicative helicase of *H. pylori* DnaB is well characterized. HpDnaB is a 488 amino acid protein. The amino acid sequence of HpDnaB showed 32% identical sequence and 57% similarity with EcDnaB. HpDnaB possesses 5'-3' helicase, ATPase and weak single and double stranded DNA binding activity (Soni et al., 2003). *H. pylori* sequence shows a unique patch of 34 amino acids in its C-terminal which is called *H. pylori* insertion (HPI). It is found that this HPI is important for hexamerization of HpDnaB (Soni et al., 2003, Stelter et al., 2012). HpDnaB can complement DnaB temperature-sensitive mutant which shows that HpDnaB is a true replicative helicase. DNA binding activity of HpDnaB is tightly regulated by its N-terminal domain which masks its DNA binding domain present within the C-terminus (Nitharwal et al., 2012).

Helicase loader gene sequence is not annotated in *H. pylori* which is an essential gene for replisome assembly in most bacteria. Overexpression of HpDnaB can bypass DnaC function in DnaC-temperature sensitive strain (Soni et al., 2005). The dodecameric structure gives indication that HpDnaB may have self loading capacity. The interesting thing about HpDnaB is that it forms double hexamer which is similar to helicases found in some eukaryotes, archaea and viruses (Stelter et al., 2012). The dodecameric structure gives indication that

HpDnaB may have self-loading capacity. The self-loading function of HpDnaB is shown *in vivo* in a heterologous system. The exact mechanism of helicase loading in *H. pylori* is not clear. Dodecameric structure of HpDnaB dissociates into single ringed primosomes after interaction with primase. The single ringed primosomes generated after interaction with HpDnaG are active in DNA binding and DNA unwinding (Bazin et al., 2015). HpDnaB interacts with several other proteins like HP0897 (hypothetical protein), HP0340 (hypothetical protein) and HP0691 (3-oxoacid CoA-transferase subunit A) (Rain et al., 2001). We have shown that Hp0897 interacts strongly with HpDnaB and functionally modulates its activity. Hp0897 helps HpDnaB to load onto DNA (Verma et al., 2016). Hp0897 shows single-stranded DNA binding activity and no ATPase activity. The ATP binding and weak ATPase activity in helicase loaders in other bacteria is required for efficient loading of DnaB onto DNA. *HP0897* gene is unique to *H. pylori.* Despite showing helicase loading function, Hp0897 show very little sequence similarity to other helicase loaders. The structure of Hp0897 is not resolved as it is very hydrophobic protein and its expression needs to be optimized in soluble fraction. The homology modeling cannot be performed due to lack of its sequence similarity with known protein structures in the protein data bank. In this case, proposed model for Hp0897 can be built by *ab initio* structure prediction. The structure prediction of Hp0897 is important because it may be possible that Hp0897 would have structurally conserved domain despite lack of similarity at the level of primary amino acid sequence. Information from crystal structure can be used for HpDnaB-HP0897 interaction by docking studies.

1.12. Rationale of the work

From the review of literature, it is observed that lot of work has been done to understand the basic mechanism of DNA replication in model organisms like *E. coli*. However,this mechanism may not be true for bacteria which inhabits in a totally different environment. These bacteria with adverse niche can evolve diverse mechanisms to adapt their immediate environmental cues. *H. pylori* which inhabits human stomach, shows many genes of DNA replication and cell division missing, which are otherwise conserved in model organism, *E. coli*.. We have recently found that the helicase loader gene which earlier was thought to be missing on the basis of gene annotation report, has been shown to be present in *H. pylori*..

ORF0897 with no sequence similarity to conserved helicase loaders performs the function of helicase loader in *H. pylori*.

We have found that Hp0897 interacts stably with HpDnaB and modulates various activities of HpDnaB. Further studies on HpDnaB and Hp0897 complex are important to understand the mechanism of loading. Accordingly the objectives for the present studies include

Structure-function analysis of HpDnaB-HpORF0897 interaction

2) Essentiality of *HP0897* gene for growth of *H. pylori*

3) Role of Hp0897 at replication origin of *H. pylori* and its putative function in cell division

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Chapter 2

Materials And Methods

2.1. Chemicals

Most of the chemicals used for this study were Molecular Biology grade and were purchased from Sigma, Merck, Qualigen and SRL. All the restriction enzymes for cloning were purchased from New England Biolabs (NEB). For cloning purpose, Phusion DNA Polymerase High fidelity enzyme was used and it was from Thermo Scientific. Unicorn 5.0 AKTA basic FPLC, Heparin-Sepharose beads were obtained from Amersham Biosciences, Uppsala Sweden. Important chemicals like Mg^{2+}-ATP, Lysozyme, Protease inhibitor, protease inhibitor, SDS PAGE reagents were purchased from Sigma Aldrich (USA). Isopropyl-1-ß-D-galactopyranoside (IPTG) was purchased from Biosciences and Biosynth AG (Switzerland). Anti 6xHis polyclonal antibodies and HRP- anti mouse/rabbit were purchased from Abcam and Santa-Cruz (USA) respectively. Antibiotics for *H. pylori* culture like Amphotericin B, Trimethoprim and Vancomycin were purchased from Sigma, VWR Life Sciences and AMERESCO respectively. Other antibiotics like Kanamycin, Chloramphenicol and Ampicillin were purchased from Hi-media (India). Horse serum and Fetal Bovine Serum used in *H. pylori* culture were purchased from Invitrogen and PAN Biotech respectively. M13mp18 ssDNA was purchased from New England Biolabs (NEB). Brain Herat Infusion (BHI) and Isovitale X were procured from BD Difco. Luria Bertani media (LB) and LB agar were procured from Hi-media. Protein ladder and DNA ladder were purchased from Thermo and NEB respectively. Dialysis membrane and PVDF membrane were purchased from Thermo Pierce and MDI respectively. Various oligos used in this study were purchased from Europhins and Integrated DNA Technologies (IDT).

2.2. Bacterial strains, plasmids and culture.

E. coli cells were grown in LB media and appropriate antibiotics were added when required (50 µg/ml Kanamycin, 100 µg/ml Ampicillin). *H. pylori* strains (*26695, P12, 217-1A, I-10, San74, SS1)* were grown on 3.7% w/v Brain Heart Infusion (BHI) agar (Difco) supplemented with 8% horse serum, 0.4% IsovitaleX, and three antibiotics (8 µg/ml Amphotericin B, 6 µg/ml Trimethoprim and 10 µg/ml Vancomycin). Culture plates were incubated at 37°C for 24–36 hr in a GasPak anaerobic system using GasPak EZ sachet (BD) or in water–jacketed thermal incubator with 10 percent CO_2 level and 80-95% humidity. The appropriate antibiotics were used for selection of transformed Hp colonies (6 µg/ml Chloramphenicol, 6

µg/ml Kanamycin). All plasmids were maintained in either *E. coli* DH-5α or DH-10β strain. *E. coli* BL21 (DE3) (Novagen) cells were used for over-expression of recombinant proteins. *E coli* strains and plasmids used in this study are given in table 1.

Table 2.1 Bacterial strains and plasmid used in this work

Strains/plasmids	Genotype/relevant characteristics	Reference
E. coli BL21 (DE3)	*F⁻ompT hsdS$_B$ (r_B⁻ m_B) gal dcm*	Molecular cloning, Sambrook et al.
E. coli DH10B	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1*ara*D139Δ(*ara, leu*)7697 *gal*U *g al*K λ⁻*rps*L *nup*G	Molecular cloning, Sambrook et al.
H. pylori 26695	26695	ATCC™ 700392D
H. pylori P12	P12	Kind gift from Dr. Niyaz Ahmad, Hyderabad
H. pylori strains	*AM1, SS1, B-28, I-3, I-10 San74, 217(1A)*	Kind gift from Dr. Ashish Mukhopadhyay, NICED Kolkata
pET28a	*T7, his, kan^R*	Novagen, Madison
pGEX6P2	Tac, GST, Amp^R	Amarsham Phsarmacia
pBluescript KS II (+)	Cloning vector	Stratagene
pMAL-c2X	Expression vector to Maltose-binding protein (MBP) fusions	Addgene
HpDnaBwt/pET28a	pET28a derivative containing 1.5 kb of *H.pylori* DnaB	Soni, R.K. *et al.*, 2003
HpDnaBN144/pET28a	pET28a vector containing 432 bp encoding 1 to 144 amino acids of HpDnaB	Kashav, T. *et al.*, 2009
HpDnaBN2/pET28a	pET28a vector containing 1062bp encoding 134 to 488 amino acids of HpDnaB	Nitharwal, R.G. *et al.*, 2007
HpDnaBN3/pET28a	pET28a vector containing 942 bp encoding 175 to 488 amino acids of HpDnaB	Nitharwal, R.G. *et al.*, 2007

HpDnaBN4/pET28a	pET28a vector containing 882 bp encoding 195 to 488 amino acids of HpDnaB	Nitharwal, R.G. *et al.*, 2007
HpDnaBDelC29/pET28a	pET28a vector containing mutant HpDnaB gene that lacks base pairs encoding 29 amino acids from extreme C terminus	Nitharwal, R.G. *et al.*, 2007
HpDnaBDelC34/pET28a	pET28a vector containing mutant HpDnaB gene that lacks base pairs encoding unique patch of 34 amino acids	Nitharwal, R.G. *et al.*, 2007
HpDnaBCDel89/pET28a	pET28a vector containing 1197 bp encoding 1 to 399 amino acids of HpDnaB	Nitharwal, R.G. *et al.*, 2007
HpDnaBCDel160/pET28a	pET28a vector containing 984 bp encoding 1 to 328 amino acids of HpDnaB	Nitharwal, R.G. *et al.*, 2007
HpDnaBC171/pET28a	pET28a vector containing 513 bp encoding 318 to 488 amino acids of HpDnaB	This study
HpDnaBC120/pET28a	pET28a vector containing 372 bp encoding 195 to 318 amino acids of HpDnaB	This study
HP0897/pET28a (wild type and mutant)	pET derivative containing 0.627 kb of *H. pylori* 0897	Verma, V. *et al.*, 2016
NTD0897/pET28a	pET28a derivative containing 345 bp encoding 1 to 115 amino acids of HP0897 gene	This study
CTD0897/pET28a	pET28a derivative containing 345 bp encoding 100 to 208 amino acids of HP0897 gene	Verma, V. *et al.*, 2016
HP0897/pMal-c2X	pMal-c2X vector containing 0.627 kb of *H. pylori* 0897	This study
NTD0897/pMal-c2X	pMal-c2X vector containing 345 bp encoding 1 to 115 amino acids of HP0897 gene	This study
CTD0897/pMal-c2X	pMal-c2X vector containing 345 bp encoding 100 to 208 amino acids of HP0897 gene	This study
GSTHpDnaB	pGEX derivative containing 1.5 kb of *H. pylori* DnaB	Soni, R.K. *et al.*, 2003
GST0897	pGEX derivative containing 0.627 kb of *H. pylori* 0897	Verma, V. *et al.*, 2016
gfpmut3/pWS311	pWS311 derivative containing gfpmut3 gene	This study
0897GFP/pWS311	pWS311 derivative containing HP 0897gfpmut3 chimera	This study
NTD0897GFP/pWS311	pWS311 derivative containing 345 bp encoding 1 to	This study

	115 amino acids of HP0897 gene and gfpmut3 gene at C terminus	
CTD0897GFP/pWS311	pWS311 derivative containing 345 bp encoding 100 to 208 amino acids of HP0897 gene and gfpmut3 gene at C terminus	This study
UPSNTD:*catGC*:DNS/ pBluescript KS II (+)	pBluescript derivative containing Chloramphenicol cassette flanked by upstream and downstream sequences of HP0897	This study
SSBGFP/pWS311	pWS311 derivative containing HP *ssb*gfpmut3 chimera	This study
ftsZGFP/pWS311	pWS311 derivative containing HP *ftsZ*gfpmut3 chimera	Kamran M, 2018

Table 2.2 Primers used in this study

S. No.	Primer	Sequence	References
1	HpDnaBC171	5'GGAATTCCATATGATGTCAGGGAGTAAAGCC ACT 3'	This study
2	HpBwt Rv BamHI	5' GCGGATCCTCAAGTTGTAACTATATCATAA 3'	Soni, R.K. *et al.*, 2003
3	FHpDnaBC120	5' CGGGATCCATGTTAGTCATTATAGGGGCAAG 3'	This Study
4	RHpDnaBC120	5' CGGAATTCTCAGAGCTGCAAATAGTCAATAAAAG 3'	This Study
5	Hp0897NFw	5' CGGGATCCATGCCAGGACCAAAACCT 3'	This study
6	Hp0897NRv	5' CCGCTCGAGGCTAGGGTGCGTGAAAT 3'	This study
7	FwHp897 CTD BamH1	5' CG**GGATCC**GCTAAAGTAGAACACCAAATC 3'	Verma, V. *et al.*, 2016
8	RvCTD0897XbaI	5' GCTCTAGAAGGGGGCGTGCGCAGCGTATTATC	This study
9	Rv0897NTDXbaI	5' GCTCTAGAGCTAGGGTGCGTGAAATAG 3'	This study
10	FwHpUPS200SacI	5' C GAGCTCGAT TTT GAT GCTTTTTGT TTC 3'	Verma, V. *et al.*, 2016

11	RvHp897 NTD BamH1	5' CG**GGATCC**TCAGCTAGGGTGCGTGAAATAGAT 3'	Verma, V. *et al.*, 2016
12	HpDNS200FwXhoI	5' 'CCGCTCGAGGAGCTGACCATGCAACTGCCG 3'	This study
13	HpDNS200RvKpnI	5' GGGGTACCTAGACTGCTCGTTATTCGCTA 3'	This study
14	Fwgfpmut3SpeI	5' GGACTAGTTAGTAAGGAGAACATATGAGTAAAGGAG AAGAACTTTTCAC 3'	This study
15	Rvgfpmut3KpnI	5´GGGGTACCTTATTTGTATAGTTCATCCATGCC 3´	This study
16	FwRBS0897SpeI	5´GGACTAGTTAGTAAGGAGAACATATGCCAGGACCAA AACCTGG 3´	This study
16	RvORF0897XbaI	5´GCTCTAGAAGGGGGCGTGCGCAGCGTAT 3´	This study
17	Fwgfpmut3XbaI	5´GCTCTAGAATGAGTAAAGGAGAAGAACTTTTC 3´	This study
18	Fw0897CTDRBS SpeI	5' GGACTAGTTAGTAAGGAGAACATATGGCTAAAGTAGAACA CCAAATC 3'	This study
19	FHPssb	GGACTAGTTAGTAAGGAGAACATATGTTTAATAAA GTGATTATGGTAG	This study
20	RHPssb	TTTACTCATTCTAGAGCCGCCGCCGCCAAAGGGGATTT CTTCTTC	This study
21	FgfpOLssb	GAAGAAATCCCCTTTGGCGGCGGCGGCTCTAGAATGAG TAAAGGAG	This study
22	0897KO1	TAATTATTCAAAAGATTGTTCAGCT	This study
23	0897KO2	CTTCCTTAGCTCCTGAAAATCTCGTCCTGGCATTC CGAGGTT	This study
24	0897KO3	TGGCAGGGCGGGGCGTGAGAGCTGACCATGCAAC TG	This study
25	0897KO4	GCTGATGCTTATCAAACAAGCC	This study

26	0897KO5	TGAGCGGCTTCACCGATTTGATAGC	This study
27	0897KO6	CTTCCTTAGCTCCTGAAAATCTCGAAGCTTTTGTATAATGCTTTC	This study
28	FCSO645	GTTTTTGGATCCATCCGAGATTTTCAGGAGCTA	This study
29	RCSO1076	AAAAATTACGCCCCGCCCT	This study
30	FCSO2243	AAGCCCTTTTGAAAGTCCTATT	This study
31	RCSO2245	CTTCCTTAGCTCCTGAAAATCTCGATCGTTCATGAAGCTCCTTT	This study
32	FCSO2246	TGGCAGGGCGGGGCGTGAAACTAATGAAACGAGAGCAATAAG	This study
33	RCSO2244	TTGCAACACATCAAAGGTCTG	This study
34	ori1Fw	GAAAATCCTTTTTTCGCATCCATTAG	This study
35	oriC1Rv	ACTCTATGAGACTAACTTTAGG	This study
36	oriC2ChIPFw	GCTTTTTATTGTTTCATTCCATCC	This study
37	oriC2Rv	GCTTATTCCTTTTTATTTTAGATTTTTG	This study

38	oriC2DNFw	GTAGAGCCGACTTTTTATGAAAC	This study
39	oriC2DNRv	CTCTTTAATTATATCGTTTAAATAATG	This study

2.3. DNA manipulations

2.3.1. Cloning of deletion mutants of HpDnaB

Deletion mutants of HpDnaB were cloned in pET28a vector for its domain mapping. The DNA fragment corresponding to the deletion mutant HpDnaBC171 (encoding 318 to 488 amino acids) was amplified by Polymerase Chain Reaction using *H. pylori* 26695 genomic DNA as a template with primers HpDnaBC171 and HpBwt Rv BamHI (Table 2.2). The amplified PCR product (513 bp) was cloned in NdeI and BamHI sites in pET28a vector backbone. Another deletion mutant HpDnaBC120 was amplified by PCR using *H. pylori* 26695 genomic DNA with primers FHpDnaB120 and RHpDnaB120 (Table 2.2). The amplified PCR product (360 bp) was ligated into BamHI and EcoRI sites in pET28a vector backbone. All the other HpDnaB deletion mutant constructs were already available in the lab.

2.3.2. Cloning of HP0897 and its deletion mutants

Deletion mutant NTD0897 was cloned into pET28a vector by PCR amplification of 1 to 345 bp of *HP0897* gene by using *H. pylori* 26695 genomic DNA as template with primers Hp0897NFw and Hp0897NRv. The amplified PCR product (345 bp) was cloned in BamHI and XhoI sites of pET28a vector backbone. This construct of NTD0897 in pET28a vector was used to express mutant protein with His_6 tag. To express HP0897 and its two deletion mutants NTD0897 and CTD0897 with Maltose binding protein (MBP) tag, these inserts were cloned in the pMal-c2Xvector backbone. HP0897 (1-627 bp), NTD0897 (1-345 bp) and CTD0897 (300-627 bp) were amplified by PCR using *H. pylori* 26695 genomic DNA as a template. For amplifying HP0897, primers Hp0897NFw/ RvCTD0897XbaI; for NTD0897, primers Hp0897NFw/Rv0897NTDXbaI and for CTD0897, primers FwHp897CTD

BamHI/RvCTD0897XbaI were used. All the primers are listed in Table 2.2. All these three constructs were cloned in BamHI and XbaI sites of pMal-c2X vector.

2.3.3. Knockout construct of HP0897

Homologous recombination method was used to knockout ***Hp0897*** gene from *H. pylori* genome. For this purpose, Hp0897 knockout construct was generated in pBluescript KS II (+) vector containing Chloramphenicol cassette at BamHI and XhoI sites (pBS::Cam) (a kind gift from G. Mukhopadhyay's lab). First of all 200 bp upstream sequence of Hp0897 plus 345 from +1 position of HP0897 gene, *i.e.*, total 545 bp fragment of DNA was amplified by PCR using 26695 genomic DNA as template and primers FwHPUPS200SacI and RvHp897 NTD BamH1 and cloned in SacI and BamHI sites of pBluescript KS II(+) vector. After cloning 545 bp upstream sequence in pBS vector (pBS-UPSNTD0897::Cam), 200 bp downstream sequence of Hp0897 was amplified by PCR using 26695 genomic DNA as template and primer pair HpDNS200 FwXhoI/ HpDNS200 Rv Kpn1. The amplified product was cloned in XhoI/KpnI sites of pBS-UPSNTD0897::Cam vector. This resulted in the final construct pBS-UPSNTD0897::Cam::DNS0897. The clones were sequenced with the appropriate primers to avoid the possibility of mutation during PCR amplification.

2.3.4. Cloning of GFP in pWS311 vector

For making GFP fusion proteins for live cell imaging, a GFP vector was designed by cloning *gfpmut3* gene in pWS311 vector. pWS122 vector contained *gfpmut3* gene but this vector was not compatible with the available *H. pylori* strains in the laboratory. Therefore, *gfpmut3* was subcloned in pWS311 vector which was compatible with and maintained stably in P12 strain in our lab conditions. *gfpmut3* gene was amplified by PCR using the pWS122 vector DNA as template and primers Fwgfpmut3SpeI/Rvgfpmut3KpnI. The forward primer Fwgfpmut3SpeI was designed to have extra sequence before start codon ATG of *gfpmut3* gene to include RBS site for expression of GFP under the control of *cagA* promoter. This extra sequence with RBS between *cagA* promoter and start codon is TAGTAAGGAGAACAT. The amplified *gfpmut3* was ligated in SpeI/KpnI sites that replaced *cagA* gene from pWS311 vector. This resulted in GFP/pWS311 vector. This GFP/pWS311 vector expressing GFP alone was used

as control for subcellular localisation experiments. *gfpmut3* was cloned under the control of *cagA* promoter.

2.3.5. Cloning of *HP0897GFP, NTD0897GFP, CTDGFP* and *SSBGFP* in pWS311 vector

For live cell imaging, GFP tag was cloned at 3' end of *HP0897* and its N and C terminal deletion mutants. For this purpose, *gfpmut3* gene was amplified by PCR using pWS122 plasmid DNA as a template with primers Fwgfpmut3XbaI and Rvgfpmut3KpnI. The amplified *gfpmut3* was ligated in XbaI/KpnI sites of pWS311 vector. After cloning *gfpmut3* at XbaI/KpnI sites in pWS311 vector, other inserts as full length *HP0897*, *NTD0897* and *CTD0897* were cloned in SpeI/XbaI sites to make *HP0897GFP*, *NTDGFP* and *CTDGFP* fusion gene constructs. For *HP0897GFP* fusion construct, *HP0897* was amplified by PCR using primers FwRBS0897SpeI / RvORF0897XbaI and 26695 genomic DNA as template. The forward primer FwRBS0897SpeI designed to have extra sequence before start codon ATG of *HP0897*gene to include RBS site for expression of *HP0897GFP* under the control of *cagA* promoter. This extra sequence with RBS between *cagA* promoter and start codon is TAGTAAGGAGAACAT. The amplified *HP0897* was ligated in SpeI/XbaI sites in pWS311 containing *gfpmut3*.

For cloning of NTDGFP fusion costruct, *NTD0897* was amplified by PCR using primers FwRBS0897SpeI/Rv0897NTDXbaI and 26695 genomic DNA as a template. The amplified *NTD0897* was cloned in SpeI/XbaI sites in continuation with *gfpmut3*.

For cloning of CTDGFP fusion construct, *CTD0897* was amplified by PCR using primers Fw0897CTDRBS SpeI and RvORF0897XbaI and 26695 genomic DNA as template. The amplified *CTD0897* was cloned in SpeI/XbaI sites in continuation with *gfpmut3*.

For cloning of *SSBGFP* fusion construct, overlap PCR method was used. Three PCR reactions were performed to clone *SSBGFP/*pWS311 construct. In first PCR reaction, *Hpssb* gene was amplified by PCR using 26695 genomic DNA as template and primers FHPssbSpeI/RHPssbOLgfp. The reverse primer RHPssbOLgfp designed in such a way that it excluded stop codon of *ssb* gene and contained $(GGC)_4$ stretches to include a linker region between *ssb and gfpmut3*. The reverse primer also included at its 5' end 15 nucleotides

complementary to *gfpmut3 for* overlap PCR. In second PCR reaction *gfpmut3* gene was amplified by PCR using primers FgfpOLssb/Rvgfpmut3KpnI and pWS122 vector as a template. The forward primer FgfpOLssb includes at its 5' end 15 nucleotides complementary to *HPssb* gene for overlap PCR extension. In third PCR, products of first and second PCR are used as template and *ssbgfp construct* was amplified by overlap PCR using primer pair FHPssbSpeI/Rvgfpmut3KpnI. The amplified product was cloned in SpeI/KpnI sites of pWS311 vector. All the primer sequences are shown in Table 2.2.

2.4. Protein purification

For pull-down assay, wild-type recombinant proteins as well as their deletions (HpDnaB, HpDnaBN144, HpDnaBN2, HpDnaBN3, HpDnaBN4, HpDnaBDelC29, HpDnaBDelC34, HpDnBCDel89, HpDNaBCDel160, HpDnaBC171, HpDnaBC120, HP0897, NTD0897 and CTD0897) were expressed with 6xHis tag in *E. coli.* BL21(DE3) strain. All recombinant proteins were expressed and purified with Ni-NTA beads as described earlier (Soni, R.K. *et al.,* 2003). Briefly, *E. coli* strain BL21 (DE3) (Novagen) harboring respective clones were grown at 37°C in LB media containing 50 mg/ml kanamycin. The bacterial cultures were induced for the expression of the recombinant proteins using 1 mM IPTG. To get the better yield of deletion mutants in soluble fraction the bacterial culture was induced for 5–6 h at 22°C in the presence of 0.2 mM IPTG followed by usual protein extraction and purification procedures as described earlier (Soni, R.K. *et al.,* 2003). Briefly, the cells were harvested after induction by centrifugation at 5000 RPM for 10 minutes. The cell pellet was lysed with lysis buffer. The lysate was subjected to sonication at 35 amplitude for 5 minutes. The sonicated sample was subjected to Triton-X 100 treatment for 1 hour at 7 RPM. After that lysate was centrifuged at 15000 RPM for 30 minutes. After separation of debris the clear supernatant was incubated with Ni-NTA beads for 1 hour at 7 RPM. After binding the beads were washed with lysis buffer containing 20 mM immadazole. After extensive washing the protein was eluted with 500 mM immidazole. GST-HpDnaB and GSTHp0897 were purified using Glutathione Sepharose-4B beads (GE-Healthcare) using the instructions supplied by the vendor. Briefly, *E. coli* cells harboring constructs of GSTHpDnaB, GSTHp0897 and GST alone were grown in LB media containing 100 µg/ml ampicillin at 37°C. The bacterial cultures were induced with 1mM IPTG at 37°C for three hours. The cells were pelleted and

frozen at -80 °C. The cells were resuspended in lysis buffer (1xPBS, 2mM EDTA, 5mM DTT, 100µM PMSF). The samples were sonicated to fragment the DNA and treated with Triton X-100. The fragmented membrane fractions and debris pelleted by centrifugation (1500 RPM/30 minutes) and supernatant was incubated with glutathione sepharose beads. The non-specific proteins were removed by washing the beads in the presence of wash buffer (1xPBS, 10mM DTT, 300mM NaCl, 100µM PMSF). The bead-bound GST fusion proteins were used as bait proteins in pull-down assay.

