3288–3303 *Nucleic Acids Research, 2016, Vol. 44, No. 7* *Published online 21 March 2016*
doi: 10.1093/nar/gkw148

'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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Received December 22, 2014; Revised February 29, 2016; Accepted March 1, 2016

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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HpDnaB is dispensable for its helicase activity whereas the C-terminal domain is essential for its enzymatic activities (9). The deletion of the N-terminus, or its engagement with an N-terminal interacting protein like DnaG, enhanced the DNA binding activity of HpDnaB profoundly, suggesting that the N-terminus might interfere with HpDnaB protein's DNA binding activity probably by folding back onto the C-terminal region (10). Interestingly, when overexpressed, HpDnaB can complement the lack of EcDnaC function in two *dnaC*^{ts} strains of *E. coli* at non-permissive temperature suggesting its probable self-loading activity at least in a heterologous system and upon overexpression (11).

Replicative helicases, in general, are assisted by helicase-loader proteins. DnaB helicase is loaded by helicase-loader DnaC in *E. coli* (12–16) and related organisms. *Aquifex aeolicus* DnaC can form helical structures and it can specifically bind to ATP-DnaA and DnaB (17). *Bacillus subtilis* encodes helicase-loader DnaI (18,19), which cooperates with a co-loader protein DnaB (not to be confused with the EcDnaB helicase) to load the replicative helicase DnaC (not to be confused with the helicase-loader EcDnaC) (20). In spite of having limited sequence similarity among the helicase loaders (mainly confined to their Walker A and B motifs), they have similar function. The *Bacillus* DnaI protein has been described as an *E. coli* DnaC homologue. However, the sequence similarity between these two proteins is confined to Walker motifs only (19). T4 gene 59 protein (loader of the bacteriophage T4 gene 41 helicase) also has limited sequence similarity to functionally related proteins (21). Recent findings emerging from electron microscopy (EM) and small angle X-ray scattering (SAXS) studies show that ATP-bound EcDnaB/DnaC complex forms a three-tiered assembly, in which DnaC adopts a spiral configuration that remodels NTD scaffolding and CTD motor region of DnaB to make a clear break in helicase ring indicating that bacterial DnaC helicase loader is a DnaB ring breaker (22). However, this conclusion has been challenged; DnaC binding has been shown to trap a spontaneously opened ring at the CTD end of the DnaB hexamer and facilitate the binding of the DnaG primase at the NTD (23). An important role of DnaC could be to generate a high DNA binding affinity of DnaB for the *oriC* region in DnaB/DnaC complex, so that it remains in the vicinity of *oriC* region.

Helicobacter pylori oriC has been previously identified *in silico* as a region localized upstream of *dnaA* and its interaction with DnaA has been characterized using different *in vitro* experiments (24–26). Using computational and experimental analysis, Zawilak *et al.* identified an additional DUE site (*oriC2*—downstream of *dnaA* gene), separated from the original one (*oriC1*—upstream of *dnaA* gene) (27,28). DnaA specifically binds to both *oriC* sequences, but DnaA-dependent DNA unwinding occurs only within *oriC2*. However, both upstream (*oriC1*) and downstream (*oriC2*) regions are required for *in vivo* initiation of *H. pylori* chromosome replication (27).

Due to lack of annotation of DnaC in *H. pylori*, the loading of HpDnaB helicase is still a mystery. Passing of DNA through a side gap at the periphery of the ring helicases may also be the one mechanism of loading of the HpDnaB helicase bypassing the need for breaking the hexameric ring. A dodecameric model of HpDnaB based on crystal structure of C-terminal domain revealed two head-to-head ring hexamers with comparatively large peripheral gaps between the individual subunits, suggesting an alternative strategy for loading HpDnaB onto forks in the absence of helicase loaders (29). How do the hexameric or dodecameric forms of HpDnaB project towards *oriC* in the absence of a helicase loader, is still an unanswered question. Additional factors may also be involved in initiation, such as HobA, (30) a structural homologue of *E. coli* DiaA (31), which forms a HobA4/DnaA4 complex required for bacterial survival (32). It is obvious that HobA and/or other factors participate in the loading of HpDnaB at *oriC* in *H. pylori*. It is not clear at this stage whether HpDnaB is loaded as a dodecamer onto double-stranded DNA (*oriC1*) or onto single-stranded DNA produced by DnaA-dependent melting region *oriC2* (33). It is plausible that HpDnaB is loaded onto a DnaA-mediated unwound DNA region on *oriC2* with the help of yet to be characterized accessory proteins. The exclusivity of HpDnaB in targeting either *oriC1*/*oriC2* is still unclear. High-throughput yeast two-hybrid protein-protein interaction studies in *H. pylori* failed to detect an HpDnaA–HpDnaB interaction minimizing the possibility of a DnaA-mediated directional loading of HpDnaB helicase (34,35).

As discussed above, overexpression of HpDnaB can bypass the requirement of DnaC protein in *E. coli* (11). It has been also reported that the overexpression of DnaC can rescue the temperature sensitivity of the *E. coli dnaB252* allele (36). The overexpression of proteins may not reflect the *in vivo* conditions of the organism. It is possible that a functional homologue of DnaC, without sharing any sequence homology with EcDnaC, is present in *H. pylori* which performs as an accessory factor helping the correct loading of HpDnaB at the origin.

Interestingly, a high-throughput yeast two hybrid assay using *H. pylori* proteins showed two interacting partners (Hp0897 and Hp0340) of HpDnaB replicative helicase (34). Like Hp0897, Hp0340 is also annotated as an unknown ORF in *H. pylori* genomic database. However, unlike Hp0897, it is less conserved in other *H. pylori* strains. Hp0340 has not been characterized further. Here, we show that Hp0897 binds to HpDnaB *in vitro* as well as *in vivo*, modulates all enzymatic activities of HpDnaB, colocalizes with HpDnaB *in vivo* and stimulates the DNA binding activity of HpDnaB profoundly. Moreover, preformation of a complex between HpDnaB and Hp0897 was necessary for the Hp0897 mediated stimulation of the DNA binding activity of HpDnaB. These results suggest a loader-like function for Hp0897 advocating involvement of a loader protein in the replicative helicase activity in *H. pylori* chromosome replication.

MATERIALS AND METHODS

Helicobacter pylori culture

Helicobacter pylori strains were grown on brain heart infusion agar (Difco, Franklin Lakes, NJ, USA) as described elsewhere (37).