2.5. GST pull-down assay

GST pull-down assay was performed to find out the minimum regions required for protein-protein interaction of HpDnaB and ORF0897 proteins,. In this assay, His_6 tagged HpDnaB and its various deletions were incubated with either bead-bound GST0897 or Bead bound GST (Control) alone in a buffer (50mM Tris pH7.5, 1mM DTT, 4% glycerol, 0.1mg/ml BSA, 5mM $MgCl_2$, 0.01% NP40, 50mM NaCl) at 4^OC for 1 hour with gentle rotation. The beads were washed thoroughly with washing buffer (50mM Tris pH7.5, 1mM DTT, 4% glycerol, 0.1mg/ml BSA, 5mM $MgCl_2$, 0.1% NP40, 600 mM NaCl) and the bound proteins were released by boiling in SDS-PAGE loading buffer followed by SDS-PAGE analysis and Western blot experiments using anti-His antibodies against His_6 residues. Similarly, to find domain of HP0897 responsible for interaction with HpDnaB, His_6 tagged HP0897 and its N- and C-terminal deletions were incubated with either bead bound GSTDnaB or bead bound GST alone as a control and processed in the same way as discussed above.

2.6. *H. pylori* extract preparation and Western blot analysis

Bacterial cell extract from different *H. pylori* strains *Hp26695,B28* and *P12* (obtained from Dr. Niyaz Ahmed, Hyderabad) were used for Western blot experiment using antibodies against HP0897. *H. pylori* cell pellet (50 µl) was washed with 1X PBS and resuspended in 200 µl of *H. pylori* lysis buffer (50 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% NP 40, 1 mM PMSF and 10 µg/ml leupeptine). Further 200 µl 2X SDS-PAGE loading buffer was added and the suspension was boiled for 10 minutes at 95ºC. The samples were resolved on 12% SDS-PAGE followed by Western blot analysis.

2.7. Immunofluorescence assay

H. pylori cells were allowed to settle and attached to poly-lysine (0.01%) coated glass slides and fixed with 4% paraformaldehyde in 1X PBS for 15 minutes at room temperature. The slides containing bacterial cells were washed with 1X PBS for three times and treated with Triton-X100 (0.3% in 1X PBS) for 25 minutes at 25^0C. Subsequently, the slides were washed with 1X PBS and blocked with 3% BSA in 1X PBS for 1 hour. Primary antibodies treatment (1:1000 dilution in 1X PBS containing 3% BSA) for anti-HpDnaB (in rabbit) and (1:500 dilution in 1X PBS containing 3% BSA) for anti-Hp0897 (in mice) were done at 25^0C for 1 hour or 4^0C for overnight. After washing with 1X PBS, cells were incubated with secondary antibodies (1:1000 dilution for Alexa fluor 594 conjugated anti-mice IgG antibodies and 1:1000 dilution for Alexa fluor 488 conjugated anti-rabbit IgG antibodies) obtained from Santa Cruz (USA). Cells were further washed with 1X PBS three times and mounted with antifade (Invitrogen). An AxioVision fluorescence microscope (Nikon) was used to capture the images. Axiovision, release 4.6 (Nikon) software was used for analysis of the images.

2.8. Chromatin Immunoprecipitation Assay (ChIP)

Bacterial cultures of wild-type 26695 strain were grown to $OD_{600}$of 1.0–1.5 or grown for overnight. Cells were crosslinked with 1% formaldehyde for 15 min at 37^OC in incubator shaker. Then, the cells were incubated on ice for 10 minutes. Subsequently, the cell were centrifuged 4000 RPM for 10 min at 4 °C and washed twice with 1X PBS which contained 1mM PMSF and protease inhibitor cocktail. Harvested cells were lysed with SDS lysis buffer (1% SDS, 10mM EDTA, 1mm PMSF, 50mM Tris-HCl, pH 8.1 and cocktail protease inhibitor). Lysozyme was added to a final concentration of 0.25 mg/ml and incubated on rocker for 30 minutes at 4^OC. Bacterial lysate was sonicated at amplitude of 35% for 15 to 20 minutes to shear the DNA. The samples were centrifuged at 13000 RPM for 10 minutes to remove debris. The clear lysate obtained was diluted with ChIP dilution buffer. 5% of the diluted sample was recovered as input. The lysate was pre-cleared by incubating it with protein-A sepharose beads (Sigma-Aldrich) for one hour. The precleared lysate was divided equally for pre-immune and immune sera. The lysates were incubated with 2.5 µl

of pre-immune and 2.5 µl of immune antibodies for overnight at 4^{O}C on rocker. Around 20 µl Protein-A-Sepharose beads, washed with ChIP dilution buffer, were added to each tube and incubated on rocker for 3 hours at 4 ^{O}C. The bead bound immune complex was washed with low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl) for 10 minutes, with high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl) for 5 minutes followed by two washes with TE (Tris-EDTA) for 10 minutes each. The proteins wereeluted with elution buffer (1% SDS, 0.1 M NaHCO3). Reversed Crosslinks were reversed by adding 20 µl of 5M NaCl and heatingat 65^{O}C for 4 hours. 10 µl of 0.5M EDTA, 20 µl of 1 M Tris-Cl, pH 6.5 and 2 µl of 10mg/ml Proteinase K were added for a sample of 500 µl. The mixture was incubated for one hour at 45^{O}C. The DNA was recovered by phenol chloroform extraction and ethanol precipitation. Pellet was washed with 70% ethanol and air dried. The resultant DNA was quantitated and used for q-PCR.

2.9. References

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Chapter 3

Structure-function analysis of HpDnaB-HpORF0897 interaction

3.1. Introduction

Helicase loading onto single-stranded DNA at the origin is an essential step during replication initiation. Once the bubble is formed at the AT-rich DNA unwinding element (DUE) by the action of DnaA protein, the next phase of replisome assembly involves loading of two ring form helicase complexes onto two melted single-stranded DNA strands (Messer, 2002). Although DNA replication proteins studied in the model organism *E. coli* are conserved among bacteria, the mechanism of helicase loading can vary among different bacterial species. Two strategies can be considered by which helicase is loaded onto ssDNA. Examples of each strategy have been found in different bacterial species (Soultanas, 2012). One way is that preformed helicase ring can be opened physically and then deposited onto ssDNA. Another method is the monomers of DnaB assemble into a ring around the melted DNA. In *E. coli*, as discussed earlier helicase enzyme is loaded on to DUE by the coordinated activity of initiator DnaA and helicase loader DnaC (Davey et al., 2002, Fang et al., 1999, Marszalek and Kaguni, 1994, Seitz et al., 2000, Wickner and Hurwitz, 1975). First of all, bidirectional replication fork is established at *oriC* with the binding of DnaA and regulatory proteins IHF, HU and Fis which leads to melting of AT-rich region within DUE near to *oriC*. Following bubble formation, DnaA assists helicase loader DnaC to load DnaB on each single-stranded DNA substrate in the proper orientation. In this process, N terminal of DnaA interacts with N terminal of DnaB and on the other hand, N terminal domain of DnaC interacts directly with C terminal RecA ATPase domain of DnaB (Figure 3.1). DnaB and DnaC interact in 6:6 stoichiometry to load onto single-stranded DNA (Kobori and Kornberg, 1982). DnaC helicase loader of *E. coli* is an ATP/ADP switch protein and belongs to the AAA+ superfamily. DnaC requires ATP to function at *oriC*. In fact, ATP bound DnaC inhibits helicase activity. Once hexameric helicase ring is loaded onto each strand of the bubble in the proper orientation, DnaC hydrolyzes bound ATP which in part is induced by completely assembled DnaB hexameric ring and ssDNA. Although, ATP is not required for DnaB and DnaC interaction and loading of DnaB, however, it is required at *oriC* as it increases the affinity of DnaB●DnaC complex for ssDNA and helps the expansion of bubble to accommodate the complex. After loading of DnaB, ATP hydrolysis by DnaC activates helicase activity of DnaB (Davey et al., 2002). In gram-positive bacteria, *Bacillus subtilis*, helicase (DnaC) loading is dependent not only upon helicase loader (DnaI), but it requires the

assistance of two additional factors DnaB and DnaD. During initiation in *B. subtilis*, the initiation protein DnaA and other helicase loading proteins assemble in the order DnaA→DnaD→DnaB (Smits et al., 2010, Smits et al., 2011). DnaD and DnaB interact with DnaC●DnaI complex to facilitate helicase loading (Velten et al., 2003). Like in *E. coli*, helicase loader DnaI binds ssDNA in ATP dependent manner and forms a stable complex with helicase (DnaC) in 6:6 ratio (Ioannou et al., 2006). Reports also suggest that helicase in *B. subtilis* assemble on the origin DNA rather than loaded as a preformed hexamer (Velten et al., 2003). Little is known about the function of accessory proteins DnaB and DnaD in replication initiation of *B. subtilis*.

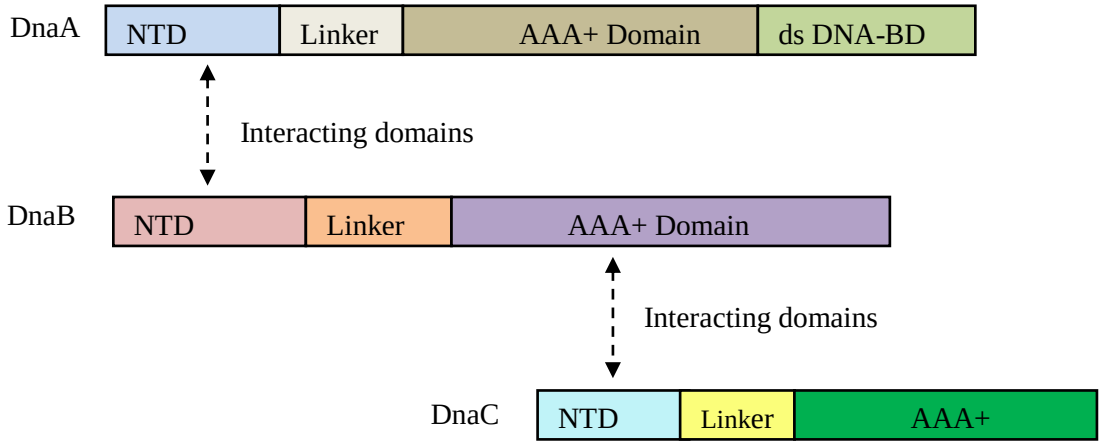


Figure 3.1. Schematic diagram of different domains of *E. coli* viz. DnaA, DnaB, and DnaC. Arrows show different domains involved in protein-protein interaction during DNA replication.

In *H. pylori*, little is known about initiation of DNA replication and loading of helicase onto ssDNA at *oriC*. It is relevant to study replication initiation as *H. pylori* differs from other bacteria in *oriC* organization and important conserved proteins of replication. Instead of a single continuous origin, *H. pylori* possess bipartite origin, which is divided into two sub-origins called *oriC1* and *oriC2*. These sub-origins have *DnaA* sandwiched between them (Donczew et al., 2012). The fundamental difference between *H. pylori*-DnaB helicase and helicases of other bacteria is that it contains the signature insertion of 34 amino acid in ATPase domain which is absent in DnaBs' of other bacteria. This unique 34 amino acid patch

is called *Helicobacter pylori* insertion (HPI). Deletion of this insertion causes DnaB to form monomers instead of hexamer and loss of helicase and ATPase activities (Nitharwal et al., 2007). Therefore, this insertion is essential for hexamerisation of DnaB. Another critical feature of HpDnaB is that it forms dodecamer in solution as compared to DnaBs in other bacteria which are mostly hexameric (Stelter et al., 2012). Two identical hexamers of HpDnaB interact head to head through N terminal domains to form dodecamer similar to replicative helicases found in eukaryotes like yeast (MCM2-7), archaea *Methanobacterium thermoautotrophicum* (MtMCM) and viruses (LTag, E1) (Stelter et al., 2012, Remus et al., 2009, Evrin et al., 2009, Valle et al., 2000, Schuck and Stenlund, 2005). Apart from these differences, *H. pylori* lacks an apparent homolog of helicase loader. A recent study has shown that HPORF0897 which is hypothetical protein that interacts functionally with HpDnaB and modulates its DNA binding, ATPase and helicase activity as helicase loader DnaC does in *E.coli* (Verma et al., 2016). Thus, HPORF0897 functionally interacts with HpDnaB and may function as a possible ortholog of *E. coli* DnaC loader whose sequence has evolved during evolution. Unlike other helicase loaders which belong to AAA+ family, HPORF0897 does not possess a conserved ATPase domain. Blast search showed HPORF0897 is unique to *H. pylori*. The functional interaction between HpDnaB and HPORF0897 raises the question of how HPORF0897 binds to HpDnaB and what are the domains within HPORF0897 that modulate HpDnaB activities. To address the mechanism for HPORF0897 stimulation of HpDnaB helicase function, we have performed structure-function analysis of the interaction between HPORF0897 and HpDnaB. In this chapter domain mapping of both the proteins has been carried out to find the minimum regions important for binding of HpDnaB with ORF0897.

3.2. Results

3.2.1 Expression and Purification of wild-type and different deletion mutant forms of HpDnaB and HPORF0897

The coding regions of *HPORF0897, HpDnaB* and their different deletion mutant forms were amplified by polymerase chain reaction using 26695 genomic DNA as template and primers listed in table 2.2 and the PCR products were cloned in pET28a vector. *E.coli* BL21 (DE3)

cells were transformed using recombinant clones. The details of expression and purification of the proteins are described in the material and method section. The induction of each protein in the presence of IPTG was checked followed by purification of the protein using $Ni^{+}2$-NTA affinity purification. The schematic diagram of all the constructs of HpDnaB, Hp0897 and their deletion mutants are shown in figure 3.2. HpDnaB is 488 amino acid protein and comprises the N-terminal domain (1-120 amino acids) and C terminal domain (175-488 amino acids) which are joined by a flexible linker region (121-174). HpDnaB144 deletion mutant contains 1-144 amino acids of HpDnaB which covers the N-terminal domain and a part of the linker region. HpDnaBN2 consists of 134-488 amino acids and includes a section of linker region and C terminal domain. HpDnaBN3 consists of 175-488 amino acids which covers complete C terminal domain and exclude linker region. HpDnaBN4 contains a portion of the C-terminal domain from 195-488 amino acids. HpDnaBDelC29 consists of 1-459 amino acids which lack 29 amino acids from the extreme C terminus of HpDnaB. HpDnaBDelC34 is comprised of full HpDnaB which excludes unique HPI region of 34 amino acids. HpDnaBCDel89 consists of 1-399 amino acids with deletion of 89 amino acids from the extreme C-terminus of HpDnaB. HpDnaBCDel160 consists of 1-328 amino acids which exclude 160 amino acids from the extreme C-terminus of HpDnaB. HpDnaBC171 is comprised of 318-488 amino acids of HpDnaB protein. One representative figure for the purification of His_6-HpDnaBΔ29 is shown in figure 3.3 (a). The purification profile of HpDnaB wild-type and all other deletion mutants are shown in figure 3.3 (b) to (g).

Further, HpDnaB, as well as HPORF0897 proteins were also expressed as GST fusion proteins. For this purpose, coding regions of *HpDnaB* and *HPORF0897* were amplified by PCR using cloned templates of the respective genes in the presence of PCR primers as shown in table 2.2 and the PCR products were cloned in the pGEX6P2 vector. The details of the expression and purification of GST fusion proteins are described in material and methods. The purification profiles of His_6-HpDnaB, His_6-HPORF0897 and their different deletion mutant forms as well as GST tagged proteins are shown in figure 3.4. The His_6 proteins were eluted from Ni^{+2} NTA beads and stored at -80 ^{O}C until use and GST tagged proteins were kept as bead-bound form. These proteins were used for pull-down assay.

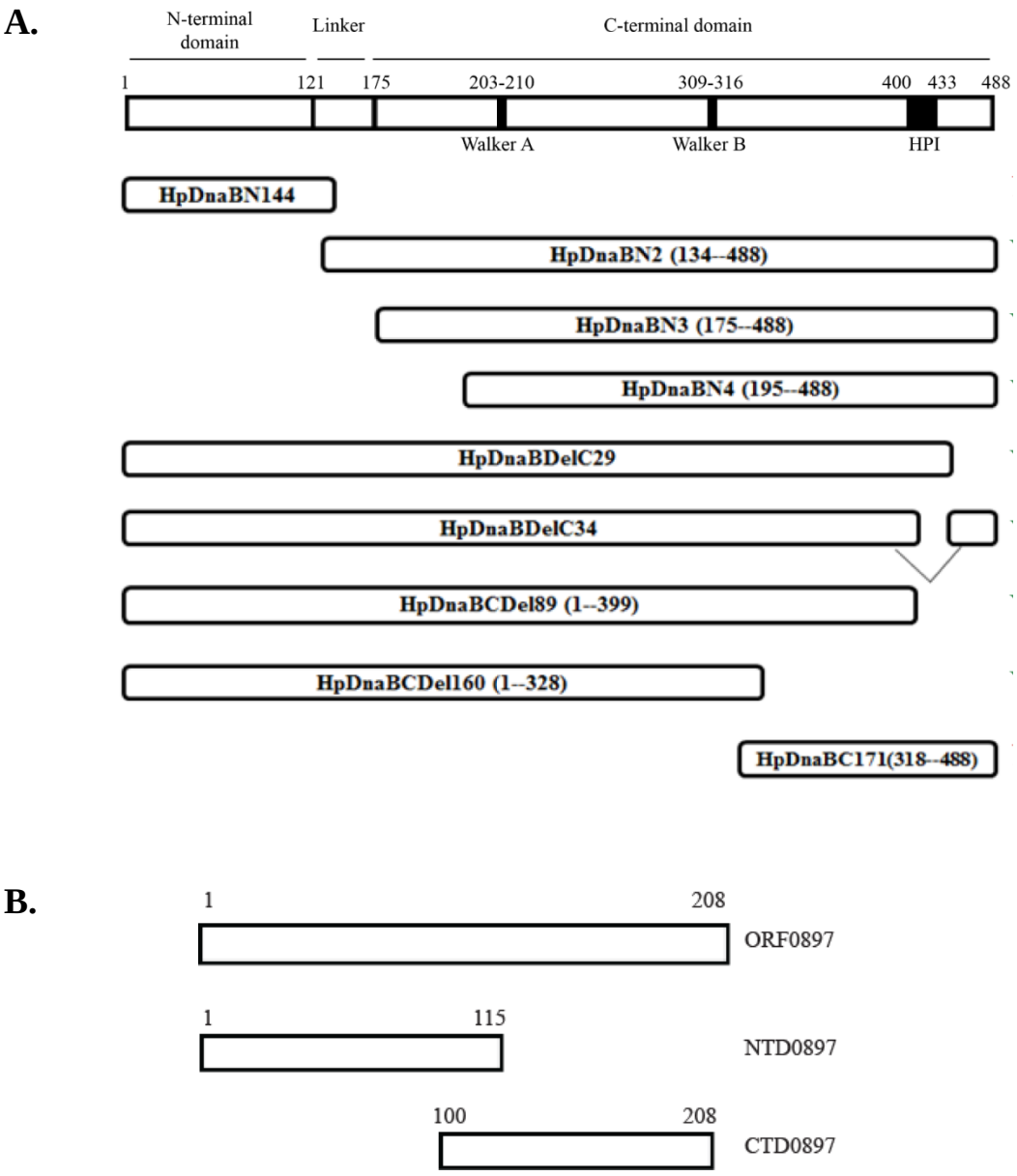


Figure 3.2 Schematic diagram of various constructs of HpDnaB and HPORF0897. Wild type and different deletion mutant forms of HpDnaB (A) and HPORF087 (B) used in pull-down assay to map interacting regions on both the proteins are shown. Amino acid co-ordinates have been marked. WALKER A and WALKER B motifs as well as *H. pylori* Insertion region (HPI) are also shown.

3.2.2. Mapping of different domains of HpDnaB for interaction with Hp0897

In order to identify the domains of HpDnaB important for interaction with Hp0897, GST pull-down assay was employed.

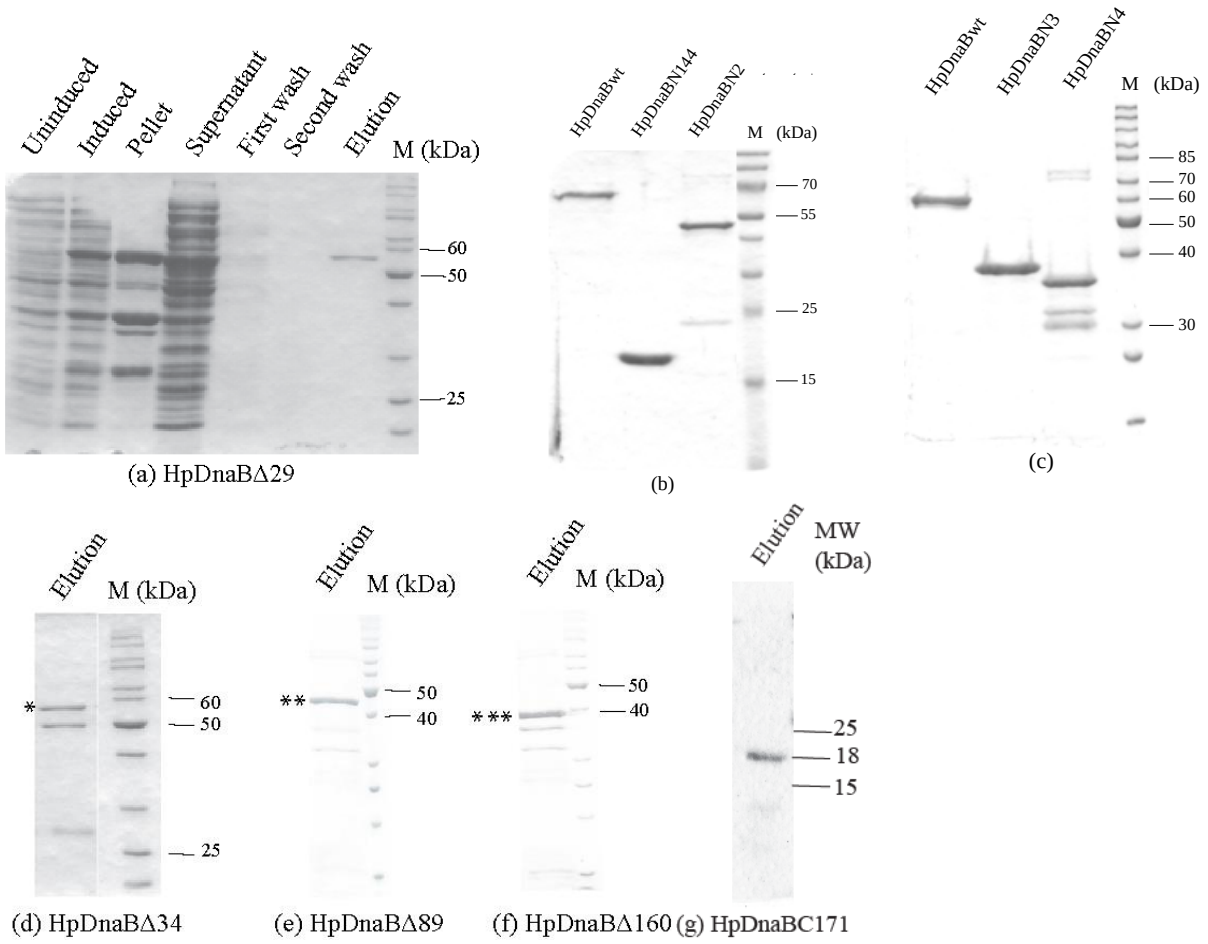


Figure 3.3 Purification profile of HpDnaB and its various deletion mutants. (a) His_6 tagged proteins were purified by Ni-NTA affinity purification and subjected to SDS-PAGE and stained with Coomassie brilliant blue. Complete purification profile of HpDnaBΔ29 is shown (a) and final elutions of wild-type HpDnaB (b) and its various deletion mutants (b) to (g) are shown. *, ** and *** indicate the major bands corresponding to respective deletion mutant proteins in (d) to (e).

In this assay, GST0897 bound to GST beads was used as bait protein and GST protein only was used as negative control. HpDnaB full length and its various deletion mutants (Figure 3.2 and 3.3) expressed as His_6-tagged recombinant proteins were used as prey proteins.