Construction of the recombinant clone of *Hp0897*

ORF0897 gene (624 bp) was amplified by polymerase chain reaction (PCR) using Phusion DNA polymerase (NEB) and genomic DNA from *H. pylori* strain *26695* as template with forward and reverse primers 5′ CGGGATCCATGCCAGGACCAAAACCTGG 3′ and 5′ CCGCTCGAGAGGGGTGCGCAGCGTAT 3′ respectively. The PCR products were digested with restriction enzymes BamHI and XhoI and ligated into the expression plasmid vector pET28a (Novagen) followed by transformation into *E. coli* DH10β competent cells. For Glutathione-S-transferase (GST) fusion protein, the PCR product was cloned into pGEX6P2 vector (Amersham Pharmacia) between the BamHI and XhoI sites. The clones were sequenced with appropriate primers to ensure absence of mutations introduced during PCR amplification. The recombinant clones for HpDnaB, EcDnaB and EcDnaC had been generated earlier (8).

Purification of recombinant protein Hp0897, EcDnaC, EcDnaB and HpDnaB

His_6-tagged and GST-tagged proteins were purified as described earlier (8). For a better yield of soluble proteins (Hp0897), the bacterial culture was enriched by 1 mM glucose, 1 mM $MgCl_2$ and induced for 10 h at 22°C in the presence of 0.2 mM IPTG followed by protein extraction and purification procedures as described elsewhere (8). The second column purification of Hp0897 and EcDnaC were performed with a 26 ml Gel Filtration column (Superdex 200 10/300 GL, GE Healthcare, Uppsala, Sweden) using a buffer containing 50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 5 mM BME, 100 μM PMSF (9). Recombinant proteins HpDnaB and EcDnaB were purified according to protocols described elsewhere (8,9) with some modifications. These column purifications of HpDnaB and EcDnaB were done using a Heparin-Sepharose6 fast flow column (GE Healthcare, Uppsala, Sweden) that had been pre-equilibrated with a buffer containing 50 mM Tris.Cl (pH 7.4), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM βME, 100 μM PMSF. All the peak fractions were analysed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and pure fractions were pooled and dialysed against dialysis buffer containing 50 mM Tris.Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 10 mM βME, 100 μM PMSF and 10% glycerol. Purified proteins as discussed above were aliquoted and stored at −80°C for biochemical assays.

Oligomerization of His_6-Hp0897

To analyse the oligomerization of His_6-Hp0897, we used the 120 ml superdex column (Hi-Load16/60 Superdex 200 prep grade, GE Healthcare, Uppsala, Sweden). For this purpose, Ni-NTA agarose-purified, concentrated 1ml protein mixture (500 μg) was loaded on calibrated 120 ml column following the protocol described in Supplementary Materials and Methods.

Immunofluorescence microscopy

Intracellular location of Hp0897 and its colocalization with HpDnaB were investigated using previously described protocols (37,38). Rabbit anti-HpDnaB (1:1000) (11) and mice anti-Hp0897 (1:500) were used as primary antibodies. In brief, *H. pylori* cells were smeared on poly-lysine (0.01%) coated glass slides and fixed with 4% paraformaldehyde in 1× phosphate buffered saline (PBS) for 15 min at room temperature followed by washing with 1× PBS for three times and treated with Triton-X100 (0.3% in 1× PBS) for 25 min at 25°C. Subsequently, the slides were washed (1× PBS) and blocked (3% bovine serum albumin (BSA) in 1× PBS) for 1 h. Primary antibodies treatment (1:1000 dilutions in 1× PBS containing 3% BSA) for anti-HpDnaB (in rabbit) and (1:500 dilutions in 1× PBS containing 3% BSA) for anti-Hp0897 (in mice) were done at 25°C for 1 h or at 4°C overnight. After washing (1× PBS), the cells were incubated with secondary antibodies (1:1000 dilutions for Alexa fluor 594 conjugated anti-mice IgG antibodies and 1:1000 dilutions for Alexa fluor 488 conjugated anti-rabbit IgG antibodies) obtained from Santa Cruz, CA, USA. Cells were further washed (1× PBS) 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.

GST pull-down and Ni-NTA pull down assay

In GST pull down assay, His_6-Hp0897 (2 μg) was incubated in the presence of bead-bound GST–HpDnaB or bead-bound GST alone (control) in pull down buffer [50 mM Tris.Cl (pH 7.5), 1 mM dithiothreitol (DTT) 4% (v/v) glycerol, 0.1 mg/ml of BSA, 5 mM $MgCl_2$, 1 mM ATP and 50 mM NaCl] at 4°C for 1 h as described elsewhere (11,37). In Ni-NTA pull-down assay, Ni-NTA bead-bound His_6-Hp0897 were incubated with purified GST tagged proteins (GST-HpDnaB, GST-EcDnaB or GST protein alone) in the pull down buffer mentioned above. Following incubation, the beads were washed thoroughly using washing buffer (pull down buffer with 300 mM NaCl) and bead bound proteins were resolved on SDS-PAGE followed by Western blot analysis using antibodies against GST.

Co-immunoprecipitation of HpDnaB and Hp0897

To test *in vivo* interaction between HpDnaB and Hp0897, co-immunoprecipitation assay was performed using *H. pylori* B-28 lysate. The *H. pylori* cell pellet (100 μl) was resuspended in 1 ml lysis buffer containing 50 mM Tris.Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF and 10 μg/ml leupeptine. The lysate was cleared by centrifugation at 12 000 × *g* and incubated with pre-immune or immune sera (anti-HpDnaB) overnight, followed by addition of protein A-sepharose beads (Sigma) for 2 h at 4°C. The beads were washed thoroughly; proteins were released by boiling in SDS-PAGE loading buffer followed by electrophoresis and identification by Western blot using anti-Hp0897 antibodies.

ATPase assay

To demonstrate ATP hydrolysis, we carried out ATPase assays using coupled pyruvate kinase/lactate dehydrogenase linked pathway as described earlier (9). To analyse the effect of Hp0897 on the ATPase activity of HpDnaB, we used a constant amount of HpDnaB (1.04 μM) and increasing concentrations of Hp0897 (mentioned in figure) in the presence of 2 mM ATP. The monomeric forms of the proteins (HpDnaB and Hp0897) were considered for the calculation of proteins concentration.

Helicase assay

To show the effect of Hp0897 on the unwinding (dsDNA to ssDNA) activity of HpDnaB, we performed Helicase assay as mentioned elsewhere (8,9). Briefly, the assay was carried out in 20 μl of reaction buffer containing 20 mM Tris.Cl (pH 7.5), 8 mM DTT, 2.5 mM $MgCl_2$, 2 mM ATP, 80 μg/ml BSA, 10 mM KCl, 4% (w/v) sucrose, 5 femto mole labelled helicase substrate and different amount of proteins in different combinations as shown in respective figures.