GST-ORF0897 immobilized to glutathione beads was incubated with HpDnaB and its various deletions in binding buffer individually as indicated in material and methods. Domains of HpDnaB precipitated with glutathione bead-bound GST-ORF0897 were detected

by running the samples on SDS-PAGE followed by Western blotting using antibodies against His_6 residues (mouse) . Either gel after transfer or PVDF

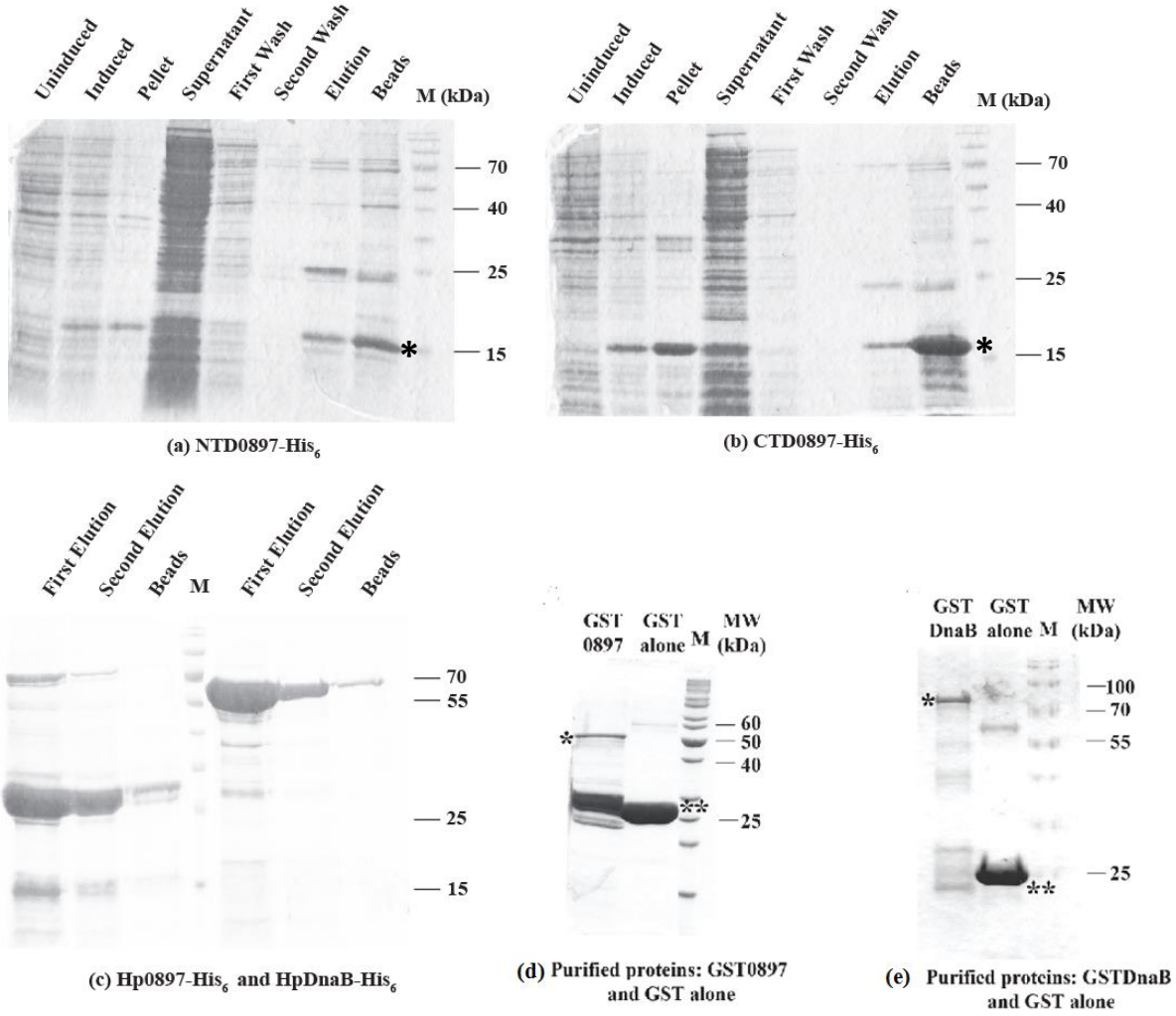


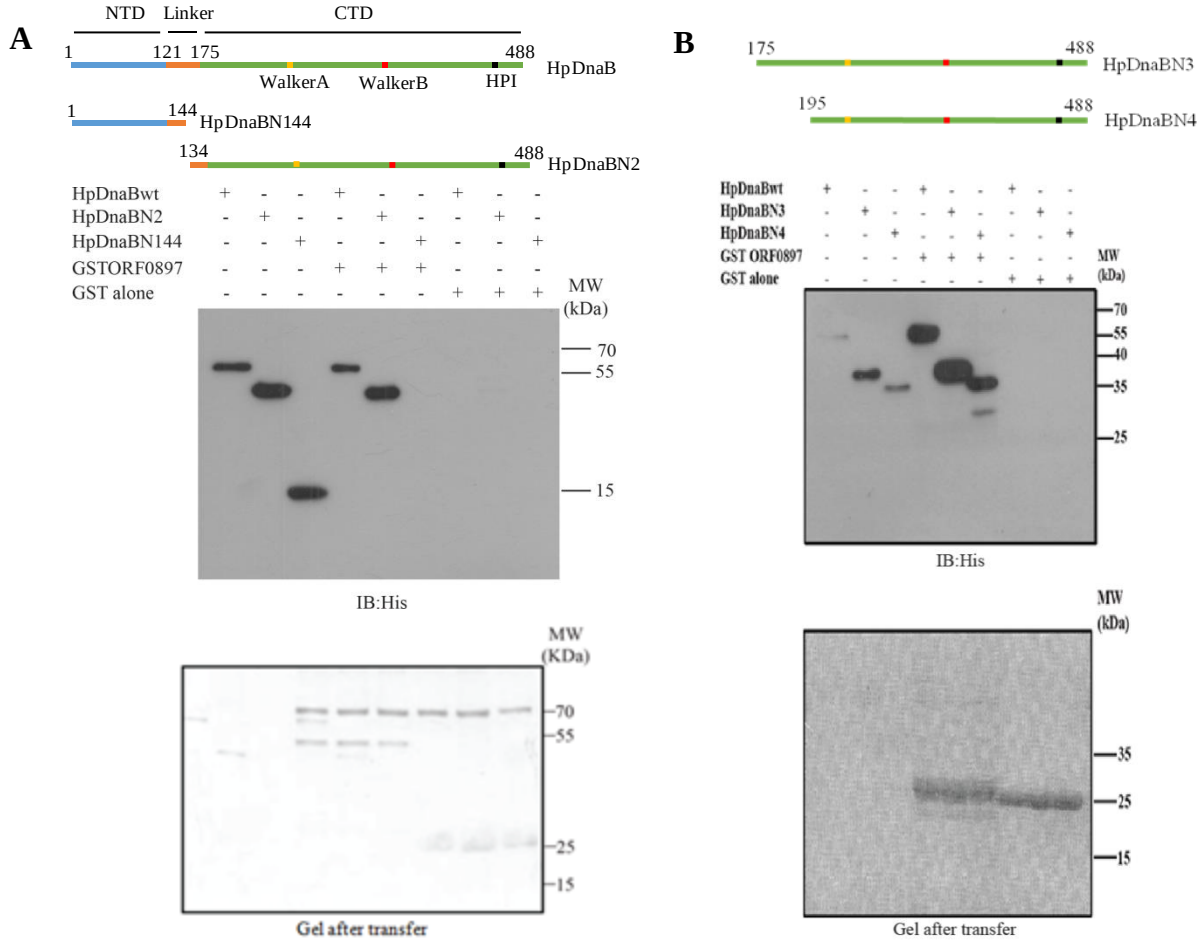
Figure 3.4 Purification profile of HpDnaB, Hp0897 and its two deletion mutants. (a) His_6 tagged proteins were purified by Ni-NTA affinity purification and GST tagged proteins were purified by glutathione beads. After elution the samples were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Complete purification profile of NTD0897-His_6 and CTD0897-His_6 are shown (a) and (b) respectively. * indicates NTD0897 and CTD0897proteins in respective gels. Hp0897-His_6 (Left to marker lane) and HpDnaB-His_6 (right to marker lane) after Ni-NTA purification are shown (c). GST tagged Hp0897, HpDnaB and GST alone after purification are shown (d) and (e) respectively. * indicates the GST-tagged full length protein and ** indicates GST alone protein.

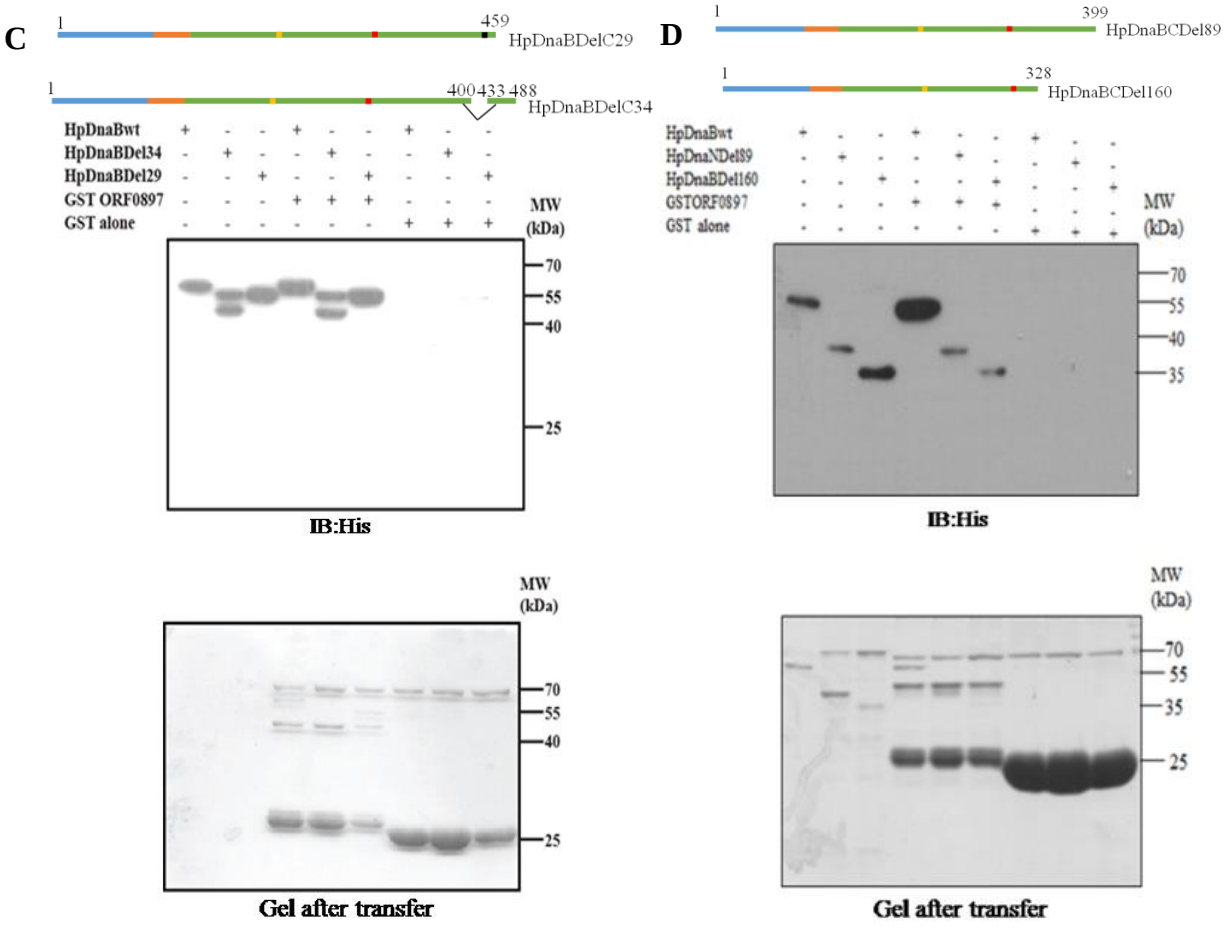
membrane after Western blotting was stained with Coomassie brilliant blue and used as loading control for various proteins (Figure 3.5).

The results showed that out of nine deletion mutants of HpDnaB two deletion mutants namely HpDnaBN144 and HpDnaBC171 did not interact with GSTHP0897 whereas all other deletion mutant forms showed interaction in the pull-down assay (figure 3.5.A-E). None of the proteins interacted with GST bound glutathione beads used as a control in the pull-down assay (figure 3.5). The results of the pull-down assay are summarised in table 3.1.

3.2.3. Mapping of Hp0897 for binding with HpDnaB

Further, we performed pull-down experiments to determine the domain on Hp0897 that interacts with HpDnaB. Due to lack of sequence similarity of Hp0897 with other helicase loaders, its N terminal and C terminal domains are not well defined. To start Hp0897 domain mapping, two domains selected and named NTD0897 and CTD0897. NTD0897 expands from amino acid 1 to 115, and CTD extends from amino acid 100 to 208 of HPORF0897.





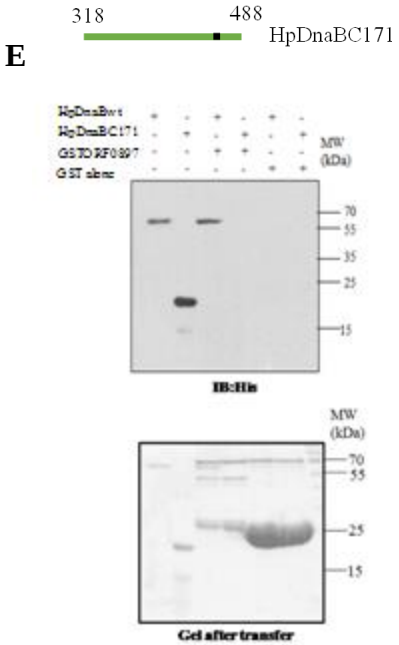


Figure 3.5 GST pull-down experiment showing interaction of HP0897 with WT and different deletion mutants of HpDnaB. (A-E) Purified GSTORF0897 was immobilized on gutathione beads and incubated with purified His_6 tagged HpDnaBwt and its various deletion mutants. After incubation, the beads were washed stringently and the eluates were resolved on SDS-PAGE. The presence of HpDnaBwt and its various deletion mutants was detected by Western blotting using antibodies against His_6 tag. For loading control either SDS-PAGE gel after transfer (A, C, D, E) or PVDF membrane after Western blotting (B) were stained with Coomassie brilliant blue.

GST proteins bound on beads were used as control for pull-down assay. The results indicated that except HpDnaBN144 and HpDnaBC171 all other deletion mutant proteins and HpDnaBwt interacted with Hp0897. No binding was found when GST alone protein-bound beads were used as a control.

While planning the boundaries of these two domains, the secondary structure of Hp0897 is also considered. The secondary structure shows 63.46% alpha helix, 7.96% extended strand and 28.85% random coil (figure 3.6 A and B).

These NTD and CTD domains of Hp0897 are designed in such a way so that their secondary structure should not disturb. These domains have an overlapping region of 16 amino acids from 100 to 115.

For pull-down assay, GSTHpDnaB bound to glutathione beads was used as bait protein and GST alone was used as negative control. Hp0897 full length and its two deletion mutants NTD0897 and CTD0897 (figure 3.4) were expressed as His_6-tagged recombinant proteins and used as prey proteins. Glutathione bead-bound GST-DnaB fusion protein was incubated with Hp0897 or its deletion mutants for 1 or 2 hours, and samples were resolved in SDS-PAGE followed by Western blotting with antibodies against His_6 residues. Results showed that both NTD0897 and CTD0897 were detected with different intensities. Input lanes showed original amounts of prey proteins used in pull-down experiment. By comparison with input lanes it was observed that NTD0897 was more in the input lane. However, its presence was less in the pulled down sample. On the other hand, binding of CTD0897 with GST-DnaB was predominant (figure 3.6.C). It implies that CTD0897 interacts strongly whereas NTD0897 interacts weakly with HpDnaB.

Due to hydrophobic nature of N terminus of Hp0897, it is difficult to express and purify the protein with reasonable yield. In order to resolve the above issue Hp0897wt and different deletion mutant proteins were expressed and purified as MBP fusion proteins as described in material and method section.

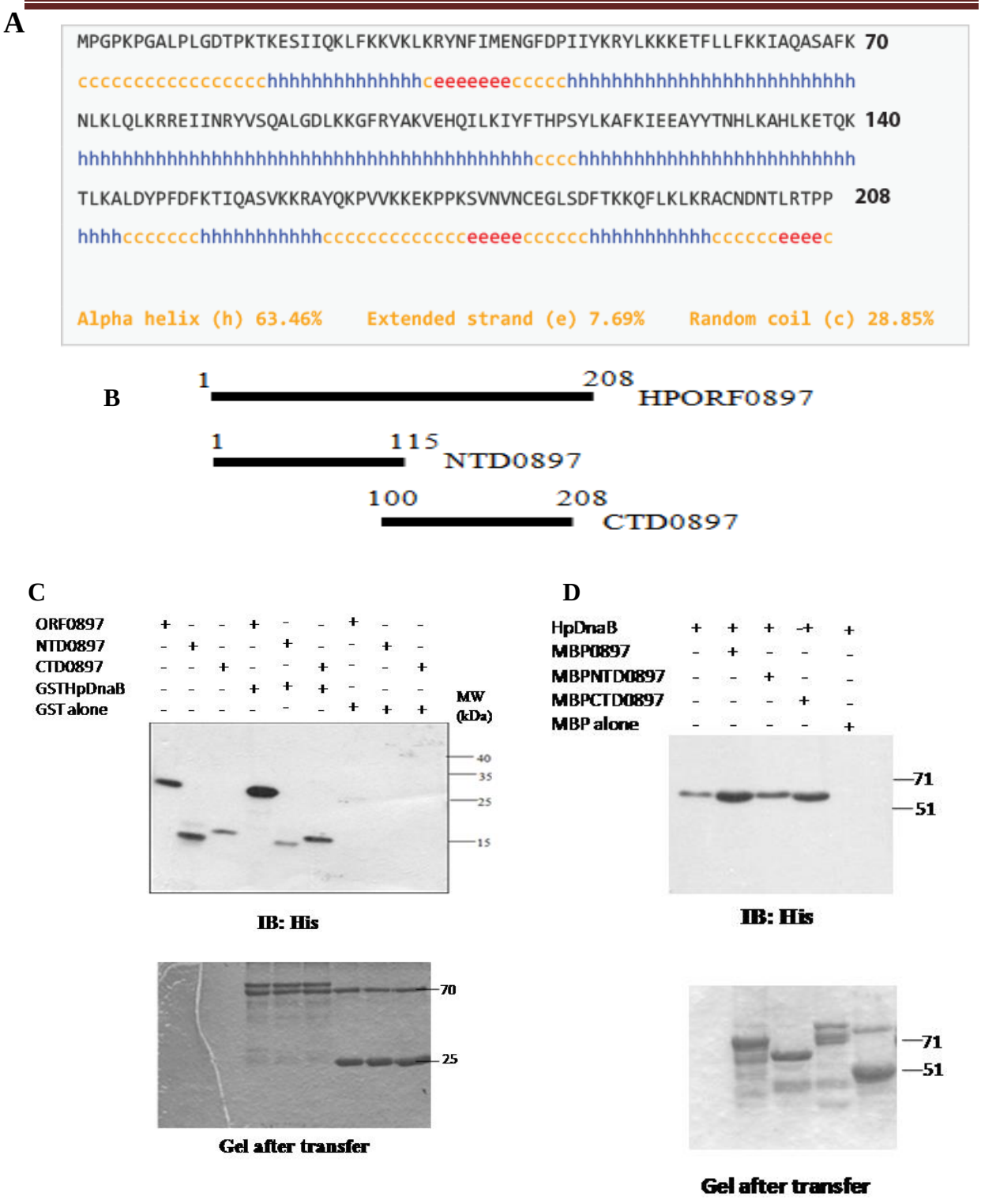


Figure 3.6 Pull-down assay for domain mapping of Hp0897 for interaction with HpDnaB (A) Secondary structure of Hp0897. (B) Schematic diagram is showing Hp0897wt, NTD0897 and CTD0897. (C) HpDnaB protein expressed as GST fusion protein and used as a bait protein in pull-down experiment and incubated with His_6 tagged Hp0897wt and its deletion mutants NTD0897 and

CTD0897. After stringent washing of the beads, eluates were resolved on SDS-PAGE, followed by Western blot analysis using antibodies against His_{six} tag. For loading control, SDS-PAGE gel after the transfer was stained with Coomassie brilliant blue. (D) Reverse pull-down experiment performed by immobilizing ORF0897, NTD0897, and CTD0897 onto beads and incubating with purified 6xHis tagged HpDnaB. Interacting HpDnaB was detected by western blot using anti His_6 antibodies.

Pull-down experiment was repeated by immobilizing MBP-0897wt, MBP-NTD0897, and MBP-CTD0897 on beads followed by incubation with His_6-HpDnaB. The bound proteins were resolved in SDS-PAGE followed by Western blot analysis using antibodies against His_6 residues. MBP protein alone bound to beads were used as control. Pull-down experiment showed comparative results for Hp0897wt and Hp0897CTD but less binding for Hp0897NTD as described above (figure 3.6.D). These results indeed conclude that Hp0897CTD interacts with HpDnaB more strongly than Hp0897NTD.

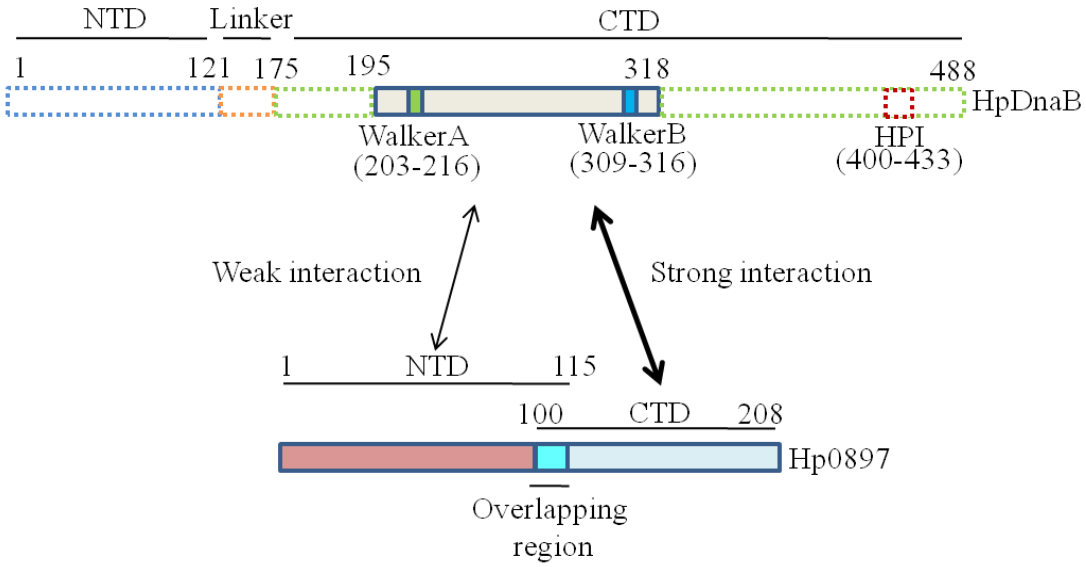


Figure 3.7 Schematic diagram showing the minimum region of HpDnaB possibly interacting with Hp0897. The minimum region of HpDnaB (195 to 318 residues) that interacts with Hp0897 is shown as filled boxes whereas rest of the part of HpDnaB with different domains that are not interacting with Hp0897 is shown as dotted lines.

Table 3.1 (Summary of pull down assay results for HpDnaBWt and different deletion mutamt proteins)

Protein	Interaction with Hp0897
HpDnaBwt	Yes
HpDnaBN144	No
HpDnaBN2	Yes
HpDnaBN3	Yes
HpDnaBN4	Yes
HpDnBCΔ29	Yes
HpDnaBCΔ34	Yes
HpDnaBCΔ89	Yes
HpDnaBCΔ160	Yes
HpDnaBC171	No

3.3. Discussion.

ORF0897 interacts functionally with HpDnaB and modulates its various activities in a fashion as helicase loader DnaC accomplishes for DnaB in *E. coli*. However, significant differences exist in sequence and structure of these proteins from *E. coli* and *H. pylori*. HpDnaB contains unique HPI domain of 34 amino acids, and structurally it is different as it exists as dodecamer instead of the hexamer (Stelter et al., 2012). At present, whether the oligomeric status of active HpDnaB after loading at fork is hexameric or remains dodecameric during its helicase function is unclear.

Hp0897 has no similarity with any known helicase loader. Helicase loaders in other bacteria belong to AAA+ family and possess ATPase domain with weak ATPase activity. No such ATPase domain is found in HPORF0897 sequence by Bioinformatics analysis. How does Hp0897 without any sequence homology with conserved helicase loaders function as

helicase loader for HpDnaB? .To understand this, we must know the domains of HpDnaB and ORF087 that interact with each other. For this purpose, *in vitro* pull-down experiment was performed to map domains of HpDnaB and HPORF0897 responsible for interaction of the two proteins. Using various deletion mutants of HpDnaB and HPORF0897 in pull-down experiment, we have found that extreme N and C terminus of HpDnaB are not required for interaction with HPORF0897. As extreme N and C terminal regions of HpDnaB are not involved in this protein-protein interaction, further deletion of N and C terminus of HpDnaB revealed that N-terminal region from amino acids 1 to 194 and C-terminal region from amino acids 329 to 488 of HpDnaB are dispensable for HpDnaB and ORF0897. It means ORF0897 requires amino acids 195 to 328 of HpDnaB and therefore ORF0897 binding domain may be present within this region of HpDnaB (figure 3.7). Both ATP binding (amino acid 203 to 210) and ATP hydrolysis (309 to 316) domains of HpDnaB are residing within this region. Hp0897 interacts with HpDnaB in *H. pylori* in a similar fashion as *E. coli* DnaC interacts with DnaB. In *E. coli* and other bacteria, helicase loader binds DnaB near to Walker and Walker B motifs in the C-terminal domain of DnaB. Therefore, *H. pylori* helicase loader binds to the C-terminal domain of HpDnaB as reported in other bacteria.

On the other hand, Hp0897 shows HpDnaB binding activity for N and C terminal regions with varying affinities. C-terminal domain of Hp0897 binds strongly to HpDnaB whereas the N-terminal domain of Hp0897 interacts weakly with HpDnaB. These results are different than model organism *E. coli* where helicase loader interacts with helicase through its N terminal domain. In *E. coli* DnaC interacts with DnaB through two regions, but both of these regions reside within the N terminus of DnaC (8-11 and 31-44 residues of DnaC).

It has been reported that the stoichiometry of DnaB and DnaC in *E. coli* is 6:6 where one monomeric unit of DnaC binds with each subunit of DnaB in hexameric DnaB. Interestingly, Hp0897 is trimeric in solution. Therefore, it will be interesting to find out stoichiometry of HpDnaB and Hp0897 which may be different from DnaB●DnaC complex in *E. coli*. Further, the crystal structure of HpDnaB and Hp0897 may shed light on the mechanism of loading of this complex onto melted origin.

3.4. References

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Chapter 4

Essentiality of HP0897 gene for growth of H. pylori

4.1. Introduction

Most of the genes involved in DNA replication are essential including *dnaC* that plays a role during initiation of replication (Kaguni, 2006) and at the sites where DNA replication is stalled (Cox et al., 2000, Manhart and McHenry, 2015). *dnaA, fis* and *DnaB* are examples of genes which have been shown to be essential for DNA replication initiation (Goodall et al., 2018).

Temperature sensitive mutant strains have been very useful for studying the function of essential genes. *DnaA* temperature sensitive (ts) mutant, *i.e., dna-59* inhibits replication at non permissive temperature (Sakakibara and Mizukami, 1980). *DnaB*ts mutants have been characterized which includes *dnaB8, dnaB252, dnaB454, dnaB70* and *dnaB43.* All of these temperature sensitive mutants arrest DNA replication at non-permissive temperatures (Saluja and Godson, 1995). *dnaB8, dnaB454* and *dnaB4 3*mutants have little ATP hydrolysis and helicase activity. *dnaB70* is defective in ATP binding. DnaB252 thought to be defective in binding with DnaC. Similarily, several temperature-sensitive mutants of DnaC gene have also been characterized which are defective in either initiation of DNA replication or elongation of DNA replication in *E. coli* (Wechsler and Gross, 1971, Sakai et al., 1974). *dna-2* mutant of *dnaC* showed defects in initiation whereas *dna-7* showed defects in elongation of DNA replication.

In case of gram positive bacteria *S. aureus* and *Bacillus subtilis,* helicase loader is *dnaI* which is required for growth and initiation of replication (Li et al., 2007). The human pathogen *M. tuberculosis* lacks an obvious homolog of DnaC, however, recently an uncharacterized gene Rv0004 has been identified which interacts with Mtb DnaB and plays a role in DNA replication. Therefore, Rv0004 is a member of DciA family of proteins which is found in bacteria with no clear homology with helicase loader protein (Mann et al., 2017) but it has a helicase operator function during initiation of replication.

The reports suggest that a functional copy of *dnaC* is required for survival of bacteria. Along with the requirement of a functional copy of *dnaC* gene, the cellular concentration of DnaC protein is tightly regulated. There is fine tuning of concentration of DnaB and DnaC proteins in the cell. A slight excess of DnaC decreases DnaB activity *in vivo* (Skarstad and Wold,

1995). This inhibition of DnaB activity by excess DnaC can be alleviated by overproduction of DnaB (Allen and Kornberg, 1991).

The presence of Hp0897 in all sequenced strains of *H. pylori* suggests some conserved function of this protein in *H. pylori*. HPORF0897 is annotated as ORF of unknown function in *H. pylori* genome database but it seems to perform essential function of helicase loader in *H. pylori* similar to DnaC protein in *E. coli*. Therefore, it will be interesting to see the status of Hp0897 gene for its essentiality in *H. pylori* for bacterial growth. So far, no temperature sensitive mutant strain for *H. pylori* replication initiation proteins has been reported.

In this chapter, we have attempted to explore the essentiality of *HP0897* gene by using conventional homologous recombination method to knockout *HP0897* gene from the genome of *H. pylori* 26695 strain.

4.2. Results

4.2.1. Designing of construct for *HP0897* knockout by homologous recombination

To knockout *HP0897*, homologous recombination method was used. Two strategies were undertaken for the generation of *HP0897* KO strain. In one method, we used closed circular DNA construct for homologous recombination (discussed in this section) and in another method we used linearized DNA construct for knockout experiment (discussed in next section). For designing closed circular DNA construct, 345 bp region of *HP0897* encoding NTD region and 200 bp upstream of start site of *HP0897* (a total of 545 bp) was used as upstream sequence (UPS) and 200 bp region downstream of *HP0897* was used as downstream sequence (DNS). For this purpose, we used a modified pBluescript (pBS) vector backbone as suicidal vector for *H .pylori* origin of replication. This vector (a kind gift from G. Mukhopadyay lab) contained chloramphenicol (CAM) cassette cloned between BamHI/XhoI sites. The vector map of pBluescript II KS (+) and schematic diagram for homologous recombination is shown in figure 4.1.

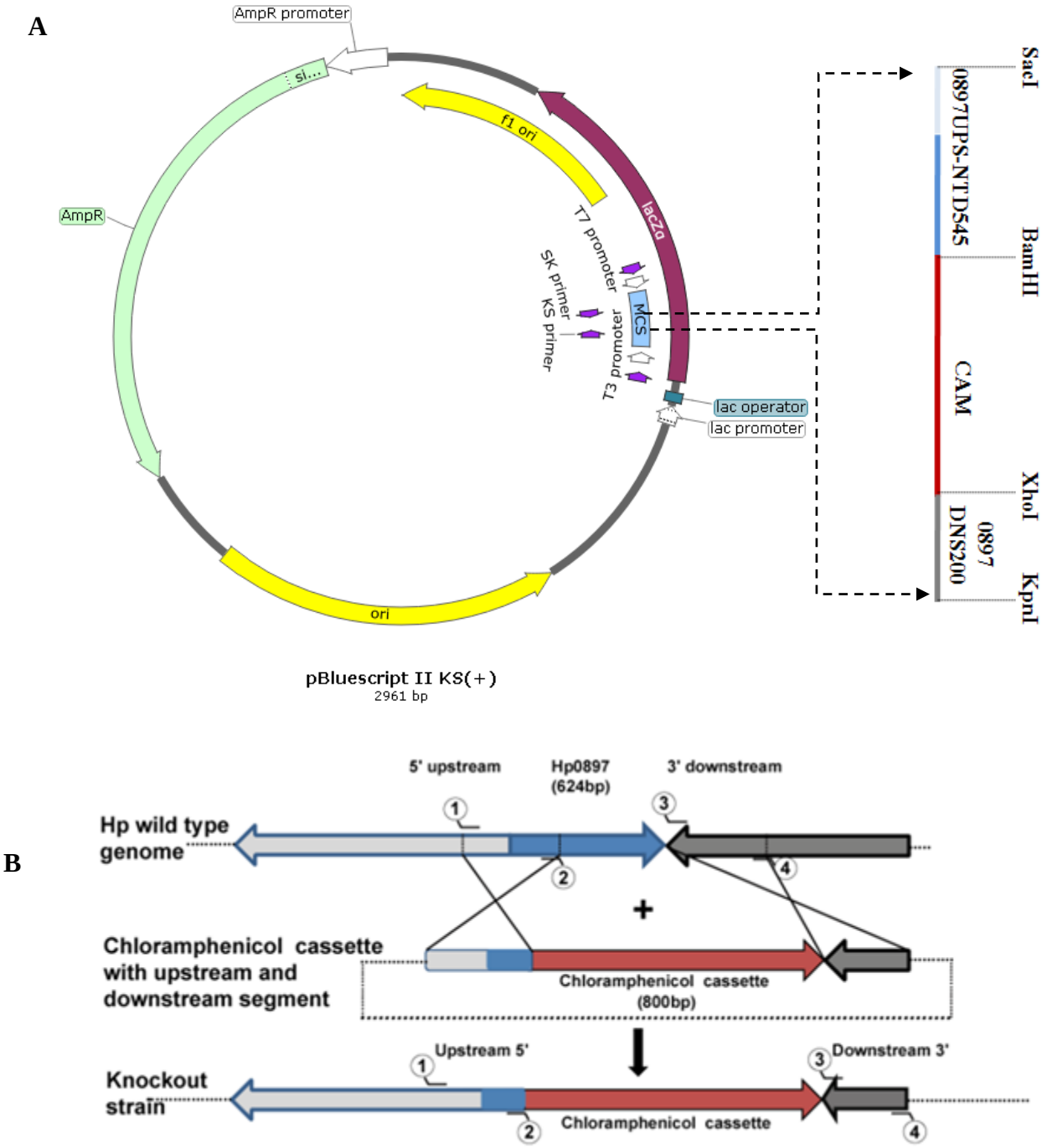


Figure 4.1 (A) Vector map of the modified pBluescript II KS (+) (a kind gift from Prof. G. Mukhopadhyay) that was used to make a construct for homologous recombination for the knockout experiment. The KO construct (UPS-NTD545::CAM:: DNS200) was cloned between SacI and KpnI sites. (B) Schematic diagram shows the strategy for *HP0897* knockout. The position of the primers 1, 2 (UPS) and 3, 4 (DNS) used for PCR amplification and cloning are marked in the figure.

For designing the circular construct, a region comprising 200 bp upstream of start site of *HP0897* and 1 to 345 bp of *HP0897* (total of 545 bp) were PCR amplified using primers FwHpUPS200 and RvHp897NTD listed in table 2.2 and 26695 genomic DNA as template (figure 4.2.a). This 545 bp PCR product was ligated in SacI/BamHI sites upstream of CAM cassette which was already cloned in BamHI/XhoI sites of pBluescript vector backbone at MCS. Next, a region of 200 bp downstream of *HP0897*gene was amplified by PCR by using primers FwDNS XhoI and RvDNS KpnI primers listed in table 2.2 and 26695 genomic DNA as template (figure 4.2.d). This 200 bp PCR product was ligated downstream of CAM cassette. The colonies were selected on ampicillin plate and screened by colony PCR (figure4.2.b and e). Positive clones were selected by restriction digestion analysis (Figure 4.2. c and f). The final construct was confirmed by sequencing.

4.2.2. Designing of construct for HP0897 KO by taking 500bp UPS and 500 bp DNS regions of *HP0897*

Initially, we took 545 bp as upstream sequence and 200 bp as downstream sequence for the designing of construct for the generation of HP0897 KO strain. 200bp upstream and downstream sequences may not be enough for homologous recombination. Further, the length of UPS and DNS of *HP0897*was adjusted to 500 bp each in order to keep UPS and DNS sequences of similar length. Subsequently, we have used UPS and DNS regions of 500 bp in the respective construct for homologous recombination to knockout *HP0897* and positive control *cagU*. *cagU,* a nonessential gene of type IV secretion system was used as a positive control for the generation of KO strain. The schematic diagram for homologous recombination strategy for Hp0897 KO strain generation has been shown in figure 4.3. The similar approach was followed for the generation of *cagU* KO strain.

The constructs for knockout of *Hp0897* (also for positive control *cagU)* were designed and amplified by overlap PCR. The strategy for overlap PCR is shown in figure 4.3. For this purpose, a region of 500 bp upstream of start site (UPS500) and 500 bp downstream of stop codon (DNS500) of *Hp0897* were amplified by PCR using primer pairs 0897KO1/0897KO2 and 0897KO3/0897KO4 respectively as listed in table 2.2 and 26695 genomic DNA as a template (figure 4.4 left).

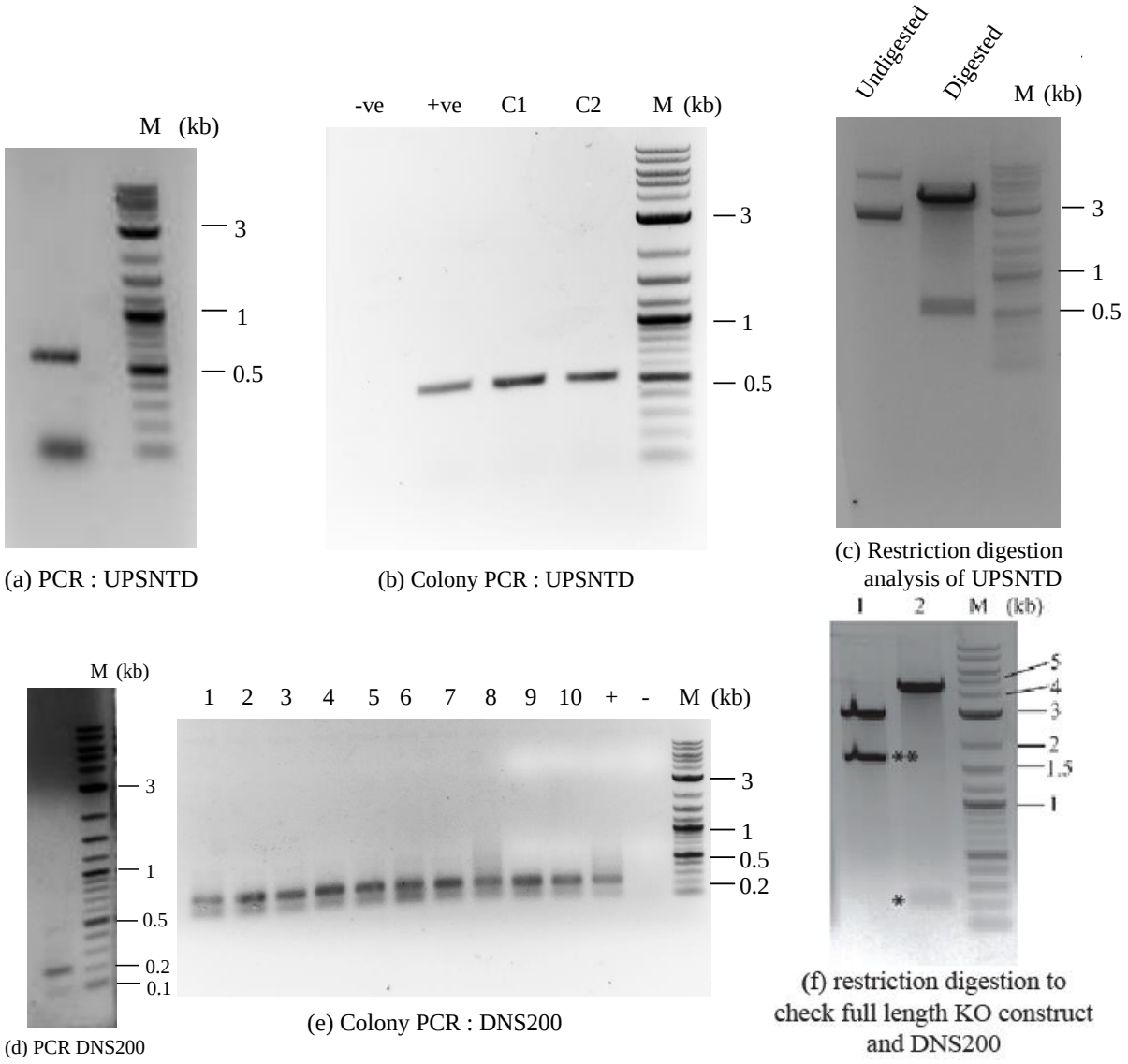
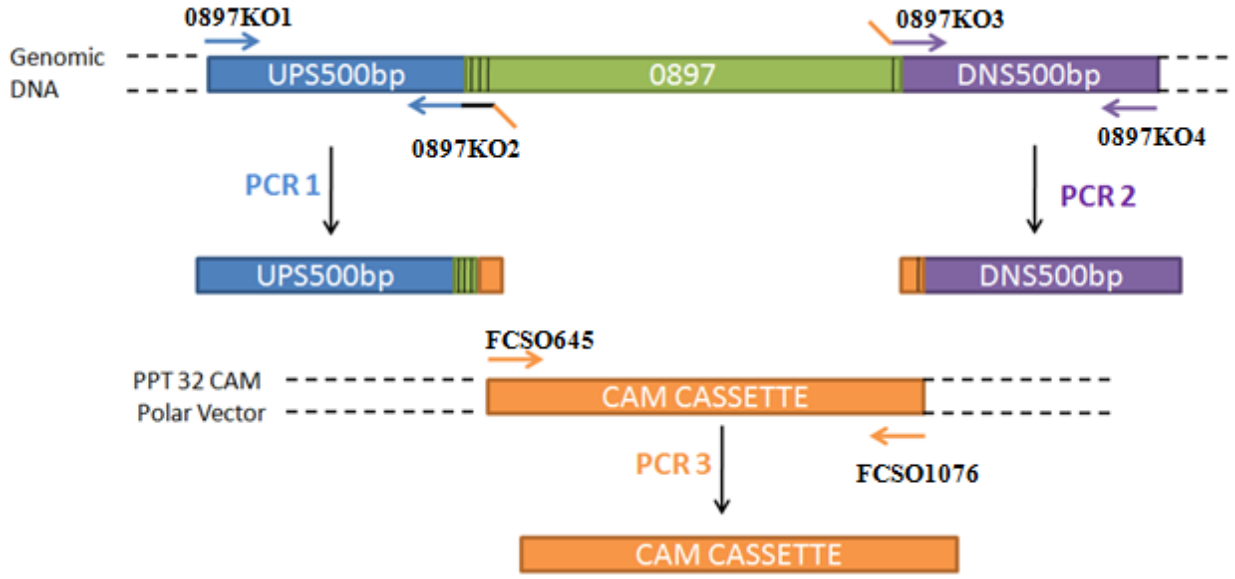


Figure 4.2 Validation of construct designed for *HP0897* Knockout.(a) A 545 bp region including 200 bp upstream region and first 345 bp of coding region of *HP0897*were amplified by PCR and ligated into pBluescript II KS(+) containing CAM cassette. (b) Colonies grown on chloramphenicol plate were subjected to colony PCR which showed C1 and C2 as positive clones. (c) Restriction digestion analysis with SacI/XhoI enzymes confirmed the presence of positive clones. (d) A 200 bp region downstream of *HP0897*gene was amplified by PCR and ligated in XhoI/KpnI downstream of chloramphenicol cassette in pBluescript II KS(+). (e) Colonies were selected on chloramphenicol cassette and subjected to colony PCR. (f) Restriction digestion analysis with SacI/KpnI(Lane1) shows fallout of complete construct ~1800 bp (marked as **) and restriction digestion with XhoI/KpnI (Lane2) shows fallout of 200bp (marked as *).

Step 1. Amplification different target DNA:



Step 2. Fusion of different DNA fragments by overlap extension PCR:

Figure 4.3. Schematic diagram for the strategy of overlap PCR to amplify constructs for *HP0897* gene knockout with increased length of complementary regions for homologous recombination. Step 1. Different targets were amplified by PCR which included 500 bp upstream and downstream sequences of *HP0897* and chloramphenicol cassette. Step 2. All the three PCR products were amplified with overlapping overhang and fused to form the final construct by overlap PCR using primers as discussed in material and methods.

The reverse primer for UPS500, *i.e.*, 0897KO2 was designed to include at its 5' end the first three codons of *HP0897* and an overhang that is complementary to 5' end of chloramphenicol cassette (CAM). The forward primer for DNS500 included at its 5' end an overhang complementary to 3' end of CAM cassette and a stop codon (figure 4.3). In third PCR reaction, the CAM cassette was amplified by using primer pair FCSO645/RCSO1076 listed in table 2.2 and pPT32 vector DNA (Kind gift from G. Mukhopadhyay Lab) that contained CAM cassette (figure 4.4 left). Finally, in the fourth PCR reaction the whole construct was amplified by overlap PCR by mixing the products of three PCR reactions, *i.e.*, UPS500, DNS500 and CAM cassette and using primer pair 0897KO1/0897KO4 (figure 4.4 middle panel).

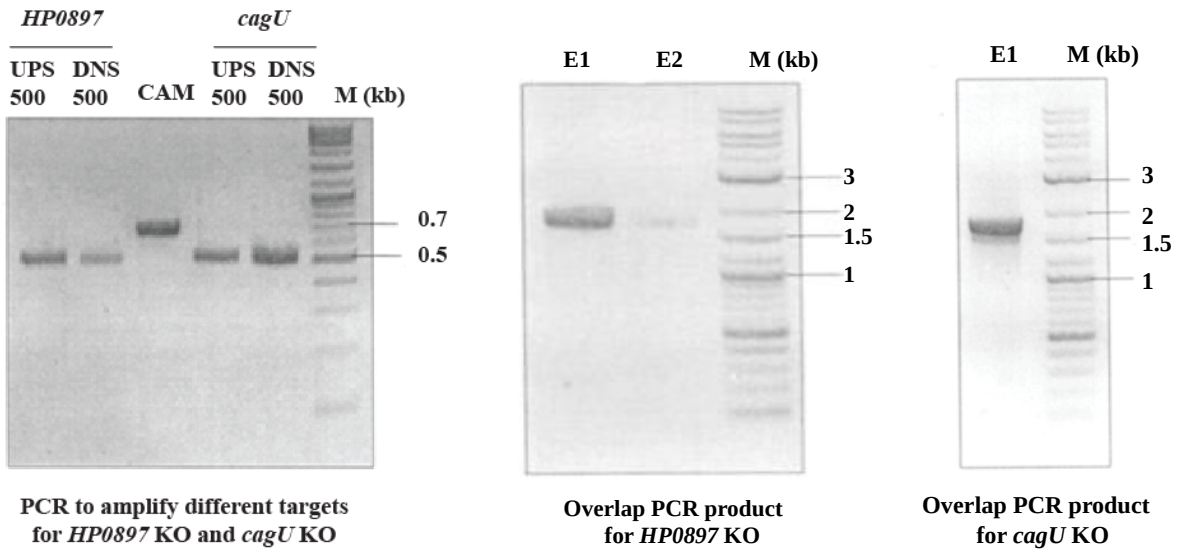


Figure 4.4 Overlap PCR method employed to design construct for *HP0897* knockout and *cagU* Knockout. The left panel shows PCR amplification of UPS500 and DNS500 of both HP0897 and *cagU* (used as a positive control) and chloramphenicol cassette. Middle and right panel shows the fusion product formed as a result of overlap PCR for *HP0897*and *cagU* KO respectively.

Similarly, the construct for positive control *cagU* was amplified by overlap PCR. A region 500 bp upstream of start site and a region 500 bp downstream of stop codon of *cagU*gene were amplified by using primer pairs FCSO2243/RCSO2245 and FCSO2246/RCSO2244 respectively (figure 4.4 left panel and right panel).

The primer RCSO2245 included at its 5' end, first three codons of *cagU* gene and an overhang that is complementary to 5' end of CAM cassette. The forward primer FCSO 2246 included at its 5'end an overhang complementary to 3' end of CAM cassette and stop codon. The CAM cassette was amplified by PCR as explained above. The final construct for knockout of cagU gene was amplified by overlap PCR using primer pair FCSO2243/RCSO2244 and template DNA which was a mix of three PCR products CAM cassette, UPS500 of cagU and DNS500 of cagU (figure 4.4 Left and right panel).

4.2.3. Construct design for partial knockout of *HP0897*

The upstream region and 5' coding region of HP0897 gene overlap with a gene that encodes Glutaminyl-tRNA. The arrangement of *HP0897* and its neighboring genes is shown in figure

4.5. To keep this Glutaminyl-tRNA gene intact, partial knockout of *HP0897* was attempted. The design of primers for partial Knockout is shown in figure 4.6 A.

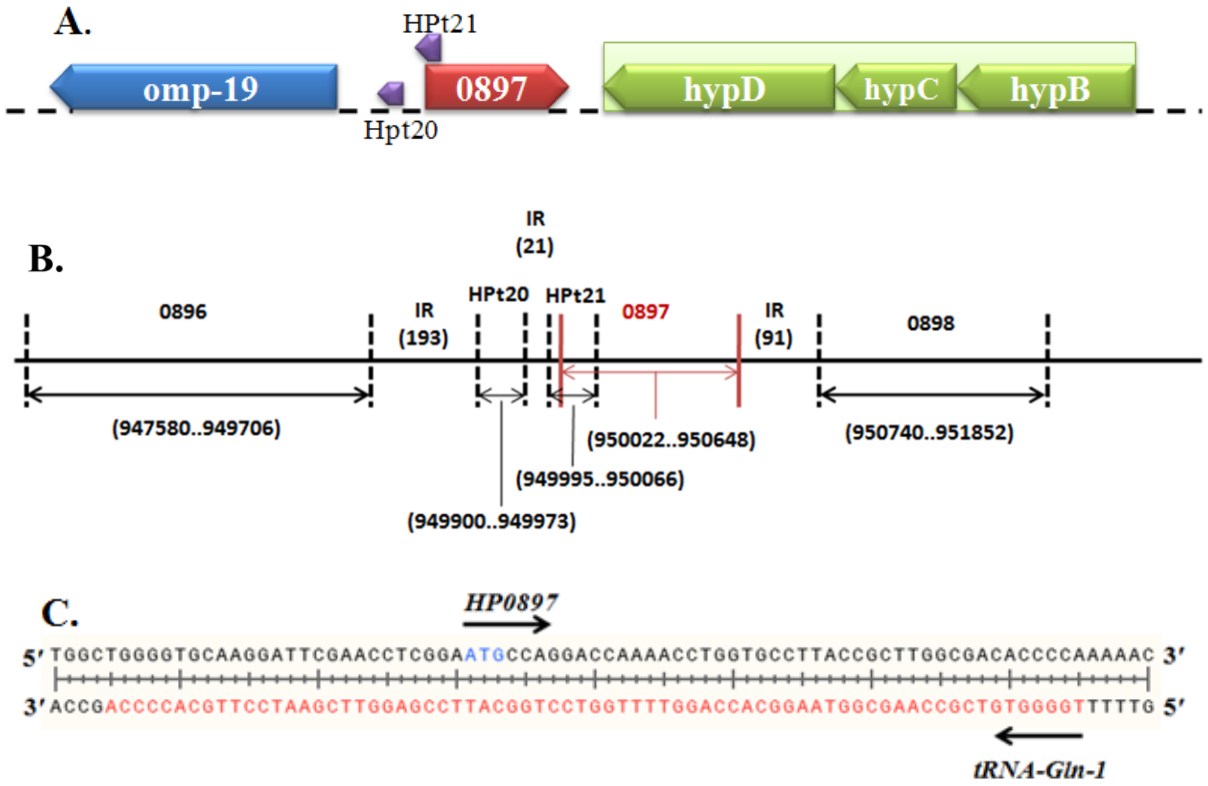


Figure 4.5 Schematic diagram showing arrangement of *HP0897* its neighboring genes.(A) *HP0897* is shown in red box is single protein coding gene at its locus and overlaps with a tRNA-Gln-1 (HPt21). (B) Position of *HP0897*locus and the other genes surrounding it in the genome are shown by respective numbers in brackets. The red solid bars represent *HP0897* and neighboring genes are shown by black dotted lines. The first 66 bp from start codon and 7 bp upstream of start site of *HP0897* gene overlaps with tRNA-Gln-1 gene (HPt21) which is 73 base pair in length. *HP0897* and tRNA-Gln-1 are present on opposite strands and are transcribed in opposite directions. Other genes present in the vicinity of *HP0897* are *HPt20* encoding tRNA- Met and *HP0896* (outer membrane protein; OMP19) which are at 5' end of *HP0897*. Towards 3' end of *HP0897, HP0898* (Hydrogenase formation protein; hypD) is present. IR represents intergenic region. (C) Nucleotide sequence of tRNA-Gln-1 (red color) overlapping with *HP0897* is shown. Arrows indicate the transcription orientations of the two genes.

For this purpose, a construct containing 425 bp upstream sequence of *HP0897* + first 75 base pairs of *HP0897* (total of 500 bp) + *CAM* cassette+ a region of 500 base pairs downstream of

stop codon of *HP0897* was amplified by overlap PCR. The region of 425 bp upstream and first 75 bp of *HP0897* coding region were amplified by PCR using primer pair 0897KO5/0897KO6 and 26695 genomic DNA as template (Figure 4.6 B). The CAM cassette and the region of 500 bp downstream of *HP0897* were amplified by PCR as discussed above.