Electrophoretic mobility shift assay (EMSA)

To explore the DNA–protein or DNA–protein–protein interaction, we performed electrophoretic mobility shift assay (EMSA) as described elsewhere (10). EMSA was performed with ssDNA-1 (Supplementary Table S2, primer no. 11) and dsDNA probes. The 70-bp PCR product (Supplementary Table S2, primer no. 1 and 3) from *HpDnaB* gene was used to make a dsDNA probe similar to the ssDNA probe. For forked DNA probes, initially we labelled the 70-nt ssDNA-1 (Supplementary Table S2, primer no. 11) similar to the ssDNA probe and annealed it with the 70-nt complementary ssDNA-2 (30-nt complementary to ssDNA-1) (Supplementary Table S2, primer no. 12). The annealed substrate was resolved on 10% Native Polyacrylamide Gel followed by elution in 1× TE (10 mM Tris, 1 mM EDTA, pH 8.0). The eluted substrate was precipitated as described elsewhere (10).

Agarose retardation assay

The ssDNA binding activities of Hp0897 and HpDnaB proteins alone and the two together were analysed by Agarose Gel Electrophoresis using M13mp18 ssDNA as a substrate which was described previously (37). The stimulation of ssDNA binding activity of HpDnaB in the presence of Hp0897 was examined using agarose gel retardation assay. M13mp18 ssDNA (∼400 ng or 0.042 μM) was incubated for 1 h at 4°C with constant amount of HpDnaB (0.45 μM) and various concentration of Hp0897 (0, 0.7, 1.4 and 2.1 μM) in DNA binding buffer (20 mM Tris.Cl (pH 8.0), 1 mM $MgCl_2$, 100 mM KCl, 8 mM DTT, 4% sucrose and 80 μg/ml BSA) in a 20-μl reaction mixture. The reaction products were resolved on 1% agarose gel after adding DNA loading dye.

RESULTS

Hp0897 is conserved in *H. pylori* strains

The ORF *Hp0897* codes for a ∼25 kDa basic protein (pI 10.76). Blast analysis of amino acid sequence of Hp0897 revealed the presence of this ORF in the *H. pylori* strains sequenced so far. However, no homologue of Hp0897 was found in other bacterial strains including *E. coli* and *B. subtilis*. Furthermore, ClustalW2.0 analysis shows high degree of sequence identity and similarity among *H. pylori* strains that have been sequenced (Supplementary Figure S1). PCR amplification using specific primers against Hp0897 and genomic DNA isolated from various *H. pylori* strains (including many Indian *Hp* isolates) showed the presence of counterparts of Hp0897 in the tested strains (Figure 1). It is interesting that Hp0897 does not share sequence homology with helicase loaders from different sources [*A. aeolicus* DnaC, *T4 bacteriophage* (T4 gp59), *E. coli* DnaC, *B. subtilis* DnaI] but shows similar molecular mass and pI value as other helicase loaders (Supplementary Table S1). These observations suggest that Hp0897 is conserved among *Hp* strains and it may serve as a helper in helicase loading for *H. pylori.*

Hp0897 exists as multimers in solution and it is expressed in *H. pylori*

A 627-bp fragment, representing the *Hp0897* gene from *H. pylori 26695* strain, was PCR amplified and cloned into the plasmid pET28a and the protein was expressed in *E. coli* BL21 (DE3) cells as described in 'Materials and Methods' section. Induction of the bacterial culture with IPTG showed expression of the recombinant protein His_6-Hp0897 (∼26kDa; Figure 2A). The protein was first purified using Ni-NTA agarose beads (Qiagen) followed by size exclusion chromatography as described in 'Materials and Methods' section. The purity of the protein is shown in Figure 2A. Size-exclusion chromatography also showed the presence of trimers of His_6-Hp0897 (∼75kDa) in solution (Figure 2B and C and Supplementary Figure S2A). Glutaraldehyde crosslinking revealed oligomeric forms of the protein that included dimers (∼52 kDa) and trimers (∼75 kDa) as shown in the Supplementary Figure S2B. Altogether, these results indicate that His_6-Hp0897 is present as multimers (dimer/trimer) in solution.

In order to check the expression of Hp0897 in different *H. pylori* strains, we raised polyclonal antibodies in mice against recombinant His_6-Hp0897 protein. The specificity of the antibodies against Hp0897 and HpDnaB (11,37,38) has been tested by western blot experiments where these antibodies could recognize specific bands against recombinant proteins as well as in the *H. pylori* bacterial cell lysate at the appropriate molecular mass regions (Supplementary Figure S2C). Pre-immune sera against Hp0897 did not recognize any such band. Western blot analysis using bacterial cell extract from different *H. pylori* strains (*Hp26695, I-10, B-28, San74* and *217-1A*) showed the expression of Hp0897 (∼26 kDa) in the *Hp* strains examined in this study, however, the levels of expression were different in different *Hp* strains (Figure 2D). The expression of replicative helicase HpDnaB as well as that of HpCagT (loading control) (39)

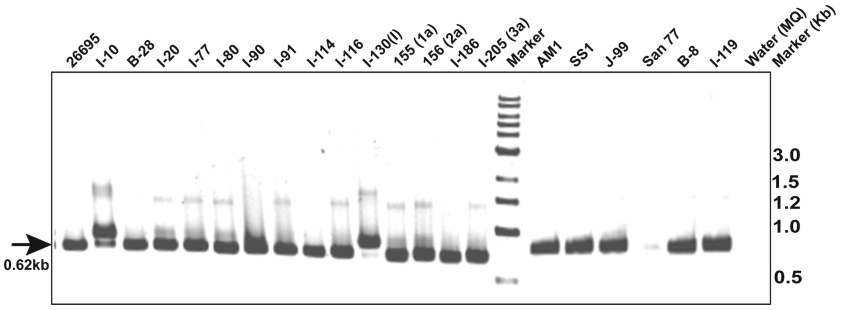

Figure 1. PCR amplification of *Hp0897* gene from genomic DNA isolated from different *Helicobacter pylori* isolates. PCR reactions were performed using specific primers (Supplementary Table S2; primer 4 and 5) and genomic DNA isolated from various *H. pylori* strains as indicated on the top of the figure. Except *H. pylori* strain 26695, all the strains were received from Dr A. Mukhopadhyay (NICED, Kolkata, India) out of which (except J99 and SS1), majority were Indian isolates. Water (MQ) was used as a negative control. The size of the corresponding bands present in DNA ladder (marker) is indicated on the right.

were checked using respective antibodies confirming that Hp0897 is expressed in *H. pylori* strains.