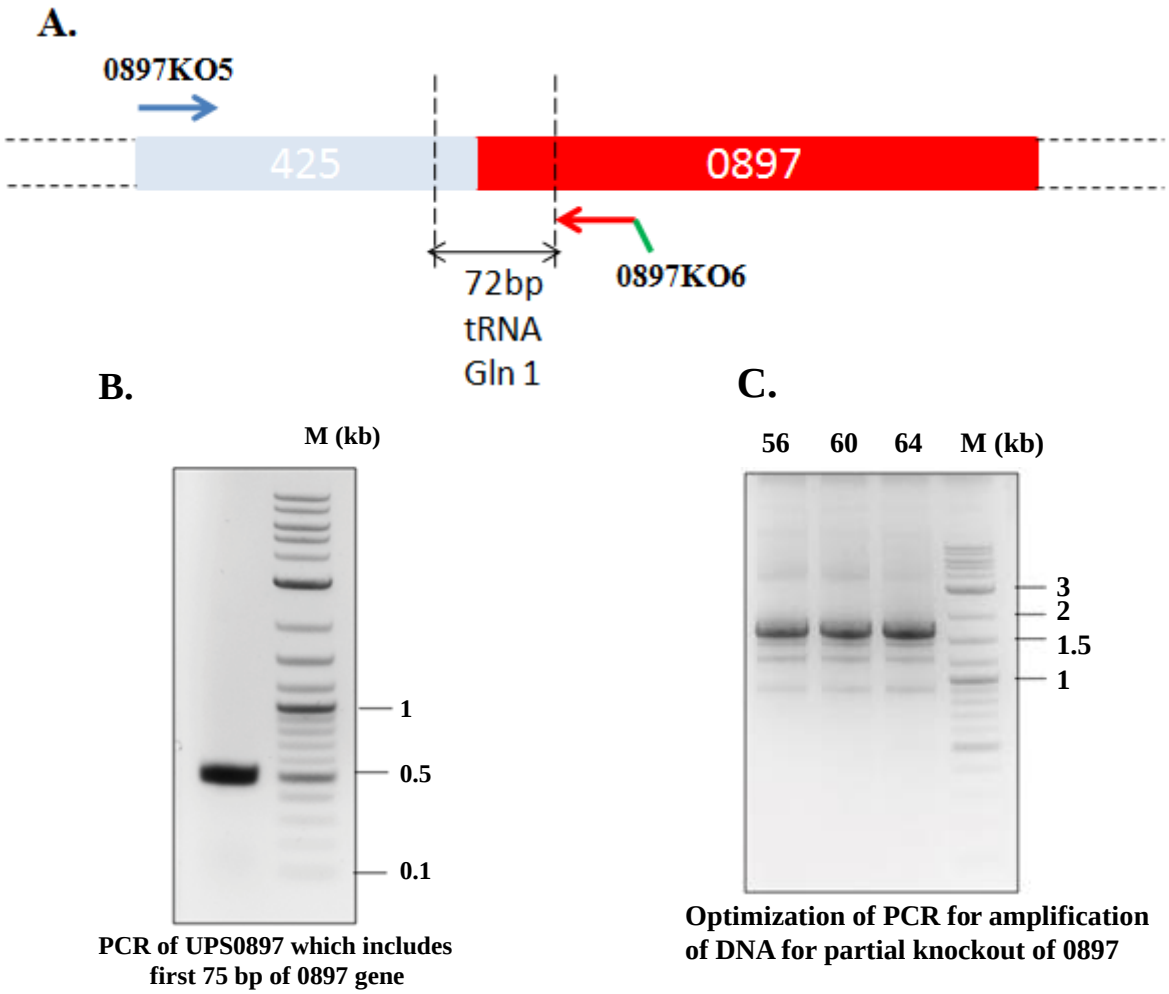


Figure 4.6 Construct design for partial knockout of *HP0897*. (A) Schematic diagram showing region of *HP0897* selected for primer design to keep tRNA-Gln-1 sequence intact after *HP0897* deletion. (B) First 75 bp of *HP0897*and 425 bp upstream sequence was chosen to design primers for overlap PCR. CAM cassette and downstream sequence (DNS500) were amplified in the same way as earlier. (C) Final construct for partial knockout of *HP0897* was amplified by Overlap PCR at different temperatures for optimization as discussed in material and methods.

The PCR products of regions UPS(425+75), CAM cassette and DNS500 were mixed and used as template for overlap PCR reaction to amplify entire construct for partial *HP0897*

knockout using primers 0897KO5 and 0897KO4 listed in table 2. The PCR amplification profile of the partial HP0897 KO construct following overlap PCR is shown in fig 4.6 C.

4.2.4. Essentiality of *Hp0897* for growth of *H. pylori*

In order to prove essentiality of *HP0897* for the survival of *H. pylori*, homologous recombination method was employed to knockout *HP0897*. In fact, this was achieved in two ways. In one method, we had used closed circular DNA construct for HP0897 KO. For this purpose, the construct for homologous recombination is cloned in pBluescript KS II (+) vector backbone carrying CAM cassette as a selection marker for Knockout strains. Schematic diagram for homologous recombination is shown in figure 4.1. In this experiment, knockout of a non essential gene *cagδ* was used as a positive control. The KO construct for *cagδ* was also a closed circular DNA made in pBS vector backbone and was a kind gift from Gauranga Mukhopadhyay's laboratory.

In second method, we have used linearised DNA constructs for knockout of *HP0897* and *cagU* genes which were made by overlap PCR as discussed above in section 4.2.2 and in material and methods. *cagU* is a component of type IV secretion system of *H. pylori* and is non-essential for growth.

To make *HP0897* KO by using closed circular DNA construct, cells of *H. pylori* 26695strain were transformed with pBS-UPS-NTD545::CAM::DNS200 construct. Under similar conditions, cells were also transformed with pBS-UPS*cagδ*500::CAM::DNS*cagδ*500 construct to knockout nonessential gene *cagδ*. pBS (plasmid alone) was also used as control. To make *HP0897* and *cagU* knockouts by using linearised DNA constructs, cells of *H. pylori* 26695 strain were transformed with 0897NTD500::CAM::0897DNS500 and *cagU*UPS500::CAM::*cagU*DNS500 linear constructs without any vector backbone.

The transformed cells were spread on the selection plates containing chloramphenicol (8µg/µl). It was observed that whenever we had attempted to knockout *HP0897* gene by any of the two methods used, *i.e.,* either by closed circular DNA constructs in pBluescript KS II (+) or by linearised DNA constructs, no colony was observed on the chloramphenicol selection plate (figure 4.7). However, in case of positive controls, sufficient colonies appeared on selection plates irrespective of the method used for knockout. As shown in

figure 4.7, large number of colonies were appeared weather it was closed circular DNA construct for *cagδ* KO or it was linearised DNA construct for *cagU*. No colony observed when cells were transformed with pBluescript KS II (+) plasmid alone.

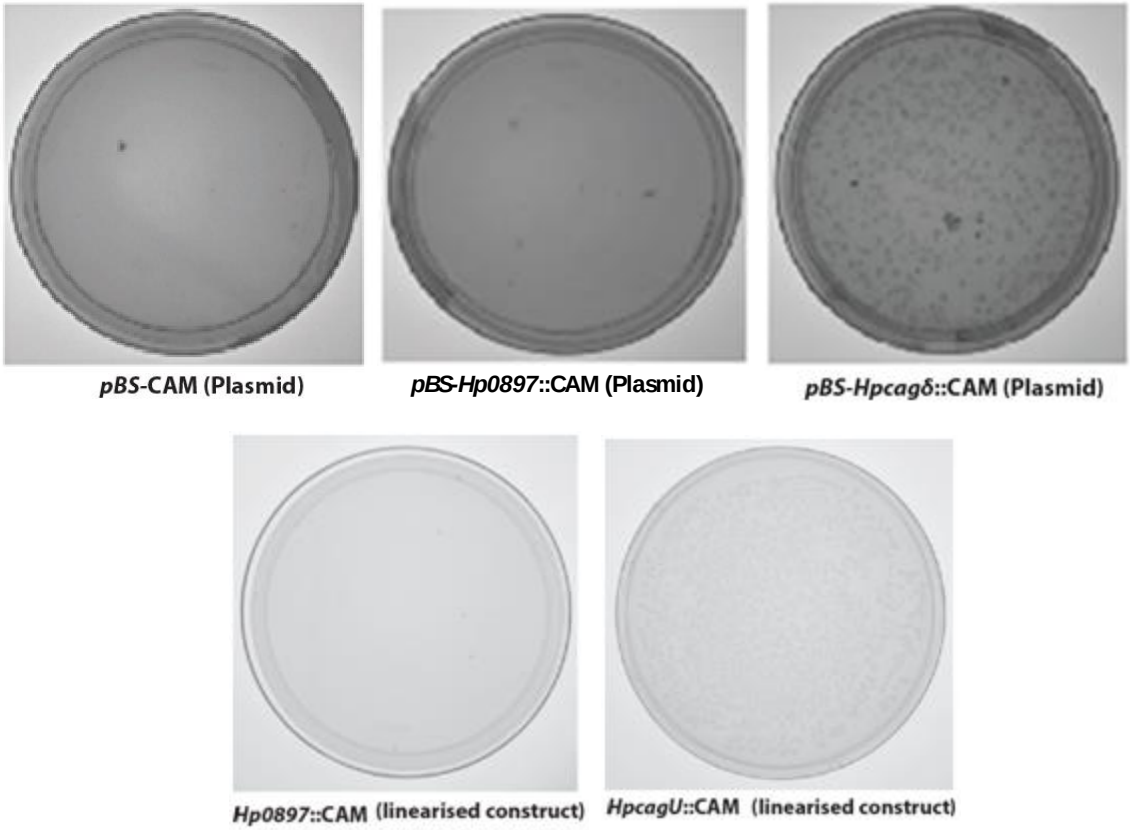


Figure 4.7 Selection of *H. pylori 26695* transformants positive for knockout experiment on selection plates. No colony was observed on BHI-chloramphenicol plate when the cells were transformed with *pBS-HP0897::CAM* or pBS plasmid alone (upper middle and left panel). *H. pylori* cells were also unable to grow on selection plate when linear KO construct was used to delete *HP0897* (lower left panel) Large number of colonies observed when cells were transformed with either *pBS-Hpcagδ::Cam* or *HpcagU::Cam* (upper right and lower right panels).

Further, we have checked weather the colonies obtained on selection plate were true for cagδ KO and cagU KO strain by checking expression of Cagδ and CagU proteins respectively. For this purpose, few colonies were selected randomly from selection plates and re-streaked on fresh chloramphenicol plates. The cells were collected from re-streaked colonies after

sufficient growth and subjected to Western blot analysis to check expression of Cagδ and CagU proteins. The transformants collected from selection plates were lysed and subjected to SDS-PAGE.

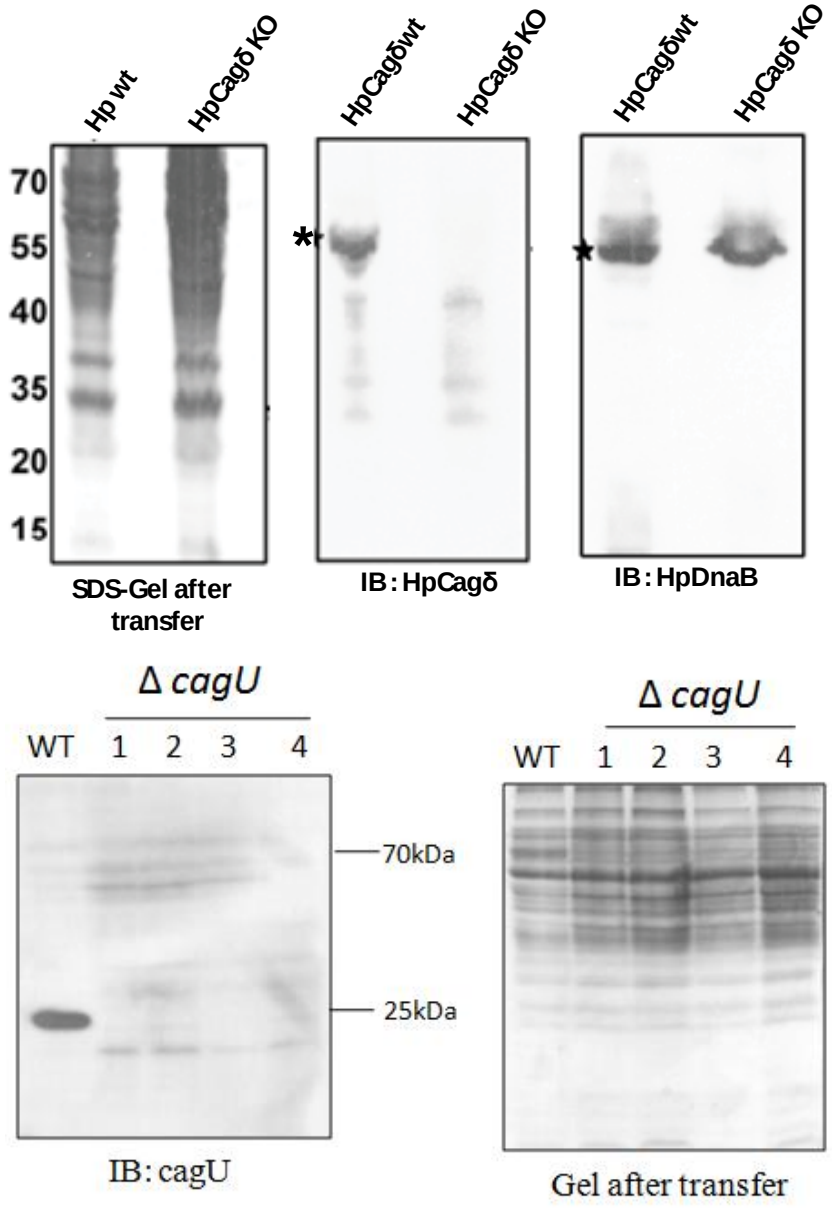


Figure 4.8 Knockout strains of *cagδ* and *cagU* were generated by homologous recombination. Lysate prepared from wild type *H. pylori* 26695 cells and from cells grown on selection plates after transformation with respective DNA construct for knockout were subjected to SDS-PAGE and analysed by Western blotting using polyclonal antibodies raised against Cagδ or CagU or DnaB. (A) Wild type strain showed band of Cagδ at 55 kDa where as knockout strain showed no clear band at respective molecular weight. SDS gel after after transfer and western blotting with antibodies raised

against HpDnaB were used for loading control. (B) Lysates from wild type and four colonies selected for *cagU* knockout were subjected to Western blotting with antibodies raised against CagU. Wild type strain showed a band at 25kDa whereas all knockout strains were true for deletion of *cagU*. The gel after transfer (right panel) was used to show similar loading of lysate in all the lanes.

Cagδ and CagU proteins were detected by Western blotting using polyclonal antibodies raised against Cagδ and CagU proteins respectively. As shown in figure 4.8 wild type *H. pylori* strain showed bands of Cagδ and CagU proteins at 55 kDa and 25 kDa respectively whereas knockout strains of Cagδ and CagU showed no band at respective sizes. Therefore, Δ*HP0897* mutant was not generated under these conditions. However, Δ*cagδ* and Δ*cagU* mutants were generated successfully that were used as positive controls for knockout experiment. The similar loading of lysate in the lane corresponding to HpWt and Hpcagδ KO strain is shown either by coomassie staining of gel after transfer or by western blot using antibody against DnaB that shows the presence of DnaB in both the lane (figure 4.8 A and B).

One interesting observation regarding *HP0897* gene is that a part of coding region towards 5' end of *HP0897*and a region of its upstream sequence overlaps with a gene on its complementary strand that encodes a Glutaminyl-tRNA (figure 4.5). Gene overlapping is a common phenomenon for rapidly evolving genomes such as viruses, prokaryotes and mitochondria (Krakauer, 2000). It is hypothesized that gene overlapping minimizes genome size and provide an opportunity for transcriptional co-regulation (Johnson and Chisholm, 2004). *H. pylori* genome encodes 36 tRNA genes that ferry 20 amino acids to ribosomes during translation of proteins. *H. pylori* encodes a single copy of *tRNA-Gln-1* gene that overlaps with *HP0897*.

It is possible that when knockout of *HP0897* was attempted by different ways as discussed above, the sole copy of *tRNA-Gln-1* gene was disrupted and cells were unable to grow because of loss of translation of cellular proteins.

To check essentiality of *HP0897*gene, overlap PCR was used to design construct for partial knockout of *HP0897* (Figure 4.6). In this way, the *tRNA-Gln-1* was not disrupted and remained intact. *H. pylori* cells were transformed with this construct DNA. However, *H.*

pylori cells after transformation could not grow on chloramphenicol plates (data not shown). These results suggest that keeping *tRNA-Gln-1* intact also did not help to get HP0897 KO strain.

Above results altogether suggest that HP0897 is an essential gene in *H. pylori* which very well justifies its essential function of helicase loader in *H. pylori.*

4.3. Discussion

DNA replication is tightly regulated at initiation so that it occurs only once per cell cycle (Zakrzewska-Czerwinska et al., 2007). DnaA along with DnaC are ATPase switches that regulate initiation of replication by binding and hydrolyzing ATP (Lee and Bell, 2000). All these factors controlling DNA replication are essential for survival of the bacteria. The indication that DnaC plays a role in initiation of DNA replication came from characterization of various temperature sensitive mutants of DnaC in *E. coli (Wechsler and Gross, 1971)*. The temperature sensitive mutants of DnaC can be characterized into two groups first that affects initiation of replication and second group that affects elongation of DNA replication which indicate DnaC may function in elongation of DNA replication also.

In accordance with above reports, Hp0897 which interacts with HpDnaB *in vitro* as well as *in vivo* and modulates its various activities (Verma et al., 2016) and may qualify as a possible homolog of helicase loader, Therefore it should be essential for *H. pylori.* The essentiality of *Hp0897* was confirmed by knockout experiment. We have used homologous recombination to delete *HP0897* by using the construct DNA in closed circular form or construct DNA in the form of linearized PCR product. For positive controls two non essential genes *cagδ* and *cagU* were used which are components of type IV secretion system of *H. pylori.* We never obtained knockout of *HP0897*as cells are not surviving when homologous recombination is used to delete *HP0897.* Under similar experimental conditions both Δ*cagδ* and Δ*cagU* strains were generated successfully. Therefore, it maybe concluded that *HP0897* is essential in *H.pylori.*

Careful examination of *HP0897* gene organization in the genome showed that it overlaps with a tRNA gene. This overlapping tRNA gene encodes for a Glutaminyl tRNA that

transports Glutamine to ribosomes for protein translation. The coding sequences for *HP0897* and *tRNA-Gln-1* are on complementary strands and these genes are transcribed in opposite directions to each other. *H. pylori* has a total of 36 tRNA genes that are required to ferry 20 different amino acids during translation of proteins. Unfortunately, there is only one copy of tRNA-Gln-1 gene that overlaps with *HP0897*. Again there is a possibility that when *HP0897* was attempted to delete, the tRNA-Gln-1 gene is also getting disrupted. This disruption would have affected protein translation of the cell. Therefore, construct DNA was designed to partially delete *HP0897* so that tRNA-Gln-1 remains intact. When *HP0897* was partially deleted by keeping tRNA-Gln-1 intact, the cells of *H. pylori* still were unable to survive. All the above experiments imply that *HP0897* is essential for the survival of *H. pylori* and cannot be deleted.

Genomic organization of *HP0897* shows it is not present in an operon with multiple genes. It is present as a single gene. As discussed above, only one tRNA gene on the opposite strand overlaps with it. Therefore there is remote possibility of polar effect so that this influences the outcome of KO strain. The failure to get a knockout may also be attributed to inaccessibility of 3'-end of *HP0897*. For that 3' replacement with GFP or any other tagged protein is required to check accessibility of 3' end.

Further experiments can be carried out by expressing *tRNA-Gln-1* and *HP0897*episomally or by integrating these two genes at other loci in the genome of *H. pylori*. Under this diploid condition for *HP0897* and *tRNA-Gln-1* genes, the attempt to delete endogenous copy of HP0897 may be performed to see its effect on survival of bacteria.

Alternatively, *tRNA-Gln-1* can be expressed episomally or from nonresident loci in the genome where it is integrated in the chromosome. Under diploid conditions *for tRNA-Gln-1*, conditional knockout of *HP0897*can be made to study its essentiality as well as function in the cell.

Being an essential gene, *HP0897* can be a novel target for drug development against *H. pylori*. Since *HP0897* is unique to *H. pylori* and conserved among *H. pylori* strains, drug designed against this molecule can be *H. pylori* specific, in this way it may be safe for micro biota of the human host.

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Chapter 5

Role of Hp0897 at Replication origin of H. pylori and its putative function in cell division

5.1. Introduction

Proteins in bacteria may locate to specific sites where they perform specific function in the cell. The knowledge about the location of a protein in the cell is often demanding to understand their function. In order to perform specific functions proteins can redistribute and localize at specific sites in the cell, for example, in *E. coli* the cell division protein FtsZ forms a ring like structure at the mid cell during cytokinesis (Bi and Lutkenhaus, 1991). Another example is of chemotaxis proteins that can assemble at the pole of cell in response to external stimuli (Alley et al., 1992). Many DNA replication initiation proteins reside at specific sites in the cell. DnaA initiator protein in different *E. coli* strains is anchored to membrane and forms a halo like appearance and it is distributed along the cell membrane (Newman and Crooke, 2000). Localization of DNA replication initiation proteins has been shown in *B. subtilis (Imai et al., 2000)*. DnaA protein in *B. subtilis* is uniformly distributed in the cytosol (Imai et al., 2000). In *B. subtilis* helicase loader is DnaC and helicase loading machinery consists of DnaB, DnaD and DnaI. Localization of helicase loader DnaI and its assistant protein DnaB have been shown with respect to *oriC* in *B. subtilis*. Both DnaB and DnaI form foci near cell poles and middle of the cell where *oriC* regions are present. In dividing cells DnaB and DnaI foci have been shown to be present at the border regions of two nucleoids. The number of *oriC* foci changes from 2 to 4 depending on the stage of cell cycle.

In *E. coli*, after the loading of DnaB to melted origin, DnaC protein dissociates from DnaB●DnaC complex (Makowska et al. 2010) after playing its role in replication initiation. DnaC can be involved in replication restart also as PriC has been shown to interact with DnaB as well as DnaB●DnaC complex directly. It is speculative that Hp0897 which is a homolog of *E. coli* DnaC in *H. pylori* may have role in DNA replication initiation and must localize at the pole of the cell near Hp*oriC*. Therefore, sub cellular localization of Hp0897 is important to explore its function in DNA replication.

Hp0897 protein shows less sequence homology with conserved helicase loaders in other bacteria and possesses a putative fatty acid binding domain towards its N terminal region ranging from amino acid residues 32 to 49 (Shivangi Verma and Suman Kumar Dhar

unpublished results). Fatty acid binding proteins (FABPs) are generally present in eukaryotes. FABPs are members of super family whose primary function is transport of fatty acids across different compartments in the cell. They are generally small proteins about 15 to 18 kDa. They are tissue specific and about 9 types of FABPs are known for example heart type FABPs (H), Intestine (I) and Brain (B), *etc.*, are FABPs found in eukaryotes(Smathers and Petersen, 2011, Chmurzynska, 2006). Proteins with fatty acid binding domains are also observed in some bacteria. For example, Fatty acid kinase (Fak) is conserved enzyme system in gram positive bacteria that consists of FakA protein that binds to ATP and FakB protein that binds to fatty acid. The acyl chain of bound fatty acid to FakB is activated by phosphorylation by FakA. Upon phosphorylation, Fak proteins incorporate exogenous fatty acids into the membrane phospholipids (Broussard et al., 2016). DegV protein of *Bacillus subtilis* shows similarity with FakB. Structural studies have revealed that DegV may bind to fatty acids (Nan et al., 2009). The crystal structure of uncharacterized protein Rv0813c *M. tuberculosis* reveals that this protein has similarity to FABPs of eukaryotes and may have role in recognition and transport of molecules in the cell (Shepard et al., 2007). The fatty acid binding domain of Hp0897 is conserved among different strains and consists of about 18 hydrophobic amino acids (Shivangi Verma and SK Dhar, unpublished data).

According to protein interactome data generated by two independent high throughput yeast two hybrid studies revealed that Hp0897 protein interacts with HpDnaB (HP1362), Arginine decarboxylase enzyme (HP0422; SpeA), HP0368 (a hypothetical protein of unknown function and HP1493, an uncharacterized protein (Rain et al., 2001, Hauser et al., 2014, Verma et al., 2016).

These protein ineractome data implies that Hp0897 may interact with DNA replication and non-replication proteins. In this way, Hp0897 may be a multifunctional protein that may have some other cellular functions apart from helicase loading.

Therefore, sub cellular localization of Hp0897 is important to gain insight into its function/s. In this chapter, sub cellular localization of Hp0897 with respect to other replication and cell division proteins is studied by immunofluorescence microscopy of fixed cells by using antibodies against respective proteins and by fluorescence microscopy of live cells by using GFP tag. For, *in vivo*, domain mapping of HP0897, N and C terminus of HP0897 fused with

GFP protein are used for fluorescence microscopy. Over-expression of HP0897 wild type and its N and C terminal domains is also studied in heterologous system *E.col* which showed similar results (data not shown). With regard to helicase loading function of Hp0897, the binding at *ori*C region is checked by Chromatin immunoprecipitation (ChIP) assay. It has been reported that *H. pylori* contains two *oriC* like regions, *oriC1* and *oriC2*. We were interested to find out which one is the functional origin *in vivo* with respect to Hp0897 loading function.