The expression of Hp0897 in different strains of *H. pylori* as shown above may suggest its important function in the bacteria. Further, to confirm the essentiality of *Hp0897*, we have made several attempts to knock out *Hp0897* from the bacterial chromosome without much success. While we got knock out *H. pylori* strains for non-essential genes like *HpcagN* and *Hpcagδ* (39,40), we never got knock out strain for *Hp0897* under the same experimental conditions (Supplementary Figure S3) suggesting that Hp0897 might be essential for viability and growth.

Direct interaction between purified HpDnaB and Hp0897 proteins

Hp0897 and HpDnaB were reported to interact with each other from a yeast two-hybrid map of protein–protein interactions in *H. pylori* (34). In order to biochemically validate the yeast two-hybrid data, we performed GST pull down assay using GST-HpDnaB fusion or GST alone bound to glutathione beads and purified His_6-Hp0897. The pull down results showed that His_6-Hp0897 bound to GST-HpDnaB specifically under our experimental conditions as compared to control GST alone (Figure 3A). We also performed GST pull down assay using GST-Hp0897 fusion or GST alone bound to glutathione beads and purified His_6-HpDnaB. The reverse pull down results also confirmed the interaction of His_6-HpDnaB with GST-Hp0897 as compared to control GST alone (data not shown). Ni-NTA pull-down assay in which His_6-Hp0897 was kept on Ni-NTA beads also validated the specific interaction between Hp0897 and HpDnaB as compared to GST-EcDnaB or GST alone (Figure 3B). These results strongly support the direct interaction of Hp0897 with HpDnaB but not with EcDnaB. We also subjected both proteins individually and in a mixture (∼1:1 molar ratio) to high resolution analytical 120 ml Gel Filtration Column (Hi-Load16/60 Superdex 200 prep grade) and the elution patterns of Hp0897 and HpDnaB alone or the mixture were followed. The mixture of HpDnaB and Hp0897 showed an extra peak towards higher molecular mass region apart from the peaks corresponding to the individual proteins (Supplementary Figure S4A) suggesting that this additional peak corresponds to the complex of HpDnaB and Hp0897. It was further confirmed by coomassie stained gel for the corresponding peak fractions that showed the presence of both the proteins (Supplementary Figure S4B). The presence of both the proteins in these fractions was also validated by western blot experiment using antibodies against His_6-tag (data not shown). The protein complex separated by gel filtration chromatography was further tested for its enzymatic activity, e.g. helicase as well as ATPase activity (Supplementary Figure S4C and D). The results indicate that the complex retains its activity following gel filtration chromatography. Furthermore, co-immunoprecipitation assay using *H. pylori* lysate indicated a clear band of Hp0897 in the lane corresponding to immune serum but not in that for pre-immune serum (Figure 3C). These results show that HpDnaB and Hp0897 interact with each other both *in vivo* and *in vitro*.

Hp0897 is associated with replisome complex

Replicative helicases form polar foci during early stages of chromosome replication in *H. pylori* cells (37,38) suggesting polar replisome assembly in *H. pylori*. In order to find out whether Hp0897 is a part of the replisome, we performed immunolocalization of Hp0897 alone or in combination with HpDnaB in *H. pylori cells*. Immunofluorescence efficiency is known to vary from experiment to experiment and we found that on average ∼20% (540 out of 2756 counted) of single nucleoid cells showed distinct foci of Hp0897 (Table 1). Majority of these 540 single nucleoid cells showed polar localization of Hp0897 (∼94%; 506 cells) as opposed to non-polar localization (∼6%; 34 cells) (Table 1 and Figure 4B). Pre-immune sera under the same experimental conditions did not show any distinct signal (Figure 4A). In order to investigate whether Hp0897 is a part of replisome, we further analysed the colocalization pattern of Hp0897 and replicative helicase HpDnaB. Approximately 4% cells (294 cells out of total 7360 cells) had both HpDnaB and Hp0897 foci in the same cell (Table 2). Among these 294 cells, ∼65% (191 cells out of 294 cells) showed colocalization of Hp0897 with HpDnaB whereas ∼35% cells (103 cells out of 294 cells) did not show colocalization (Table 2 and Figure 4C and D; Supplementary Figure S5). Out of the 191 cells with colocalized Hp0897 and HpDnaB foci, majority of cells showed polar localization (∼92%; 176 cells)

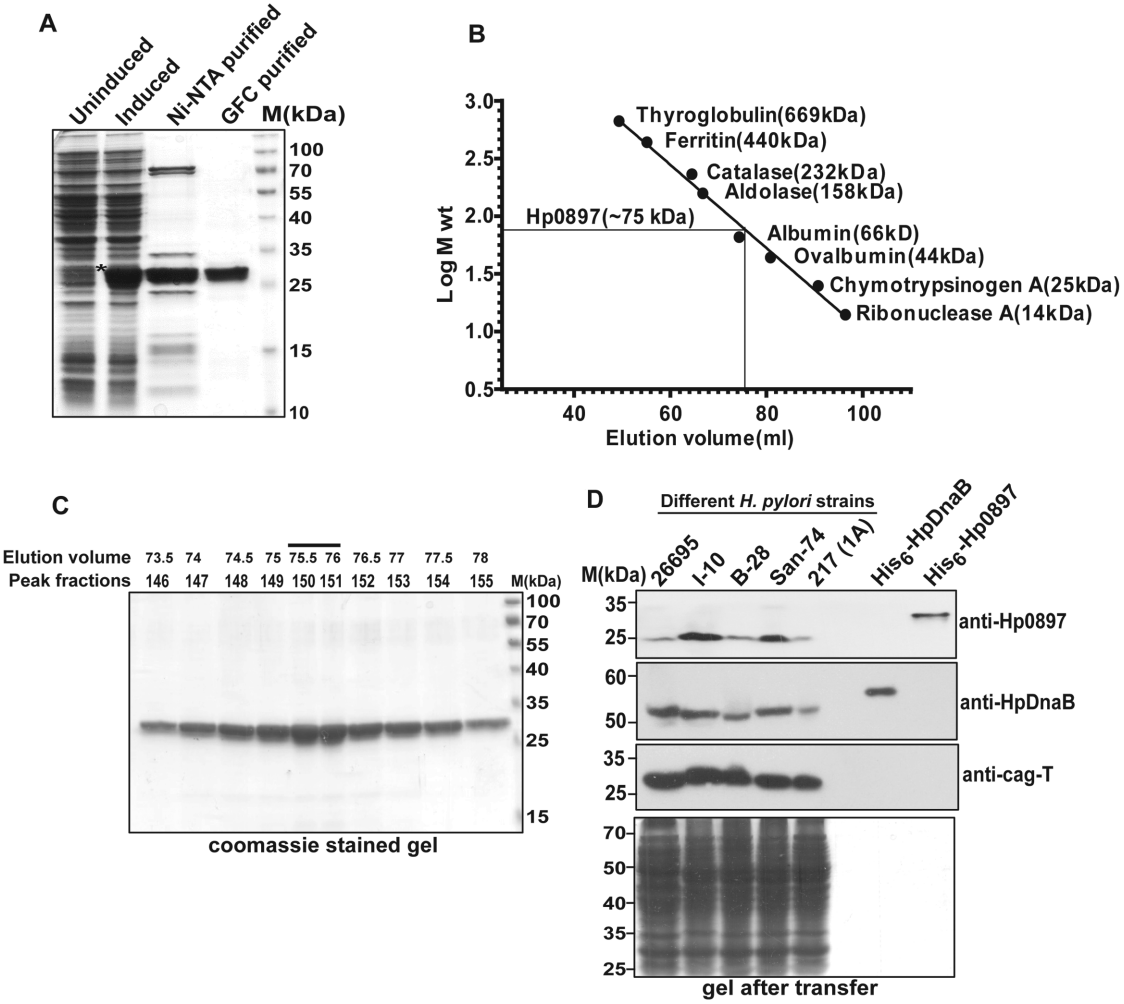

Figure 2. Purification of His_6-Hp0897, its oligomerization status and expression pattern in different *Helicobacter pylori* strains. (**A**) The coomassie stained gel shows the expression and purification of His_6-Hp0897. The uninduced and induced lanes correspond to lysate obtained from bacterial culture grown in the absence and presence of IPTG. The protein was purified using Ni-NTA column followed by gel filtration chromatography (GFC). The Ni-NTA purified (∼5 μg) and GFC purified (∼3 μg) proteins were loaded in SDS-PAGE followed by coomassies staining. The purity of the gel filtration chromatography purified protein was >95%. The molecular mass markers are shown on the right. (**B**) Size-Exclusion Chromatography of His_6-Hp0897 protein. His_6-Hp0897 protein, along with the marker proteins, were subjected to Hi-Load16/60 Superdex 200 prep grade 120 ml gel filtration column (GE Healthcare) followed by elution of the protein. Calibration of the column was done by molecular mass standards and plotting a standard graph using log of molecular mass (standards) against corresponding retention volume. Molecular mass of His_6-Hp0897 was deduced (∼75 kDa) from the plot suggesting its oligomeric nature. (**C**) SDS-PAGE analysis. SDS-PAGE analysis of peak fractions following Size Exclusion Chromatography of His_6-Hp0897 protein. (**D**) *In vivo* expression of Hp0897 in different *H. pylori* strains using antibodies against Hp0897. The western blot experiment shows expression of Hp0897 in different *H. pylori* strains. Antibodies against HpDnaB were used as a positive control for other replication protein and antibodies against HpCagT were used as loading control. Lower panel shows coomassie stained gel after transfer.

whereas only ∼8% (15 cells) showed midcell foci. These results further corroborated the possibility of Hp0897 as a part of replisome complex.

The shift of the HpDnaB signal from the polar region towards the middle of the cell has been interpreted to suggest that the replication machinery was assembled near the pole at initiation but then moved towards the mid-cell during elongation (38). The majority of colocalization (∼92%; 176 cells out of 191) of HpDnaB and Hp0897 were seen near the pole (Table 2 and Figure 4C) and non-polar colocalized foci of Hp0897 and HpDnaB were rare (<1%) suggesting the association of Hp0897 with replisome to occur predominantly at replication initiation. Out of non-colocalized 103 cells (∼35% of cells having both signal), a significant number of cells (∼58%; 60 cells) showed polar localization of Hp0897 signal but HpDnaB showed different localization (either polar or towards middle or at the middle; Table 2 and Figure 4D; also see Supplementary Figure S5 I, II and III) suggesting that Hp0897 and HpDnaB foci do not move together from pole proximal to the pole-distal middle region of the cell. Among the non-colocalized cells (103 cells), some (∼42%; 43 cells out of 103 non-colocalized cells) cells showed Hp0897 and HpDnaB signals at both the poles. Significance of such localization is not clear at present.