5.2. Results

5.2.1. Hp0897 is a component of replisome complex

DNA replication starts at the middle of the cell in *E. coli*. The replisome remains fixed at the center of the cell, but the duplicated *oriC*s move towards opposite poles of the cell and reside at the edge of nucleoid (Lemon and Grossman, 1998, Roos et al., 1999). Similarly, in *B. subtilis*, the duplicated origins soon after duplication at the middle move apart and occupy opposite poles of the dividing cells. These studies imply that a mitotic spindle-like apparatus may be present that pulls the duplicated *oriCs* towards opposite poles (Webb et al., 1997, Webb et al., 1998). In *H. pylori* the *oriC* is located at one pole of the cell irrespective of the flagellar end. The replication starts at one pole of the cell where *oriC* is present and gradually replisome moves towards the middle of the cell. The duplicated *oriCs* do not separate and move together towards the middle of the cell. After termination of replication at midcel the two chromosomes resolve and move apart into daughter cells. The *oriC*s move parallel and not in the opposite direction. (Sharma et al., 2014, Sharma et al., 2009). Residence of *oriC* and initiation of replication at the cell pole suggests polar localization of Hp0897. To find out association of Hp0897 with replisome, sub cellular localization of Hp0897 was studied by immunofluorescence microscopy of Hp0897 and its co-localization with HpDnaB in fixed *H. pylori* cells. A total of 2756 cells with a clear signal were analyzed for studying pattern of Hp0897 and HpDnaB localization in the cell. It is observed that 20% of these cells (540 out of 2756 counted) showed single nucleoid cells with discreet foci of Hp0897 (Table 5.1). In majority of these 540 cells, Hp0897 was localized at one pole of the cell (94%; 506 cells) and in 6% (34 cells) cells Hp0897 is observed at non polar locations (Table 5.1 and figure 5.1.B).

Under similar conditions, immunofluroscence with pre-immune sera did not show any distinct signal (figure 5.1.A). In order to probe Hp0897 as a part of replisome, co-localization of Hp0897 with replicative helicase HpDnaB was studied. Generally, 4% (294 cells out of total 7360 cells) of the cells showed foci of both Hp0897 and HpDnaB in the same cell. Amid 294 cells, Hp0897 was co-localized with HpDnaB in ~65% (191 cells out of 294) cells whereas ~35% cells (103 cells out of 294 cells) did not show colocalization (Table 5.2 and Figure 5.2.A and B). Out of 191 cells in which Hp0897 and HpDnaB were colocalised, ~92 %; 176 cells showed polar localization of these duo proteins whereas only in 8% (15) cells, these two proteins were co-located at the middle of the cell. These results further authenticate the possibility of Hp0897 as a part of replisome complex.

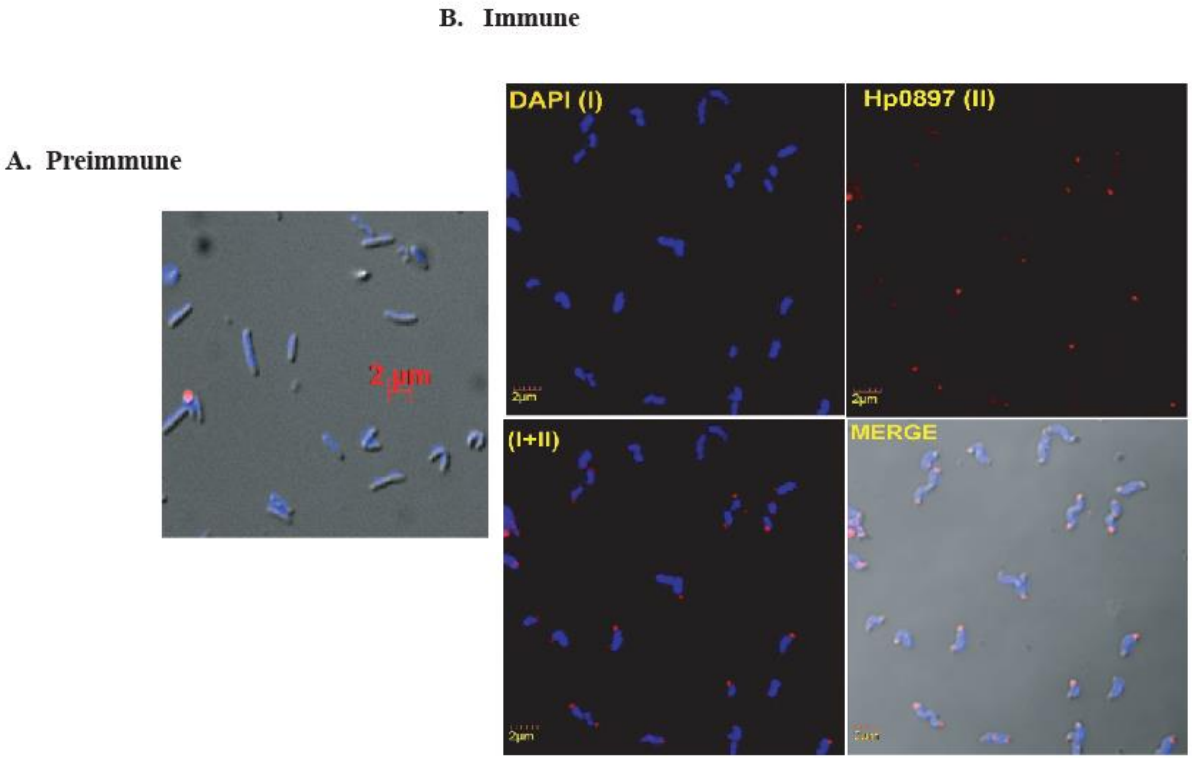

Figure 5.1. Sub cellular localization of Hp0897 in fixed *H. pylori* cells. Localization of Hp0897 in B28 strain of *H. pylori*. Poly-L-lysine coated coverslips containing *H. pylori* cells were treated with pre-immune sera (A) or antibodies against Hp0897 (B) followed by Alexafluor 594 (red fluorescence) conjugated secondary antibodies as described in 'Materials and Methods' section. In panel B, Hp0897

localizes at the pole of the cell. In cells treated with pre-immune sera no signal was observed. DAPI staining shows nucleoid position in the cell.

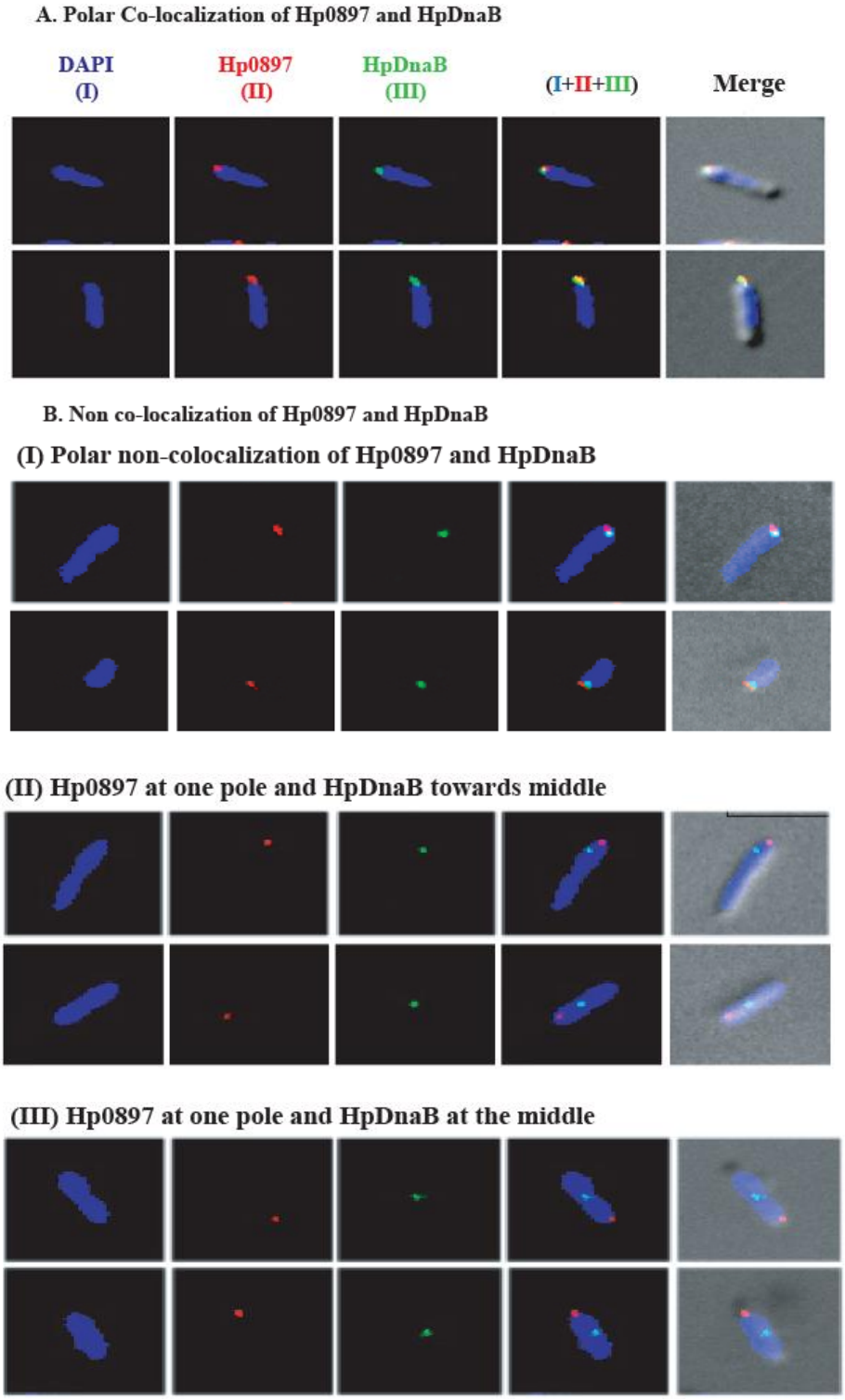

Figure 5.2 Co-localization study of HP0897 with HpDnaB in *H. pylori* cells. Co-localization of Hp0897 and HpDnaB proteins in *H. pylori* cells. For detecting Hp0897 mice anti-Hp0897 (1:500 dilution) and for HpDnaB rabbit anti HpDnaB (1:1000 dilutions) antibodies were used. Alexafluor 594 conjugated anti-mice (red) antibodies and Alexafluor 488 conjugated anti-rabbit (green) were used as secondary antibodies (1:1000 dilution). Merge panels towards the right show signals for DAPI, Hp0897, HpDnaB as well as phase. The results indicate co localization of Hp0897 and HpDnaB at the pole proximal region. (B) Non-colocalization of HpDnaB and Hp0897. Non-colocalized cells with Hp0897 at one pole and different localization of HpDnaB at the pole (I), towards middle (II) or at middle (III) have been shown.

HpDnaB signal was not fixed at the pole as Hp0897 in majority of cells. The HpDanB showed a shift in signal from pole to middle of the cell. This shift clarified the movement of replisome complex at the pole and then its movement towards middle of the cell during elongation. Polar co-localization of HpDnaB and Hp0897 dominated among other patterns (~92%; 176 cells out of 191) (Table 5.2 and Figure 5.2.A) and non-polar co localized foci of Hp0897 and HpDnaB were rare (<1%). These observations suggest that Hp0897 is mainly associated with replisome at the time of initiation of replication.

Table 5.1 Percentage of Helicobacter pylori cells with polar or non-polar foci of Hp0897 and HpDnaB respectively in single nucleoid cells

Protein Name	Polar (%)	Non Polar (%)	Total Examined cells
Hp0897	~20% (540 cells have signal)		2756 (Total cells)
Hp0897	**~94%** (506 cells)	**~6%** (34 cells)	**540 (single nucleoid cells containing signal)**
HpDnaB	~15 % (417 cells have signal)		2756 (Total cells)
HpDnaB	**~87%** (362 cells)	**~13%** (55 cells)	**417 (single nucleoid cells containing signal)**

In a fraction of cells (~103; 35 % of cell having both the signals for HpDnaB and Hp0897) 60 cells showed Hp0897 at the pole but HpDnaB at the middle of the cell or towards the middle (Table 5.2 and Figure 5.2.B). These results indicated that Hp0897 and HpDnaB foci do not move together from pole proximal to the pole-distal middle region of the cell. Out of 103 non-colocalised cells, ~42% (43 cells out of 103 non-colocalized cells) exhibited Hp0897 and HpDnaB foci at both the poles. At present, implication of such localizations is not clear.

5.2.2. Hp0897 preferentially binds to *oriC2* during initiation of replication

Unlike *E. coli*, the origin of replication in *H. pylori* is divided into two sub origins named *oriC1* and *oriC2*. The two sub origins are separated by *DnaA* gene in between them (Figure 5.3.A). DNA unwinding element (DUE) is present near to oriC2 where replisome machinery assembles during origin firing. DnaA binds to both *oriC1* and *oriC2* regions, but DnaA dependent unwinding of AT-rich region occurs at *oriC2*. It is found that DnaA has comparatively more affinity at *oriC1* as compared to oriC2 (Donczew et al., 2012).

Table 5.2 Co-localization pattern of Hp0897 and HpDnaB foci

<table>
<tr><th colspan="4">Total number of cells observed: 7360</th></tr>
<tr><td colspan="4">Total number of single nucleoid cells having distinct HpDnaB + Hp0897 signal: ~4 % (294 cells)</td></tr>
<tr><td colspan="2">Total cells with co-localised signals:
~65% of 294 cells =191 cells</td><td colspan="2">Total cells with non co-localised signals:
~35% of 294 cells =103 cells)</td></tr>
<tr><td>Polar co-localised
: ~92% (176 cells)</td><td>Middle
co-localised: ~8% (15 cells)</td><td>*Total: ~58% (60 cells)

(a) Both polar: ~27% (28 cells out of 103 cells);
(b) Hp0897 polar and HpDnaB towards middle: ~18% (19 cells out of 103 cells);
(c) Hp0897 polar and HpDnaB at middle: ~13% (13 cells out of 103 cells)</td><td>Hp0897 and HpDnaB at opposite pole:
~42% (43 cells)</td></tr>
</table>

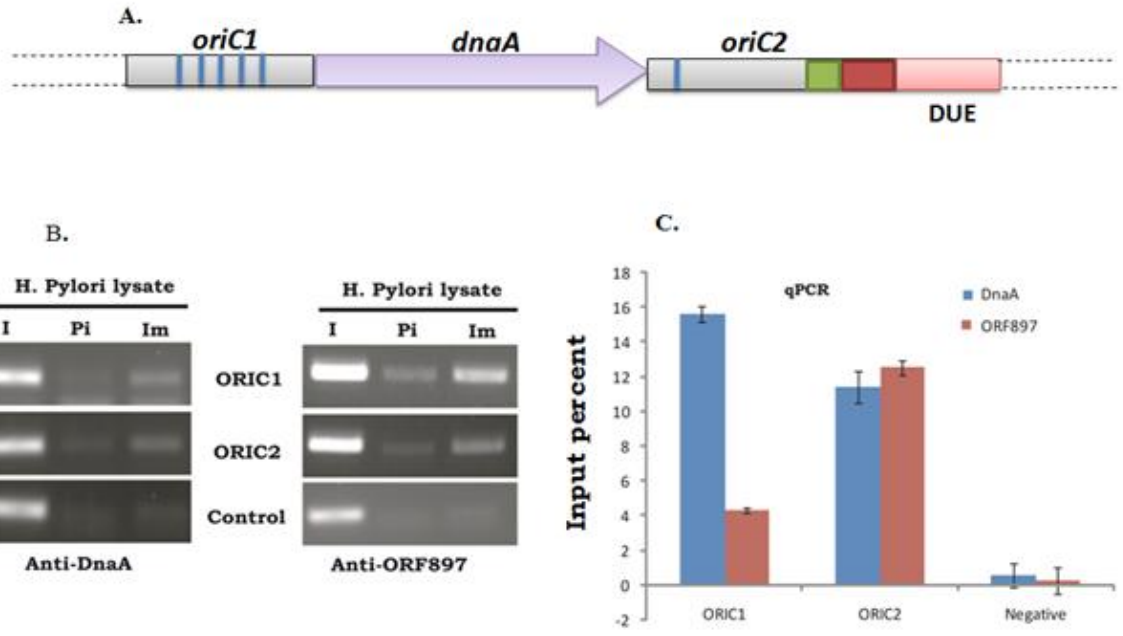

Figure 5.3 Hp0897 binds preferentially at *oriC2*. (A) Schematic diagram of *H. pylori oriC* region. *H. pylori* origin is composed of two sub origins *oriC1* and *oriC2*. *dnaA* gene separates the *oriC1* and *oriC*. Blue Bars represent DnaA boxes within *oriC1* and *oriC2* and DUE is towards 3' end of *oriC2*. (B) ChIP followed by semi-quantitative PCR showed binding of DnaA (left panel) and Hp0897 (right panel) at *oriC1, oriC2, and* Non-*oriC* region (control) respectively. (C) ChIP followed by quantitative PCR showed that binding of HpDnaA was more at *oriC1* than at *oriC* whereas Hp0897 was more at No binding of HpDnaA and Hp0897 was observed at non-*oriC* region (negative control).

It has been shown earlier that Hp0897 has the property to bind single-stranded DNA *in vitro* as shown earlier (Verma et al., 2016) and in the present study it is observed that Hp0897 resides at the pole and it is a part of replisome as shown by immunolocalization microscopy with DnaB. Therefore, it is possible that Hp0897 is localized at *oriC* DNA *in vivo*.

In order to confirm the binding of Hp0897 at *oriC*, Chromatin immunoprecipitation (ChIP) was followed by semi-quantitative and quantitative PCR reactions. Binding of DnaA protein at *oriC* was analyzed as a positive control for ChIP experiment. For negative control, the binding of both the proteins were analyzed at a non-*oriC* region which is about 1.5 kb downstream of *oriC2*.

ChIP followed by semi-quantitative PCR experiments showed the presence of Hp0897 at both *oriC1* and *oriC2* sites (figure 5.3.B). However, ChIP followed by quantitative PCR showed that the binding of Hp0897was higher at *oriC2* where replisome assembly takes place (figure 5.3.C). Hp0897 did not bind with non *oriC* DNA in ChIP experiment (Figure 5.3.B and C). HpDnaA was bound to both *oriC1* and *oriC2*. Nonetheless DnaA showed more binding at *oriC1* as compared to *oriC2*. This result is in accordance with the previous study that showed binding of DnaA at both *oriC* (Donczew et al., 2012). Preferential binding of Hp0897 at *ori*C2 indicates its possible role in helicase loading during replication initiation. These *in vivo* results are in accordance with our previous study where it has been shown that Hp0897 is a putative helicase loader protein in *H. pylori*.

5.2.3. Designing of a GFP vector for episomal expression of different proteins in *H. pylori*

We have shown the localization of Hp0897 in the fixed *H. pylori* cells. In order to locate the position of Hp0897 in growing live cells, we planned to express Hp0897 as GFP fusion protein. However, no suitable expression vector for GFP expression was available. In order to construct GFP expression vector, *gfpmut3* gene was cloned in pWS311 vector (Kind gift from Dr. Niyaz Ahmad, Hyderabad). *gfpmut3* is one of the several mutant forms of *gfp* genes which can fold properly at low pH and low oxygen conditions (Hansen et al., 2001). Since *H. pylori* is a microaerophilic bacteria, *gfpmut3* was used to express this gene in *H. pylori*. *gfpmut3* gene was amplified by PCR using pWS122 vector (Kind gift from Dr. Niyaz Ahmad, Hyderabad) as a template and primers Fwgfpmut3Spe1/Rvgfpmut3Kpn1 (Table 2.2 in material and method section). The forward primer designed to include 15 nucleotides between SpeI site and ATG (TAGTAAGGAGAACAT) with a ribosome binding site (RBS) for translation of the *gfp* gene. The amplified PCR product was cloned in SpeI and KpnI sites of pWS311 vector under the control of *cagA* promoter. The strategy for cloning of *gfpmut3* in pWS311 vector is shown in figure 5.4.

In order to make 0897GFP fusion, two step cloning was employed. In the first step, *HP0897* gene was amplified by PCR by using 26695 genomic DNA as template and primer pair FwRBS0897SpeI/ RvORF0897XbaI and cloned in SpeI/XbaI sites in the pWS311 vector. The forward primer was designed to include 15 nucleotides between SpeI site and ATG (TAGTAAGGAGAACAT) with ribosome binding site (RBS) for translation of the *HP0897*. In second step, *gfpmut3* gene was amplified by PCR by using primers Fwgfpmut3XbaI/Rvgfpmut3KpnI and pWS122 vector DNA as a template and cloned in XbaI/KpnI sites in pWS311 vector carrying *HP0897* gene in SpeI/XbaI sites (Figure 5.5). Similarly, *NTD0897* and *CTD0897* were cloned in pWS311 vector containing *gfpmut3* as discussed in detail in material and methods. *ssbgfp* was cloned in pWS311 by overlapping PCR as discussed in material and methods.

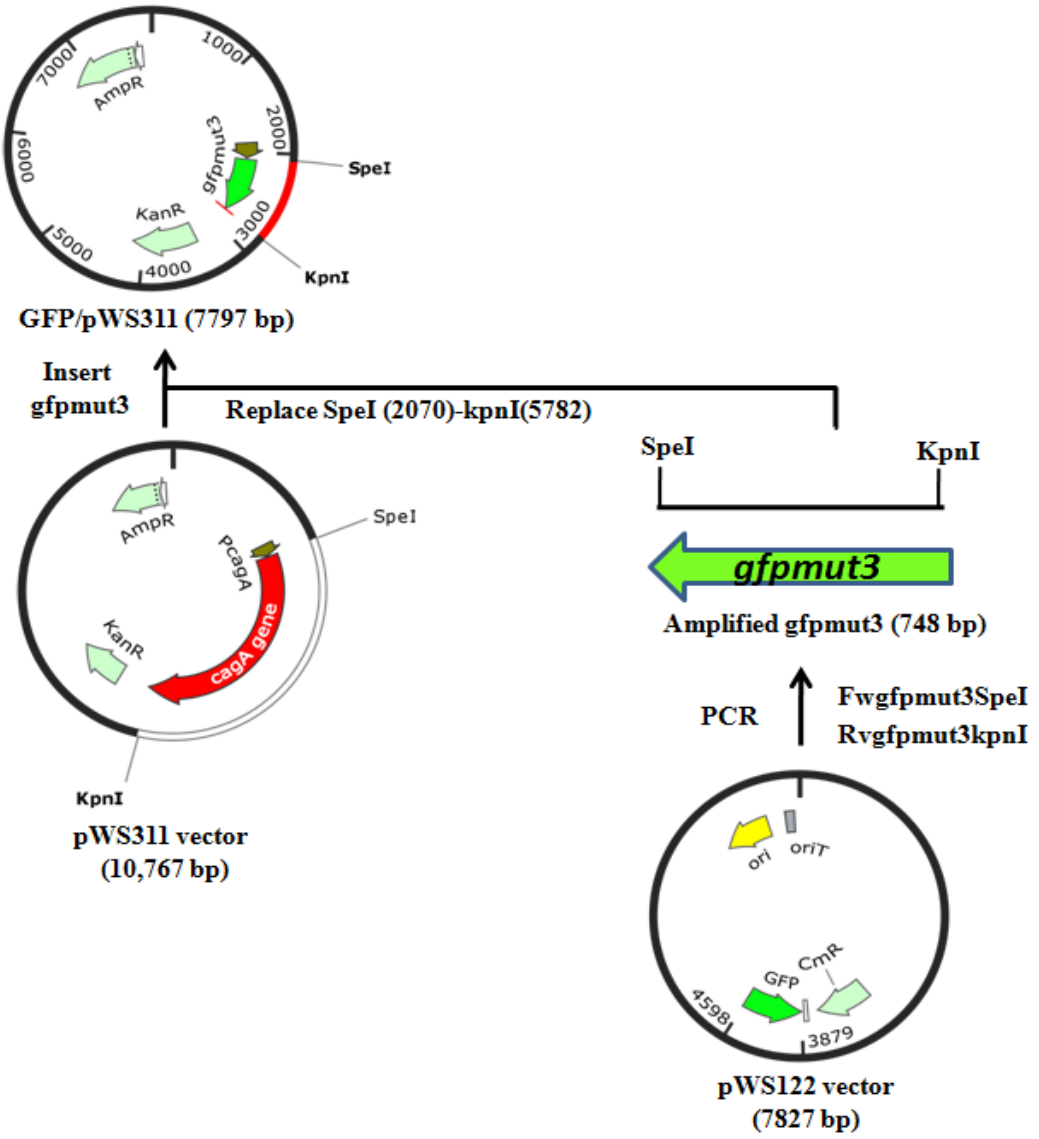

Figure 5.4. Schematic diagram of cloning strategy for designing GFP vector for *H. pylori.* *gfpmut3* gene was amplified by PCR and cloned in pWS311 vector by replacing *cagA* gene from *pWS311* vector.