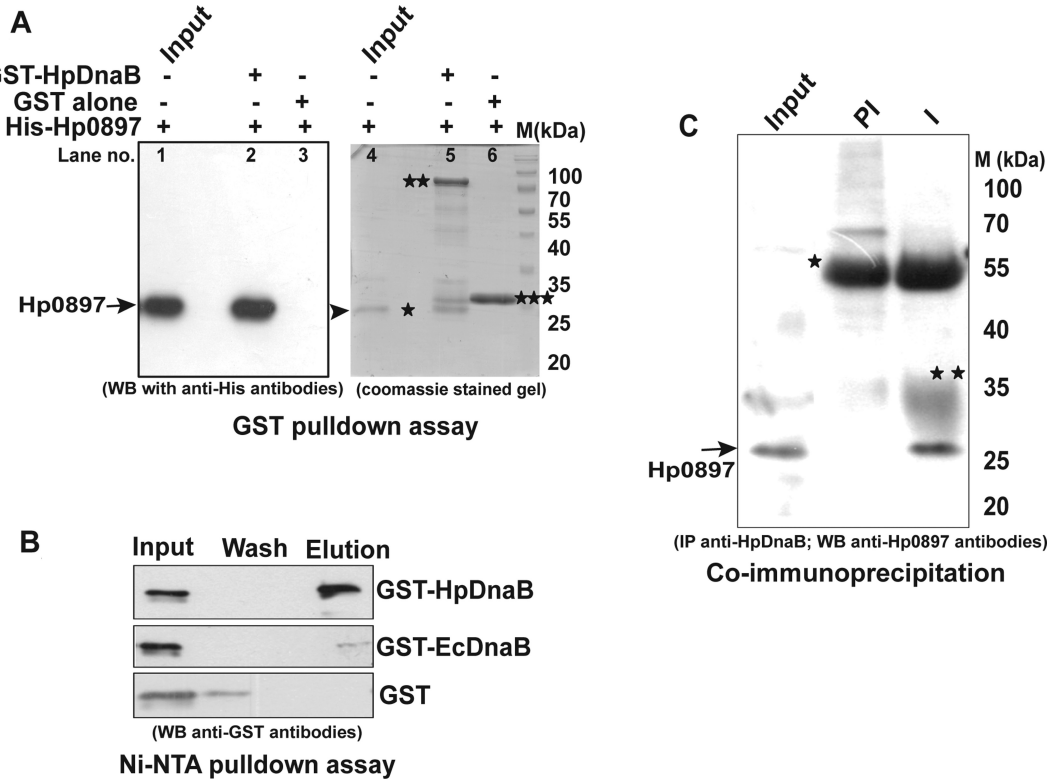

Figure 3. HpDnaB and Hp0897 interaction. (**A**) GST Pull-down assay. GST-agarose bead bound GST-HpDnaB or GST proteins were incubated with purified His_6-Hp0897. After wash, the bead bound proteins (GST-HpDnaB; lanes 2 and 5 and GST; lanes 3 and 6) were released by boiling with SDS-PAGE loading buffer. One set of experiment was subjected to western blot analysis using anti-His antibodies (left panel). To see the interaction in coomassie gel, the released proteins were also subjected to SDS-PAGE followed by coomassie staining (right panel). Lanes 1 and 4 contain His_6-Hp0897 protein as input (5%). In coomassie stained gel, '*' indicates the presence of His-Hp0897 (in the input lane as well as in GST-HpDnaB pull down lane 5), '**' indicates GST-HpDnaB and '***' shows GST alone protein. Hp0897 was present both in the input (lanes 1 and 4) as well as in the pull down samples (lanes 2 and 5). (**B**) Ni-NTA Pull down assay. His_6-Hp0897 bound to the Ni-NTA bead was incubated with purified GST tagged proteins (GST-HpDnaB, GST-EcDnaB) or GST protein alone. After incubation, bead bound proteins were eluted and subjected to western blot analysis using antibodies against GST. Input lane (5%) contains GST-tagged or GST alone proteins. The results indicate interaction between HpDnaB and Hp0897 proteins. (**C**) Co-immunoprecipitation assay. *Helicobacter pylori* cell lysate was used for immunoprecipitation experiment using pre-immune (PI) or immune (I) sera against HpDnaB followed by western blot analysis using antibodies against Hp0897. Molecular mass markers are shown on the right. The results show specific band corresponding to Hp0897 (indicated by arrowhead) in immune lane (I) but not in pre-immune lane (PI). '*' and '**' indicate the heavy chain and light chain of the immunoglobulins respectively. The input (5%) is also shown.

Table 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)**

Hp0897 modulates the ATPase and helicase activity of HpDnaB

Establishment of *in vitro*, as well as, *in vivo* interaction of Hp0897 with HpDnaB raises the issue of its functional relevance. We examined whether the ATPase and DNA unwinding activities of HpDnaB were influenced by Hp0897. The ATPase assay was performed using a constant amount of HpDnaB and increasing concentrations of Hp0897 in the presence of 2mM ATP. Initially, at lower concentration of Hp0897, the ATPase activity of HpDnaB increased marginally but at higher concentration, Hp0897 inhibited the ATPase activity (Figure 5A) in analogy with the effect of EcDnaC on the ATPase activity of EcDnaB (41). Hp0897 alone did not show any significant ATPase activity under our experimental conditions (Figure 5A). Neither, the ATPase activity of PfGyraseB (a type II topoi-

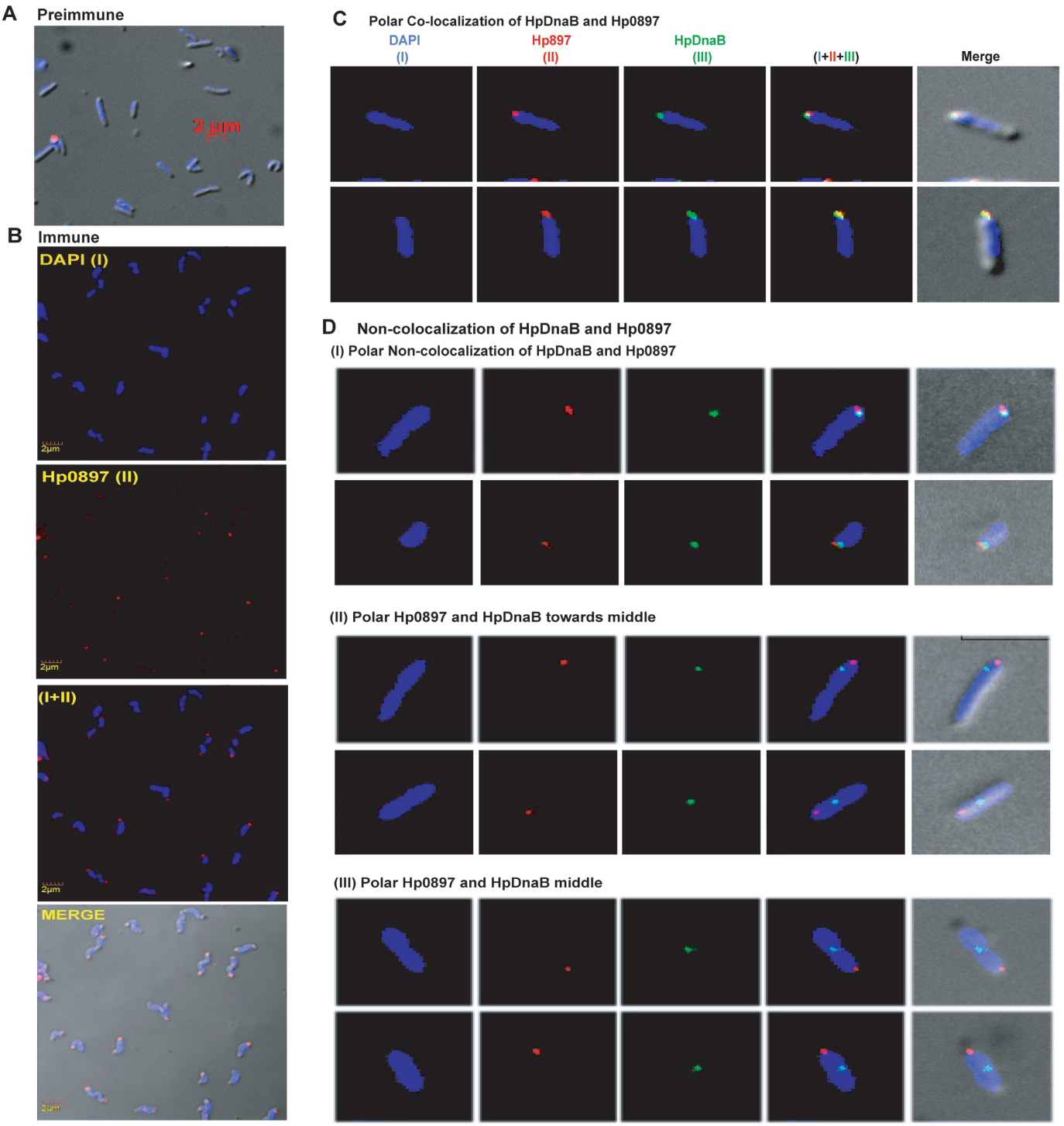

Figure 4. Localization of Hp0897 and Colocalization with HpDnaB in *Helicobacter pylori*. (**A** and **B**) Immunolocalization of Hp0897 protein in *H. pylori B28* strain. Glass slides containing *H. pylori* cells were treated with pre-immune sera (A) or antibodies against Hp0897 (**B**) followed by Alexafluor 594 (red fluorescence) conjugated secondary antibodies as described in 'Materials and Methods' section. Panel B shows polar localization of Hp0897 in *H. pylori* cells. No signal was obtained in pre-immune sera treated *H. pylori* cells. DAPI shows the nucleoid staining. (**C**) Co-immunocolocalization of HpDnaB and Hp0897 proteins in *H. pylori* cells. *H. pylori* cells were treated with rabbit anti-HpDnaB (1:1000 dilutions) and mice anti-Hp0897 antibodies (1:500 dilutions). Alexafluor 488 conjugated anti-rabbit (green) and Alexafluor 594 conjugated anti-mice (red) antibodies were used as secondary antibodies (1:1000 dilution). Merge panels show images containing signals for DAPI, Hp0897, HpDnaB as well as phase. The results indicate colocalization of HpDnaB and Hp0897 at the pole proximal region. (**D**) 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.

Table 2. Colocalization pattern of Hp0897 and HpDnaB foci

Total number of cells observed: 7360			
Total number of single nucleoid cells having distinct HpDnaB + Hp0897 signal: ~4 % (294 cells)			
Total cells with co-localised signals: ~65% of 294 cells =191 cells		Total cells with non co-localised signals: ~35% of 294 cells =103 cells)	
Polar co-localised : ~92% (176 cells)	Middle co-localised: ~8% (15 cells)	*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)	Hp0897 and HpDnaB at opposite pole: ~42% (43 cells)

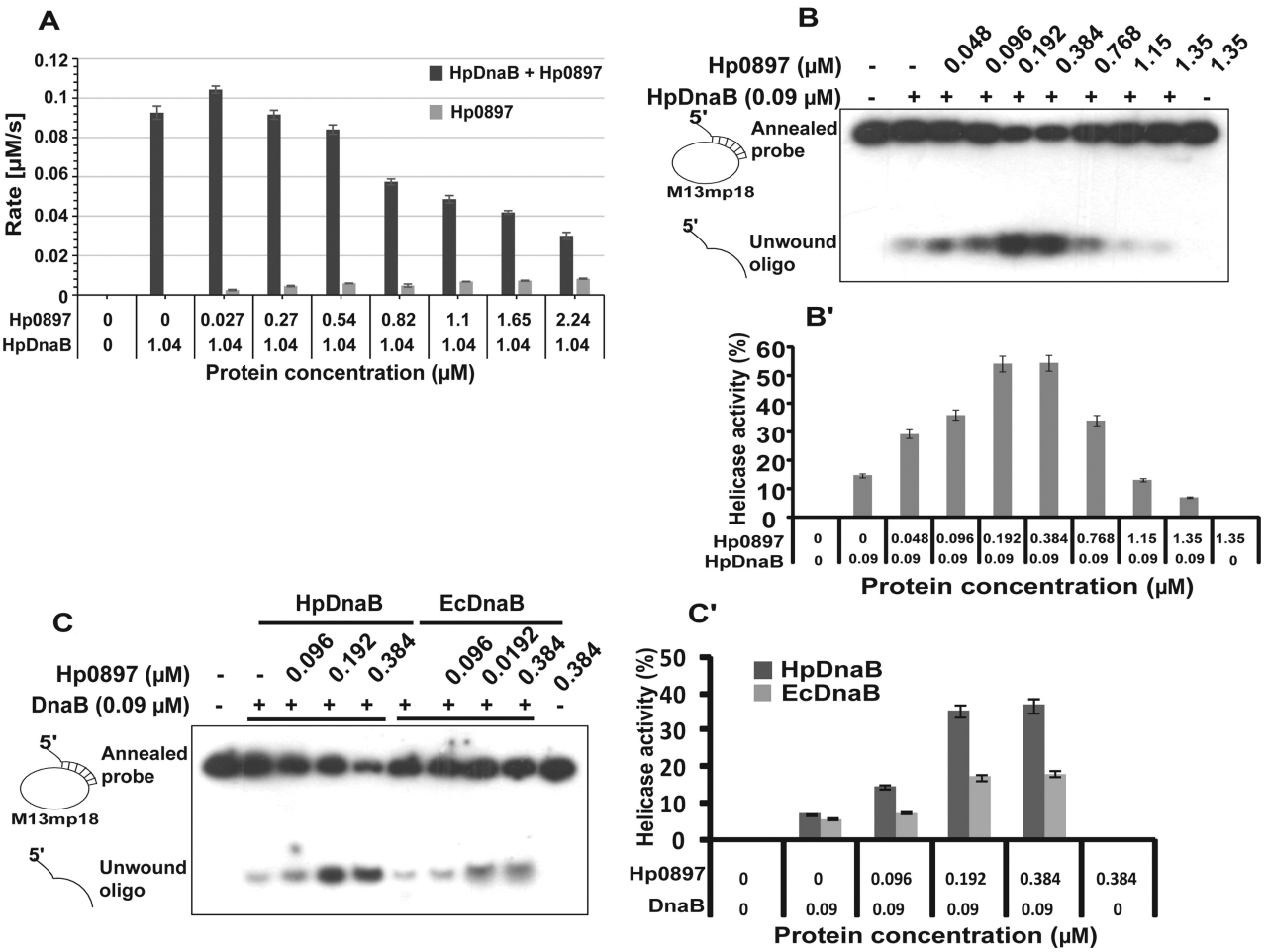

Figure 5. Modulation of HpDnaB helicase and ATPase activity in the presence of Hp0897. (**A**) The ATP hydrolysis rate (2 mM ATP) was measured in a reaction volume of 70 μl containing 1.04 μM of HpDnaB with increasing concentration of Hp0897 as shown in the figure. ATP hydrolysis rates were plotted against protein concentration. Similarly ATP hydrolysis rate was also measured for Hp0897 alone under the same experimental conditions. ATPase assays were performed in triplicates. Error bars show standard deviation of the mean (Mean ± SD). (**B**) Helicase assays were carried out with the constant amount of DnaB helicase and various amounts of Hp0897 as shown in the figure. The displacement of the synthetic 29-mer from complex of 29mer-M13mp18 by the helicase with various concentration of Hp0897 are shown. (**C**) Comparison of the helicase activity of HpDnaB and EcDnaB with increasing amount of Hp0897. (**B'** and **C'**) show the quantitative analysis of the (B) and (C) respectively. Helicase assays were performed three to four times and similar results were obtained. Panels B and C are the representative images of those experiments. The intensity of the bands for each image was quantified in triplicates by ImagesJ software. Error bars showed in panels B' and C' are standard deviation from the mean (Mean ± SD).