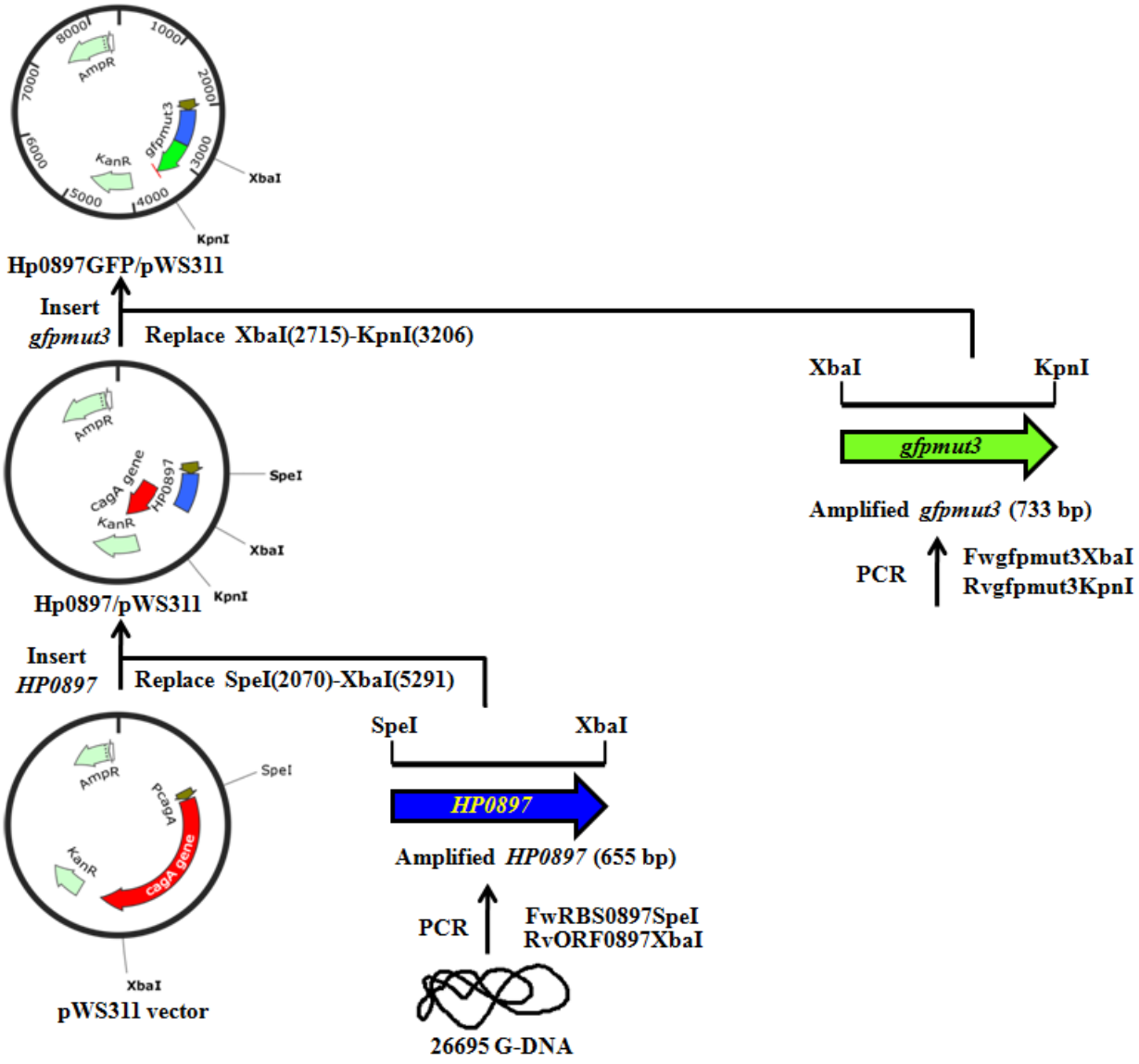

Figure 5.5 Schematic diagram showing the cloning of *HP0897-GFP* construct in the pWS311 vector. *HP0897-GFP* fusion was made by two-step cloning. In one step *HP0897* gene amplified by PCR and cloned in the pWS311 vector by replacing a portion of *cagA* gene from the pWS311 vector. In the second step, the remaining part of the *cagA* gene was replaced by cloning *gfpmut3* gene at 3' end of *HP0897*.

5.2.4. Localization of Hp0897 as GFP fusion protein in live *H. pylori* cells

The polar localization of Hp0897 in fixed cells and its binding to origin region prompted us to analyze subcellular localization of Hp0897 in live cells. For this purpose, Hp0897GFP fusion protein was expressed as episomally from the pWS311 vector. As a control GFP protein alone was also expressed episomally. Both GFP alone and Hp0897GFP gene were under the control of *cagA* promoter (P_{cagA}). The expression of GFP protein alone in *H. pylori P12* strain was checked using Western blotting (Figure 5.6.A) and fluorescence microscopy (figure 5.6.B). Western blot experiment using antibodies against GFP clearly showed the expression of GFP in *H. pylori* lysates. Fluorescence microscopy of live cells showed the diffused expression pattern of GFP protein distributed all over the cells (figure 5.6.B). When Hp0897GFP was expressed episomally from pWS311 vector under the control of P_{cagA} in P12 strain, Hp0897GFP expression was found to be higher than endogenous expression of Hp0897 because of higher copy number of plasmid pWS311 and strong P_{cagA} (figure 5.7.A). The over expression of Hp0897 led to certain visible phenotypic changes in *H. pylori* cells which provided useful information regarding Hp0897 function. The phenotypic changes included cell death due to over production of Hp0897GFP, cell elongation and multiple foci of Hp0897GFP in the cell (figure 5.7.B). Furthermore, the sub cellular distribution patterns of Hp0897 were different from that of endogenous Hp0897 in fixed cells. When localization of Hp0897 was analyzed at endogenous level in fixed cells, majority of cells showed polar localization of Hp0897. Cells expressing episomal Hp0897GFP, showed polar as well as non-polar localization of Hp0897GFP protein. However, the majority of cells showed multiple foci of Hp0897GFP in elongated cells.

The number and localization pattern of Hp0897GFP foci were highly variable among the given population of the cells. The number of Hp0897GFP foci was ranging from 1 to 5 per cell. The percentage of cells having different numbers of foci varied significantly in different experiments. Since overexpression of Hp0897 yielded elongated cells with multiple foci of Hp0897, it may not reflect the true characteristics of the endogenous protein. Hence, we did not perform thorough localization studies of Hp0897GFP in live cells. 3' replacement of endogenous copy of Hp0897 with GFP will be required for this purpose.

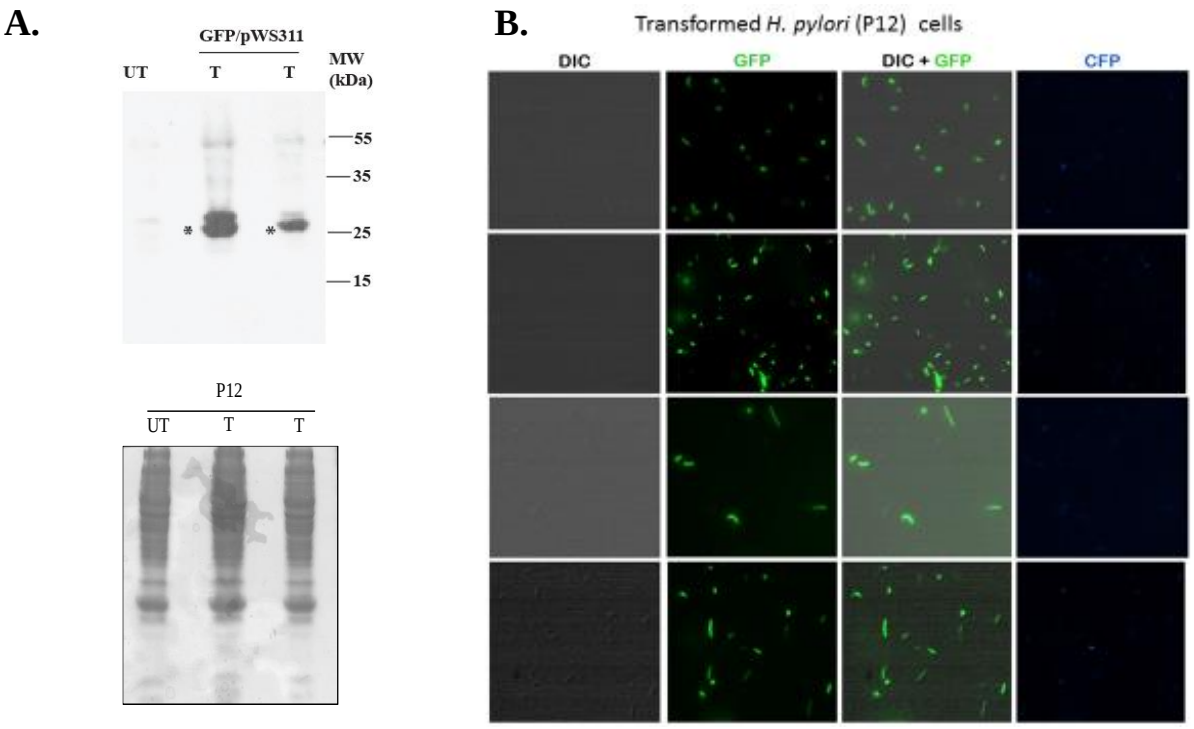

Figure 5.6 Expression of GFP protein alone in strain P12.GFP protein over-expressed episomally under the control of cagA promoter in strain P12. A) Western blot of lysates of untransformed (UT) vs transformed (T) cells with GFP antibodies (Stars denote bands of GFP protein). (B) *H. pylori* P12 cells expressing GFP alone showing diffused GFP signal throughout the cell.

Table 5.3 Average cell length of *H. pylori* P12 cells over producing GFP alone and Hp0897GFP.

Construct	GFP alone	0897 GFP
Cells showing signal	79	26
Average cell length (µm)	2.9	4.7
SD	0.9	1.5
Min	1.8	2.6
Max	5.5	8.0

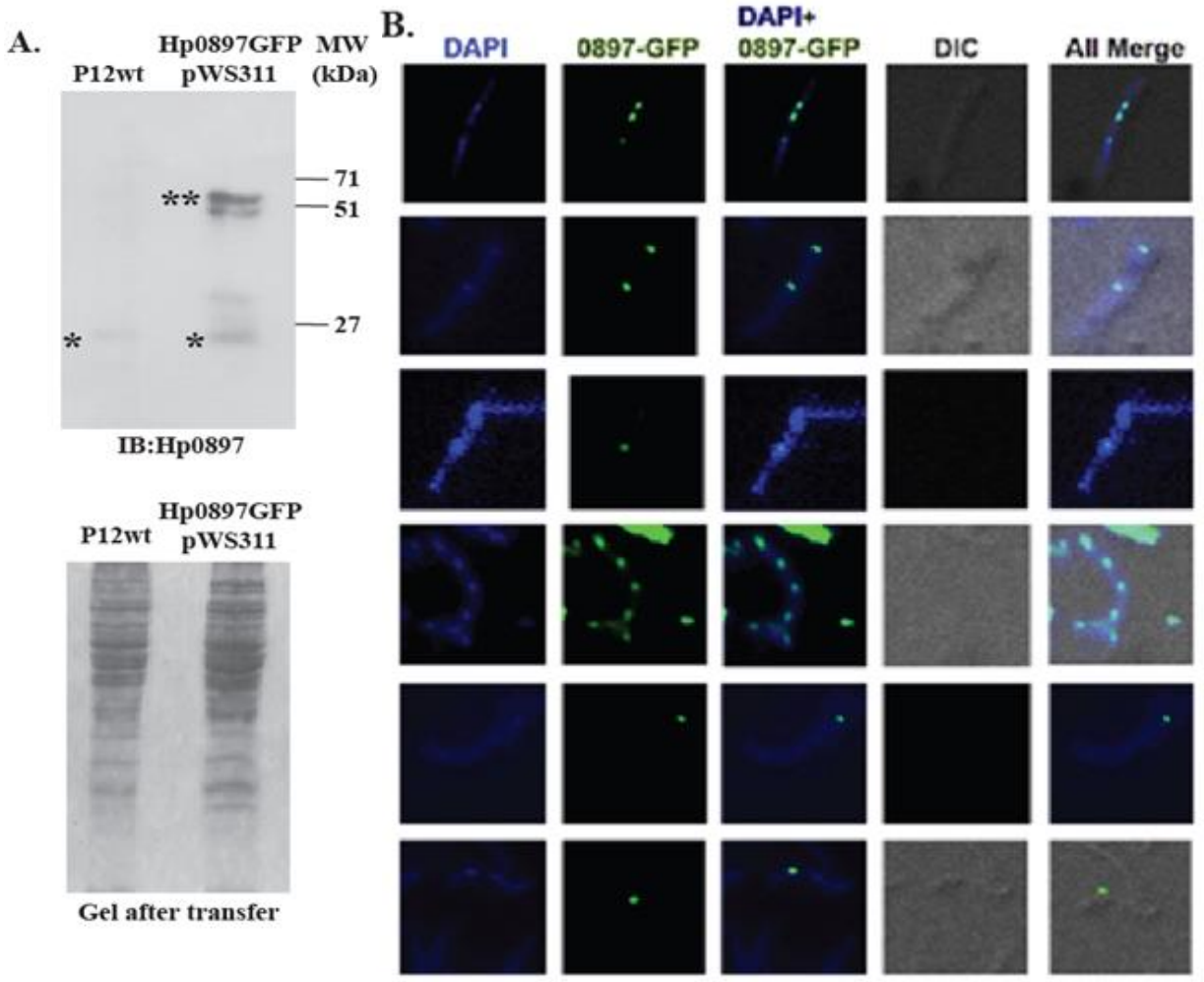

Figure 5.7 Expression of Hp0897GFP fusion protein in P12 strain of *H. pylori.* (A) Cells of *H. pylori* P12 strain were transformed with HP0897GFP/pWS311 construct and positive colonies were selected by growing transformed cells on Kanamycin plates. Positive colonies were further confirmed by western blot analysis using antibodies raised against Hp0897. The bottom panel shows the gel after transfer as loading control. (B) Fluorescence micrographs of *H. pylori* cells expressing HP0897GFP showed different patterns of Hp0897GFP foci and cell elongation phenotype.

The phenotypic changes included cell elongation and cell death due to over production of Hp0897GFP. Further, the *H. pylori* cells transformed with Hp0897GFP/pWS311 were very short lived as they could not grow beyond first subculture. This implies that Hp0897 over expression is toxic for cell growth. Second, average cell length of *H. pylori* cells was increased under the influence of the increased cellular concentration of Hp0897. The average

cell length of wild-type *H. pylori* cells is 2.5 µm (Jones et al., 1985, Goodwin et al., 1985). The average cell length of *H. pylori* cells overproducing Hp0897GFP was increased to 4.7 µm. *H. pylori* cells over producing GFP alone protein did not show considerable increase in cell length. The average cell length of GFP alone cell line measured was 2.9 µm (Table 5.3). Moreover, *H. pylori* cells expressing GFP alone did not die upon subculture and they grew for many generations.

5.2.5. Over-production of NTD and CTD domains of Hp0897 showed similar foci distribution patterning as Hp0897GFP but different cell elongation phenotype

Over expression of Hp0897GFP yielded cell elongation phenotype. We were interested to explore which domain (N-terminal or C-terminal) of Hp0897 is responsible for this phenotypic change. For this purpose NTD0897 and CTD0897 were expressed as GFP fusion protein in pWS311 vector like the wild type protein. Results indicated that like Hp0897GFP, overproduction of NTDGFP domain of Hp0897 was toxic for cell growth and cells died after first subculture. Cell elongation phenotype was more prominent in NTDGFP overproduced cells. Average cell length of *H. pylori* cells over expressing NTDGFP was 10.7 µm which is four times as compared to average cell length of GFP alone over expressing cells, *i.e.*, 2.9 µm. Over expression of CTDGFP did not affect the average cell length drastically under the same experimental conditions. Average cell length of the cells with CTDGFP overproduction was measured to be 3.2 µm (Table 5.4).

Table 5.4 Average cell length of *H. pylori* P12 cells over producing NTDGFP alone and CTDGFP.

Construct	GFP alone	NTD0897GFP	CTD 0897 GFP
Cells showing signal	79	37	50
Average cell length (µm)	2.9	10.7	3.2
SD	0.9	10.7	0.9
Min	1.8	3.7	1.6
Max	5.5	26.9	6.2

The average cell length of *H. pylori* cells transformed with different constructs (GFP/pWS311, 0897GFP/pWS311, NTDGFP/pWS311 and CTDGFP/pWS311) were further plotted as shown in figure 5.8.

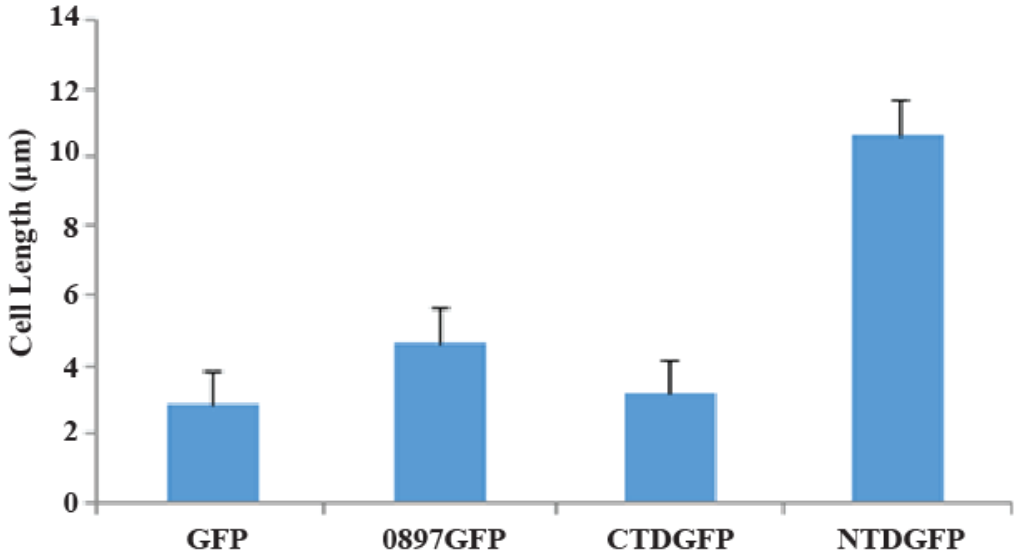

Figure 5.8 Graphical representation of the cell length of *H. pylori* cells overexpressing 0897GFP, NTD0897, CTD0897 and GFP alone.

Further, the expression of CTD0897GFP in *H. pylori* cells was evaluated by western blot analysis using *H. pylori* bacterial lysates in the presence of antibodies against GFP as shown in figure 5.9.A.

Fluorescence microscopy analysis of NTD0897GFP and CTD0897GFP overexpressing *H. pylori* cells showed majority of cells with multiple foci like the wild type protein (figure 5.9.C and D). No foci were found in GFP only expressing cells (figure 5.9.B). It is interesting that over expression of CTD-Hp0897 did not alter the average cell length dramatically but it yielded multiple foci suggesting that over expression of wild type Hp0897 or NTD or CTD may not reflect the true localization of the endogenous protein.

5.2.6. Overexpression of Cell division protein FtsZ showed elongation phenotype but not that of DNA replication protein HpSSB

To conclude the phenotypic changes observed in *H. pylori* cells due to over expression of Hp0897GFP, effect of overproduction of cell division and DNA replication proteins were analysed. FtsZGFP, a cell division protein and SSB, a DNA replication protein were overexpressed in *H. pylori* cells in the same way as Hp0897 under the control of *cagA* promoter. The expression of HpSSBGFP in transformed cells of *H. pylori* P12 strain was confirmed by Western blotting using antibodies against GFP and HpSSB proteins as shown in figure 5.10.A. Expression of HpftsZGFP was also confirmed by western blot (data not shown). Production of HpFtsZGFP led to filamentation of the cells as observed in case of Hp0897GFP overproduction (figure 5.10.B; top and bottom panel respectively). The filamentation phenotype upon overexpression of FtsZ is in accordance with previous results (Dziadek et al., 2003). In contrast to Hp0897, the cells were not lysed when FtsZ was over expressed. Cell elongation phenotype was not observed when HpSSB overproduced (figure 5.10.B; middle panel). These results suggested that one possible reason for cell elongation phenotype observed due to overproduction of Hp0897GFP may be attributed to failure of the cells to divide after replication. Like ftsZ over expression phenotype, this implies that Hp0897 may also have role in cell division.

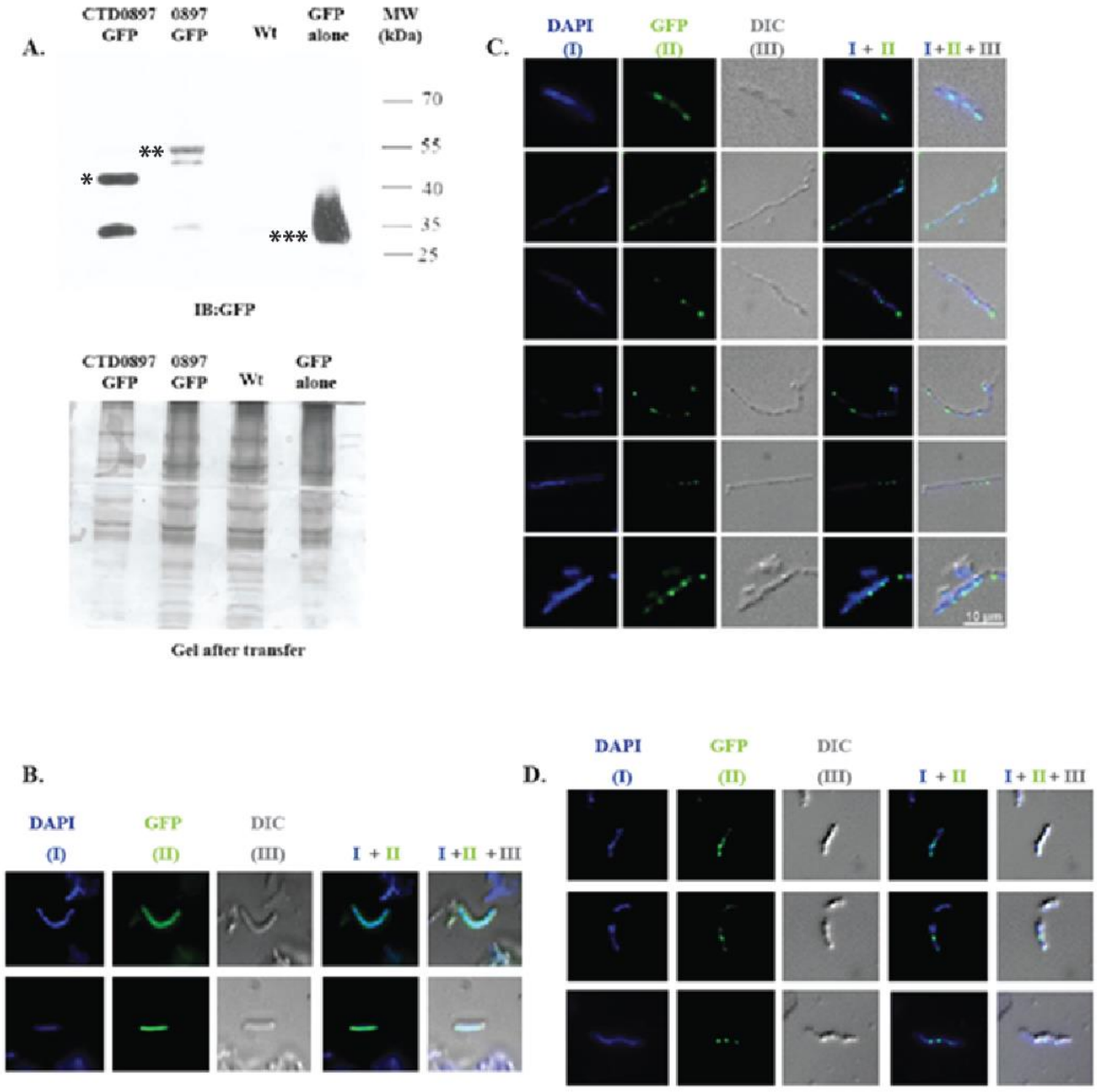

Figure 5.9 Over expression of NTDGFP and CTDGFP in *H. pylori* cells and their localization. (A) *H. pylori* P12 cells were transformed with NTDGFP/pWS311, CTDGFP/pWS311, 0897GFP/pWS311 and GFP/pWS311 construct DNAs. Positive clones were selected on kanamycin plates and confirmed by western blotting with antibodies against GFP protein (Western blot data for NTD is not shown here). The expression of individual proteins can be seen. Wt stands for untransformed bacteria. (Single, double and triple stars denote CTDGFP, 0897 GFP and GFP alone respectively).Coomassie stained gel after transfer is shown as loading control. (B) GFP alone protein did not localize at particular position and is diffused throughout the bacteria. In each panel DAPI staining is shown for nucleoid and GFP for respective protein localization, DIC and merge images of

DAPI and GFP and in last lane combined images of all the channels. (C) Overproduction of NTDGFP led to cell elongation and various multiple foci of NTD0897 can be seen in these elongated cells. (D) CTDGFP did not cause cell elongation but it led to multiple foci of CTDGFP.

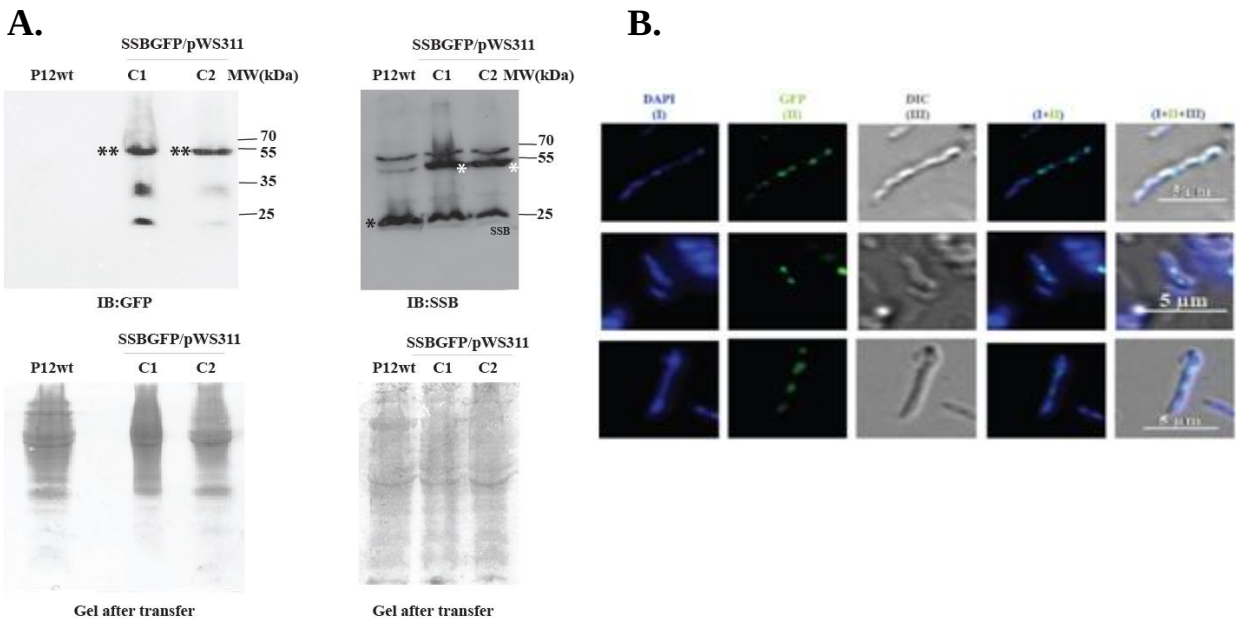

Figure 5.10 Overproduction of HpFtsZ but not HpSSBb showed elongation phenotype (A) *H. pylori* P12 cells transformed with SSBGFP/pWS311 construct were grown on Kanamycin plate and positive clones were confirmed by Western blotting by using antibodies against SSB (right panel; white and black stars denote SSBGFP and endogenous SSB respectively) and GFP proteins (left panel; double stars denote SSBGFP). Coomassie-stained gels after transfer were used as loading control. (B) Fluorescence microscopy images of *H. pylori* P12 cells with overexpression of HpFtsZGFP (top lane), HpSSBGFP (middle lane) and Hp0897GFP (bottom lane). Elongation phenotype and multiple foci of respective proteins were formed in case of HpftsZ and Hp0897 but not in the case of HpSSB.

5.3. Discussion

Proteins in the cell often have specific addresses where they perform their specific jobs. In eukaryotes, proteins may have their residence in particular organelles to carry out specific cellular functions. In prokaryotes also, the cytosol is highly organized and dynamic (Murat et al., 2010). Protein localization is useful in tracking the function of a protein by classifying proteins according to their location and their colocalization with other proteins of known

function. There are several examples of bacterial proteins which are highly organized in the cell. For example, FtsZ forms a ring-like structure at middle of the cell in *E. coli* (Bi and Lutkenhaus, 1991), accumulation of chemotactic proteins in response to chemical stimuli(Ames and Parkinson, 2006) and MinCDE system localizes at the cell poles whereas show pole to pole oscillation (Shih et al., 2003). These observations suggest that proteins involved in various processes reside at particular locations in the cell and their distribution is not uniform.

Subcellular localization of Hp0897 was studied in fixed as well as live cells which revealed some important aspects about its functions in the cell. The localization studies of Hp0897 showed that it may have multiple functions apart from helicase loading. In fixed cells Hp0897 located in most of the events at the pole of the cell. In a very small number of cells, it also showed nonpolar localization. It has been reported that Hp*oriC* region localizes at either pole of the cell independent of the flagellar end (Sharma et al., 2014). Similar localization of Hp0897 at one pole co-localized with HpDnaB helicase cells reveals that Hp0897 may be a component of replisome that assembles at *oriC* which resides at polar region during initiation of replication.

H. pylori is unique in that it possesses bipartite origin of replication. *HporiC* has two suborigins (*oriC* and *oriC2*) which are separated by *dnaA* gene in between them. ChIP-PCR experiments were performed to study the binding of Hp0897 at two suborigins. The results revealed that Hp0897 have preferential binding towards *oriC2* where bubble formation occurs followed replisome assembly.

In previous studies, it has been shown that Hp0897 interacts with and modulates HpDnaB activities. Recent studies with immunofluorescence and ChIP experiments point out that Hp0897 resides at the pole, preferentially within *oriC2* region and it may be a part of replisome complex during initiation of replication where it performs helicase loading function.

The helicase loading function of Hp0897 is also supported by the co-localization studies of Hp0897 with HpDnaB helicase in the cell. Hp0897 co-localized with HpDnaB at polar region of the cell where it may interact with HpDnaB and helps in loading on to melted origin for the start of replication.

Clue for other functions of Hp0897 came from overexpression studies of Hp0897. Indication for a role of Hp0897 in cell division became obvious when Hp0897 was episomally expressed under the control of *cagA* promoter. This overproduction of Hp0897 in the cell had lead to phenotypic change in the cell. The cells were elongated and they showed multiple foci of Hp0897 with multiple patterns of localization and cell toxicity which led to cell death after one or two generations. For comparison, overexpression of HpFtsZ (a cell division protein) and that of HpDnaB, (a replication protein) were also analyzed. Overproduction of HpSSB showed no visible phenotypic change. Under similar conditions, HpFtsZ showed filamentous and elongated cells. Cells get elongated under altered levels of Hp0897 and HpFtsZ due to failure to divide the cells. It seems that alteration of Hp0897 affects cell division process which implies its possible role in this process.

Analysis of Hp0897 sequence for motif search by bioinformatics tools like Scan Prosite showed that it possesses cytosolic fatty acid binding (FAB) domain at its N terminus region. If this FAB domain is functional in fatty acid binding, then this protein may have some role in cell wall synthesis by transporting fatty acid at the site of cell division. If it is true, then new cell wall synthesis at the site of division can be analyzed under the regulated expression of Hp0897. Therefore the overexpression of Hp0897 may indeed affect the cell division process and may lead to cell elongation as it has been reported here.

These studies imply that Hp0897 is a multifunctional protein that may be involved in DNA replication and cell division of *H. pylori*. While the C-terminus region of Hp0897 may be engaged in helicase loader function, the N-terminus may be involved in cell division. The various interacting partners of Hp0897 can be detected by co-immunoprecipitation by using antibodies against Hp0897 followed by mass spectrometry. Knowledge of its interacting partners may be useful to confirm its multiple functions.

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Summary

Summary

DNA replication is a fundamental process that is conserved among all forms of life and faithfully duplicates the genomic information and transfers this genetic information equally into the progeny. To accomplish this process, battery of proteins is involved. These proteins generally perform their function in a sequential manner at a specific time during the cell cycle. Therefore, replication and cell division proteins are highly dynamic.

Generally, bacteria possess a single *oriC* which contains an AT-rich DNA unwinding element (DUE), one IHF (integration host factor) binding site and 12 DnaA binding sites of various affinities. *H. pylori* possess bipartite origin which is subdivided into *oriC1* and *oriC2*. DUE is present near to *oriC2*. DNA replication starts as DnaA (initiator protein) binds to specific sites (DnaA boxes) at *oriC* (origin of chromosomal replication). DiaA protein stimulates multimerization of DnaA and co-operative binding of DnaA to *oriC*. In *H. pylori*, HobA is the homolog of DiaA protein. After binding to *oriC*, DnaA and IHF generate a topological strain that leads to melting of nearby AT-rich DNA unwinding element (DUE). IHF bends the DNA and helps in the formation and stabilization of the nucleoprotein complex. Along with IHF the other two proteins, HU and Fis also enhance initiation of replication by DnaA protein. Hda protein interacts with DnaA after initiation and coverts ATP-DnaA to inactive ADP-DnaA and thereby preventing over initiation of *oriC*, so that origin firing occurs once per cell cycle. The single strands generated are the sites of replisome assembly. After melting of DUE, DnaC (helicase loader) protein loads DnaB helicase ring on to each strand of the melted DUE. Once helicase is loaded on to DNA, DnaG (primase) interacts with DnaB and DnaC is released by the hydrolysis of ATP bound to DnaC. It is proposed that there are two ways of helicase loading; either ring breaking or ring making. In the case of ring breaking, six molecules of DnaC interact with six molecules of DnaB. DnaB●DnaC complex formation leads to conformational change that opens the hexameric ring so that one DNA strand enters the opened ring through the open end of the hexameric ring and the other strand is excluded from the inner core of the ring. In another way, DnaC helps to assemble different monomers of DnaB around the melted DNA strand to form a hexameric ring. When DnaB is in complex with DnaC, it is tightly bound to DNA, and it cannot move along the forked DNA. But as soon as the DnaC is released by interaction of DnaB with DnaG, the interaction of DnaB with single-stranded DNA becomes weak, and DnaB slides along the DNA to unwind duplex DNA ahead of the replication fork. After

synthesis of the short primer by DnaG, DNA polymerase starts amplifying leading and lagging strands simultaneously.

In *H. pylori,* helicase loader is not annotated which is conserved in most of the bacteria. Helicase needs the assistance of one or more proteins for loading on to single-stranded DNA. The reports have suggested that *H. pylori* helicase may have self-loading capacity onto DNA. When HpDnaB was overexpressed in *E. coli* DnaC temperature-sensitive strains, it allowed these mutant strains to grow at non-permissive temperatures. HpDnaB supported the growth of mutant *E coli* cells which were defective in DnaC. The status of helicase loading in *H. pylori* itself is not well understood. Recently, a hypothetical protein Hp0897 has been shown to be a possible homolog helicase loader in *H. pylori.* Hp0897 interacts with HpDnaB and modulates its DNA binding, helicase and ATPase activities. *HP0897* is unique to *H. pylori* and its sequence is conserved in most of *H. pylori* strains. HP0897does not show any sequence similarity with helicase loaders in other bacteria. Exploring HP0897 functions in more detail would be intersting. Therefore, in the present study, we focused on studying the Hp0897 and HpDnaB interaction by *in vitro* pulldown asasy and to find out the minimum regions of both the proteins involved in the DnaB●DnaC complex formation. Further, we have also checked the essentiality of Hp0897 protein for the survival of *H. pylori.* To gain insights into function/s of Hp0897 we have done detailed sub cellular localization studies of Hp0897 and other replication proteins in fixed cells as well as live cells. We have also checked binding of Hp0897 protein at *oriC* region of *H. pylori.* These results have suggested that Hp0897 plays an important role in helicase loading and may have a function in cell division also.

In the first chapter, *in vitro* pull-down assay was performed to find out minimum regions of HpDnaB and Hp0897 that are required for DnaB●DnaC complex formation. For domain mapping of HpDnaB (total length 488 amino acids), various deletion mutants of HpDnaB were expressed with $(His)_6$ tag in *E. coli* cells. These deletion mutants of HpDnaB were purified and used as prey proteins in the GST pull-down assay. Hp0897 protein was expressed with GST tag and used as the bait protein. GST alone protein was used as negative control. In pull-down reaction, GST-Hp0897 was immobilized on the glutathione beads and incubated with different deletion mutants of $(His)_6$-tagged HpdnaB. The protein bound beads

were washed with high salt wash buffer and then subjected to SDS-PAGE followed by Western blotting using antibodies against $(His)_6$. The results have shown that out of nine deletions of HpDnaB, seven had interacted with Hp0897 which means these regions of HpDnaB are required for interaction with DnaB. The remaining two deletions of HpDnaB did not show interaction with Hp0897. After compiling the *in vitro* pull down assay data, a common region from amino acids 195 to 318 has emerged that may be involved in complex formation with Hp0897.

Similarly, domain mapping of Hp0897 was performed by using deletion mutants of Hp0897 as prey proteins. Two deletion mutants of Hp0897 protein (total length 208 amino acids) that is NTD0897 (1 to 115 amino acids) and CTD0897 (100 to 208 amino acids) were expressed with $(His)_6$ tag in *E. coli* cells. The HpDnaB was expressed with GST tag in *E. coli* cells and used as the bait protein. The GST alone protein was used as negative control. The results indicated that both NTD0897 and CTD0897 deletion mutants of Hp08987 had interacted with HpDnaB but CTD0897 had shown more strong interaction whereas NTD0897 had interacted weakly with HpDnaB.

In the second chapter, the essentiality of the Hp0897 gene for the physiology of *H. pylori* was checked by various attempts to knockout *HP0897* gene from the genome. For this purpose, Homologous recombination method was used to delete *HP0897*. *HP0897* deletion had been done in two ways. Either by using circular DNA constructs or linear DNA fragments. For circular DNA construct to generateHp0897 KO, the construct was cloned in a vector backbone pBluescript KS II(+); pBS. The pBS vector is a modified vector that contains chloramphenicol cassette (CAM) as a selection marker between BamHI and XhoI sites (a kind gift from G. Mukhppadhyay's lab). The 545 bp upstream region (200 bp upstream of the start site of *HP0897* and 1 to 345 bp of the coding region of *HP0897*) of *HP0897* were cloned in 3' site of CAM and 200 bp of *HP0897* were cloned in 5' site of CAM. For positive control, pBS/UPS500 *cagδ*::CAM::DNA500*cagδ* construct was used to knockout *cagδ* gene. *cagδ* is a component of the type IV secretion system of *H. pylori* and is non-essential. *cagδ* KO construct was a kind gift from G. Mukhppadhyay's lab.

For linear DNA construct for the generation of *HP0897* KO, primers were designed and used in overlap PCR to amplify CAM cassette which was flanked by 500 bp UPS and 500 bp DNS

regions of *HP0897* at its 5' and 3' respectively. Similarly for positive control, linear construct to knockout non essential gene *cagU* was designed by overlap PCR. Again, primers were designed to amplify CAM cassette along with 500 bp UPS and 500 bp DNS regions of *cagU* at the 5' and 3' sites of CAM cassette by overlap PCR.

H. pylori 26695 strain was transformed with the circular constructs for knockout of *HP0897* and *cagδ* or linear DNA fragments for knockout of *HP0897* and *cagU* . pBS vector backbone was also transformed as a negative control. The bacteria were selected on chloramphenicol plates. No colony was grown on selection plate when *HP0897* knockout was attempted either by using circular DNA construct or by linear DNA fragment. In case of negative control, pBS vector backbone only, no colony wasseen on selection plate. Under similar conditions, for both positive controls, *cagδ* KO and *cagU* KO, a large number of colonies appeared on chloramphenicol plates irrespective of the nature of the DNA construct whether it was circular or linear DNA construct.

Interestingly, *HP0897* overlaps with a tRNA gene that encodes for Glutaminyl tRNA. The *HP0897* and *tRNA-Gln-1* gene are present on complementary strands and are transcribed in opposite directions. This *tRNA-Gln-1* gene is 72 bp in length and out of 72 bp, the 66 bp overlaps coding strand of *Hp0897* at its 5' end and remaining six bp overlaps with the upstream region of start codon of HP0897. *H. pylori* genome encodes a single copy of *tRNA-Gln-1*. It is possible that this gene was disrupted when HP0897 KO was attempted. Disruption of this tRNA gene will stall translation of cellular proteins and hence cell death. So, to keep this tRNA-Gln-1 gene intact, partial deletion of *HP0897* was attempted. The constructs for partial HP0897 KO was designed by overlap PCR. The primers were designed in such a way that the tRNA gene was not disrupted while rest of *HP0897* gene was deleted. Even when partial deletion of *HP0897* was attempted, no colony was obtained on selection plate. These results have shown that neither complete nor partial deletion of *HP0897* is possible under these conditions. These results imply that *HP0897* is essential gene of *H. pylori.*

In the third chapter, the role of Hp0897 protein at *oriC* and during cell division has been shown by fluorescence microscopy and Chromatin immunoprecipitation (ChIP). The subcellular localization of Hp0897 protein in the cell was analyzed by immunofluorescence

microscopy of fixed *H. pylori* cells. The *H. pylori* cells were permeabilized and treated with pre-immune sera or immune sera against Hp0897 protein. The Alexa fluorophore-conjugated antibodies were used as secondary antibodies to detect the primary antibodies. The microscopy results revealed that HP0897 is present at one pole in the majority of cells analyzed. The co-localization of Hp0897 with HpDnaB was analyzed in fixed cells by using antibodies raised against both HpDnaB and Hp0897 proteins. The results indicated that Hp0897 colocalized with HpDnaB at the polar region of the cell. Hp0897 resided at the pole whereas HpDnaB had shown non-polar localization apart from polar ones. HpDnaB was present at the pole, near to pole and at the middle of the cell suggesting dynamic movement of replisome from pole to midcell region in *H. pylori*. Following loading of DnaB, Hp0897 may not be a part of active replisome.

In *H pylori*, it has been shown that chromosomal origin is located at one pole of the cell. To confirm whether Hp0897 binds to *oriC* while it resides at the pole of the cell, ChIP-qPCR was performed by using antibodies against Hp0897. For positive control, antibodies against HpDnaA were used to check binding of HpDnaA on *oriC*. The q-PCR was performed to check binding of Hp0897 and positive control DnaA at *oriC1, oriC2* and non-*oriC* region. The results indicated the binding of HpDnaA at both *oriC1* and *oriC2* as reported in earlier studies. Hp0897 also showed binding at both *oriCs,* however, Hp0897showed preferential higher binding towards *oriC2* where origin firing occurs during initiation of replication.

Localization of Hp0897 was also studied in live *H. pylori* cells. For this purpose, GFP vector was designed and coding region of Hp0897 was cloned in this GFP vector. Hp0897GFP was overexpressed episomally under the control of *cagA* promoter. Western blotting and microscopy confirmed the expression of GFP alone as well as Hp0897GFP. The results had shown that GFP alone protein was uniformly distributed throughout the cell whereas Hp0897GFP showed distinct foci. It was observed that overexpression of Hp0897 led to visible phenotypic changes in the cell. The average cell length became double and most of the cells were elongated, multiple foci of Hp0897 with different localization patterns were observed and cell death was also observed. The similar phenotype was obtained when cell division protein FtsZ was overexpressed under similar conditions. But no phenotypic change observed when a replication protein SSB was overexpressed in the same way.

From these results, it can be concluded that Hp0897 is a possible homolog of helicase loader. For performing the loading function Hp0897 interacts stably with HpDnaB. For this interaction, a region of 195 to 318 amino acid residues of HpDnaB is required. This region also contains Walker A and Walker B motifs. It is interesting that in *E. coli*, DnaB interacts with DnaC through its two regions from amino acid residues 295 to 304 and from 431 to 435. The region 295 to 304 lies between Walker A and Walker B motifs. Like in *E. coli*, the region of HpDnB interacting with Hp0897 was mapped near Walker A and Walker B motif. The difference is that HpDnaB has a single interaction site for helicase loader Hp0897. On the other hand Hp0897 interacts through NTD and CTD regions and moreover, the CTD region interacts strongly with full-length HpDnaB. In case of *E. coli* also two regions of helicase loader DnaC (8-11 and 31-44 amino acid residues) have been reported to involve in interaction with DnaB. However, these two interacting regions are present within the N-terminus of DnaC.

Hp0897 gene is essential for the survival of *H. pylori* and at least one functional copy is required for growth of the bacteria. It is difficult to make Hp0897KO strain as it overlaps with essential tRNA-Gln-1 gene which has only one copy in the genome. So deletion of Hp0897 gene disrupts this overlapping tRNA gene and may halt the translation machinery of the cell. The partial KO of *Hp0897* was not successful where tRNA gene was kept intact. Therefore, Hp0897 gene might be essential or 3' end of *HP0897* gene is not accessible for homologous recombination. To check the accessibility of 3' end, GFP tag or any other suitable tag can be integrated at 3 ' end of *HP0897* by 3' replacement.

Another approach can be a complementation assay to make *HP0897* KO. A mutant strain can be generated with two copies of *HP0897* and *tRNA-Gln-1* genes. This mutant strain which will be merodiploid for *Hp0897* and *tRNA-Gln-1* can be used to delete endogenous *HP0897*.

The conditional KO for *HP0897* can be made in a mutant strain which is merodiploid for *tRNA-Gln-1* gene.

In *H. pylori,* it is reported that *oriC* region is present near the polar region of the cell irrespective of the flagellar end. Polar localization of Hp0897 implies its association with *oriC* region which was confirmed by ChIP-qPCR. Binding of Hp0897 with *oriC* reveals its

function at the initiation of replication. Co-localization of Hp0897 with HpDnaB at cell pole indicates its helicase loading function during replisome assembly at *oriC2*.

Like cell division protein HpfFtsZ, overexpression of Hp0897 led to cell elongation and inhibited cell division. The defects in cell division due to overproduction of Hp0897 imply that it may have some role in the cell division process also. The N terminal domain of Hp0897 has been shown to contain fatty acid binding signature motif (Shivangi Verma and SK Dhar unpublished data). The presence of fatty acid binding motif may impart property of fatty acid transportation by Hp0897 at the site of cell division that needs to be explored further.

Therefore, these observations indicate that Hp0897 plays a role of helicase loading in *H. pylori* and may have a role in cell division also. Multifunctional nature of Hp0897 necessitates to find out different interacting proteins of Hp0897 by co-immunoprecipitation followed by Mass Spectrometric analysis.

Being an essential gene and a part of two important processes of DNA replication and cell division which are required for *H. pylori* proliferation, Hp0897 can be a potential therapeutic target.

Publications

Publications

Verma, V; **Kumar, A**; Nitharwal, RG; Alam, J; Mukhopadhyay, A; Dasgupta, S and Dhar SK; (2016) **Modulation of the enzymatic activities of replicative helicase (DnaB) by interaction with Hp0897: A possible mechanism for helicase loading in *Helicobacter pylori*. *Nucleic Acids Research*** 44(7):3288-3303.

Book chapter

Verma V, **Kumar A** and Dhar SK (2016) **Helicobacter pylori: An overview of the Infection, Pathogenesis and Molecular cell biology of DNA replication and cell division.** In Prof. Assis Datta and Prof. V.P. Sharma (Editors), Recent Advances in Communicable and Non-Communicable Diseases. The National Academy of Sciences, Allahabad India (NASI). Capital Publishing Company New Delhi, India. ISBN: 978-93-81891-31-5.

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'Modulation of the enzymatic activities of replicative helicase (DnaB) by interaction with Hp0897: a possible mechanism for helicase loading in *Helicobacter pylori*'

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ABSTRACT

DNA replication in *Helicobacter pylori* is initiated from a unique site (*oriC*) on its chromosome where several proteins assemble to form a functional replisome. The assembly of *H. pylori* replication machinery is similar to that of the model gram negative bacterium *Escherichia coli* except for the absence of DnaC needed to recruit the hexameric DnaB helicase at the replisome assembly site. In the absence of an obvious DnaC homologue in *H. pylori*, the question arises as to whether HpDnaB helicase is loaded at the *Hp*-replication origin by itself or is assisted by other unidentified protein(s). A high-throughput yeast two-hybrid study has revealed two proteins of unknown functions (Hp0897 and Hp0340) that interact with HpDnaB. Here we demonstrate that Hp0897 interacts with HpDnaB helicase *in vitro* as well as *in vivo*. Furthermore, the interaction stimulates the DNA binding activity of HpDnaB and modulates its adenosine triphosphate hydrolysis and helicase activities significantly. Prior complex formation of Hp0897 and HpDnaB enhances the binding/loading of DnaB onto DNA. Hp0897, along with HpDnaB, colocalizes with replication complex at initiation but does not move with the replisome during elongation. Together, these results suggest a possible role of Hp0897 in loading of HpDnaB at *oriC*.

INTRODUCTION

Helicobacter pylori is a gram-negative, slow-growing, spiral-shaped bacterium which infects more than half of the human population. *H. pylori* infection is of growing concern today because of its crucial role in the pathogenesis of chronic gastritis, peptic ulcer diseases and in the multi-step carcinogenic processes of gastric cancer, the fourth most common cancer worldwide. Epidemiologically, over 3 billion people are infected by this bacterium and develop persistent stomach inflammation, which lasts for decades unless treated with antibiotics (1,2).

DNA replication and its control are the keys to bacterial proliferation, pathogenesis and virulence. However, our knowledge of DNA synthesis mechanisms and their control in slow growing pathogenic bacteria like *H. pylori* is still in its infancy. Chromosomal DNA replication is a tightly controlled process, regulated by a battery of proteins. In the model system *Escherichia coli,* replication initiation takes place at the unique site *oriC* by binding of DnaA–adenosine triphosphate (ATP) protein complex at DnaA boxes leading to unwinding of the AT-rich sequences defined as the DNA unwinding element (DUE) (3–6). Subsequently, two oppositely oriented complexes of DnaB–DnaC are recruited at this opened region of *oriC* resulting in further separation of the DNA double strands. Interaction of DnaG primase with DnaB helicase followed by primer formation triggers the release of DnaC from DnaB and activation of its DNA-dependent adenosine triphosphate hydrolysis (ATPase) and DNA unwinding activities (7).

The organization of replication genes in *H. pylori* differs from the model gram-negative bacterium *E. coli*. Unlike in *E. coli*, the *HpdnaA* gene is located 600 kb away from the *dnaN–gyrB* genes and the homologues for *EcrecF* and *EcdnaC* are apparently absent (8). *H. pylori* encodes a unique replicative DnaB helicase that has been characterized both *in vitro* and *in vivo* and found to complement the helicase function in a *dnaB*^{ts} mutant strain of *E. coli* at non-permissive temperature (8). The N-terminal domain of

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Helicobacter pylori: An overview of the infection, pathogenesis and molecular cell biology of DNA replication and cell division

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1. Introduction

Helicobacter pylori were previously named as *Campylobacter pyloridis*. After 16S ribosomal RNA gene sequencing, it was placed in its own genus *Helicobacter*. *H. pylori* is a microaerophilic, slow growing and Gram-negative bacterium found in the stomach. It has three shapes: curved or s shape (0.5-0.9 μm wide and 2-4 μm long), rod shape bacillary and round shape coccoid (Fig. 1). *H. pylori* were identified by Robin Warren and Barry Marshall in 1982, who found that it was present in the patients with chronic gastritis and gastric ulcers. Initially, there was a debate whether the bacteria would cause ulcers. It is very surprising that ~80% of individuals infected with the bacterium are asymptomatic [1]. Infection is more prevalent in developing countries, and the incidences of the infection of *H. pylori* are decreasing

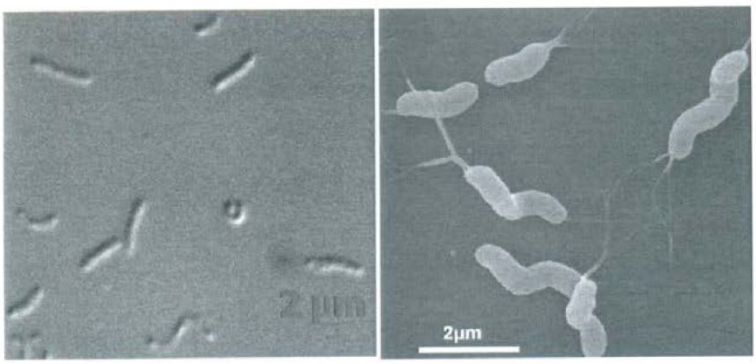

Fig. 1. *Helicobacter pylori*, as seen under (I) Light microscopy (Carl Zeiss fluorescence microscope): Image was taken in SCMM, JNU, New Delhi and (II) Electron microscopy: Image courtesy Dr. Nina Salama, Fred Hutchinson Cancer Research Center, Seattle, WA [3].

History

As mentioned above, *H. pylori* was first discovered in the stomach of patients suffering with gastritis and stomach ulcers in 1982. Initially, there was a strong debate whether any bacterium could live in the human stomach. In the decades of 1970s, researchers thought that lifestyle problems and stress would lead to peptic ulcer disease or gastritis. Previous to the research of Marshall and Warren, some other scientists also found spiral shaped bacteria in human stomach lining, but they were unable to culture it [1].

In 1979, J. Robin Warren noticed small curved bacteria and associated inflammation on the surface of many gastric biopsies taken from antrum of the stomach. Over the next two years, he thoroughly examined the gastric biopsies with the bacteria from several patients and

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