somerase from *Plasmodium falciparum* which has intrinsic ATPase activity) (42) was modulated by Hp0897 (Supplementary Figure S6A) under the same experimental conditions suggesting that the modulation of HpDnaB ATPase activity by Hp0897 was species specific. One of the important hallmarks of the replicative helicases is their DNA-dependent ATPase activity that is the basis of their helicase function. We have reported earlier that the ATPase activity of HpDnaB can be stimulated several folds in the presence of ssDNA (9). Hp0897 inhibited the ATPase activity of HpDnaB both in the absence or presence of DNA (Supplementary Figure S6B and C). However, the inhibition of HpDnaB ATPase activity is significantly higher in the presence (∼40%) than in the absence (∼10%) of DNA.

We examined the effect of Hp0897 on DNA unwinding activity of HpDnaB (0.09 μM) by monitoring the release of a radiolabelled 29-nt ssDNA oligomer annealed to M13mp18 ssDNA in the presence of different amounts of Hp0897 as shown in Figure 5B. We found that Hp0897 stimulated the helicase activity of HpDnaB at lower concentrations. However, at a higher concentration of Hp0897, the helicase activity was inhibited significantly (Figure 5B). It is worth mentioning here that *Ec*DnaC has a similar effect on *Ec*DnaB helicase activity (41). Furthermore, we investigated whether the stimulation of helicase activity in the presence of Hp0897 was specific for HpDnaB only. We find that the stimulation of helicase activity of HpDnaB is much higher (7–8-fold) in the presence of Hp0897 as compared to the marginal stimulation of EcDnaB helicase activity (<3-fold) in the presence of Hp0897 under similar experimental conditions (Figure 5C) suggesting the specificity of Hp0897 for HpDnaB helicase. Thus, Hp0897 modulates the enzymatic activities of HpDnaB and that such modulations are specific for *H. pylori*.

DNA binding affinity of HpDnaB increases in the presence of Hp0897

HpDnaB shows weak DNA binding activity by itself (10). The loading of replicative helicases is usually assisted by proteins with helicase loader activity like *E. coli* DnaC or *B. subtilis* DnaI. We hypothesized that Hp0897 might help recruitment of HpDnaB to DNA by modulating the affinity of the protein complex for DNA. In order to examine the effect of Hp0897 on DNA binding activity of HpDnaB, we performed EMSA and agarose gel retardation assay for HpDnaB in the absence or presence of increasing amounts of Hp0897. We have used ∼70-nt radio-labelled ssDNA, ∼70-nt radio-labelled forked DNA as well as ∼70-nt ds-DNA as probes for the EMSA experiment. The results indicate that the DNA binding activity of HpDnaB was robustly stimulated in the presence of Hp0897. With increasing concentration of Hp0897, supershifted bands appeared with higher mobility shifts (Figure 6A). This stimulation of DNA binding activity was a combined effect of HpDnaB and Hp0897 as Hp0897 alone showed lower migrating DNA–protein complex under same experimental conditions (see Supplementary Figure S6D). Again, the stimulation of ssDNA binding activity by Hp0897 was specific for HpDnaB, as the ssDNA binding activity of EcDnaB was not modulated at all in the presence of Hp0897 under the same experimental condition (Figure 6A). The DNA binding activity of HpDnaB was stimulated by Hp0897 for forked structure DNA and dsDNA as well (Figure 6B and C).

Stimulation of the ssDNA binding activity of HpDnaB by Hp0897 was further validated by agarose gel retardation assay using M13mp18 ssDNA as a substrate. M13mp18 ssDNA (∼400 ng or 0.042 μM) was incubated with constant amount of HpDnaB (0.45 μM) and various concentration of Hp0897 as indicated in Figure 6D. The results show that the band corresponding to the M13 ssDNA was not retarded in the presence of HpDnaB alone but with increasing concentration of Hp0897, it was retarded significantly indicating that HpDnaB–Hp0897 complex had higher affinity towards ssDNA than HpDnaB alone (Figure 6D). Hp0897 alone also showed ssDNA binding activity but the retardation of the corresponding band is not that prominent compared to the band corresponding to the lane containing both HpDnaB and Hp0897 (Figure 6D). The DNA binding activity of Hp0897 was further characterized. Hp0897 showed ssDNA binding activity that could be supershifted using specific antibodies against Hp0897 but not using pre-immune sera (Supplementary Figure S6D-II). However, we could not find any significant dsDNA binding activity of Hp0897 under our experimental conditions (Supplementary Figure S6D-III). These results suggest that although both Hp0897 and HpDnaB individually show moderate DNA binding activity, complex of HpDnaB and Hp0897 shows more robust DNA binding activity that may be attributed to the loading of HpDnaB onto DNA with the help of Hp0897.

However, the results as shown above (Figure 6) do not give direct evidence whether loading of HpDnaB on DNA is enhanced in the presence of Hp0897. In order to address this issue, we have checked the binding of these two proteins to biotinylated DNA (followed by pull down by streptavidin beads) separately as well as for the complex under the same experimental conditions. We find more HpDnaB binding to DNA in the presence of Hp0897 compared to its binding in the absence of Hp0897 under the same experimental conditions (Supplementary Figure S7A). The loading experiments were repeated few times. The increased loading of HpDnaB in the presence of Hp0897 was quantified and demonstrated accordingly (Supplementary Figure S7B) suggesting ∼40% more loading of HpDnaB. These results (Figure 6 and Supplementary Figure S7) together suggest that the loading of HpDnaB is indeed enhanced in the presence of Hp0897.

Pre-complex formation of HpDnaB–Hp0897 enhances the overall DNA binding activity

Above results show that Hp0897 enhances the DNA binding activity of HpDnaB. However, it is not clear whether HpDnaB or Hp0897 binds to ssDNA separately or a pre-complex formation of HpDnaB and Hp0897 is required to enhance DNA binding affinity of the protein complex (Figure 7A). In order to resolve this issue, we performed EMSA by adding the ssDNA probe to the proteins (HpDnaB and Hp0897) after pre-incubating them for different time periods (short or long pre-incubation) and then loading the re-

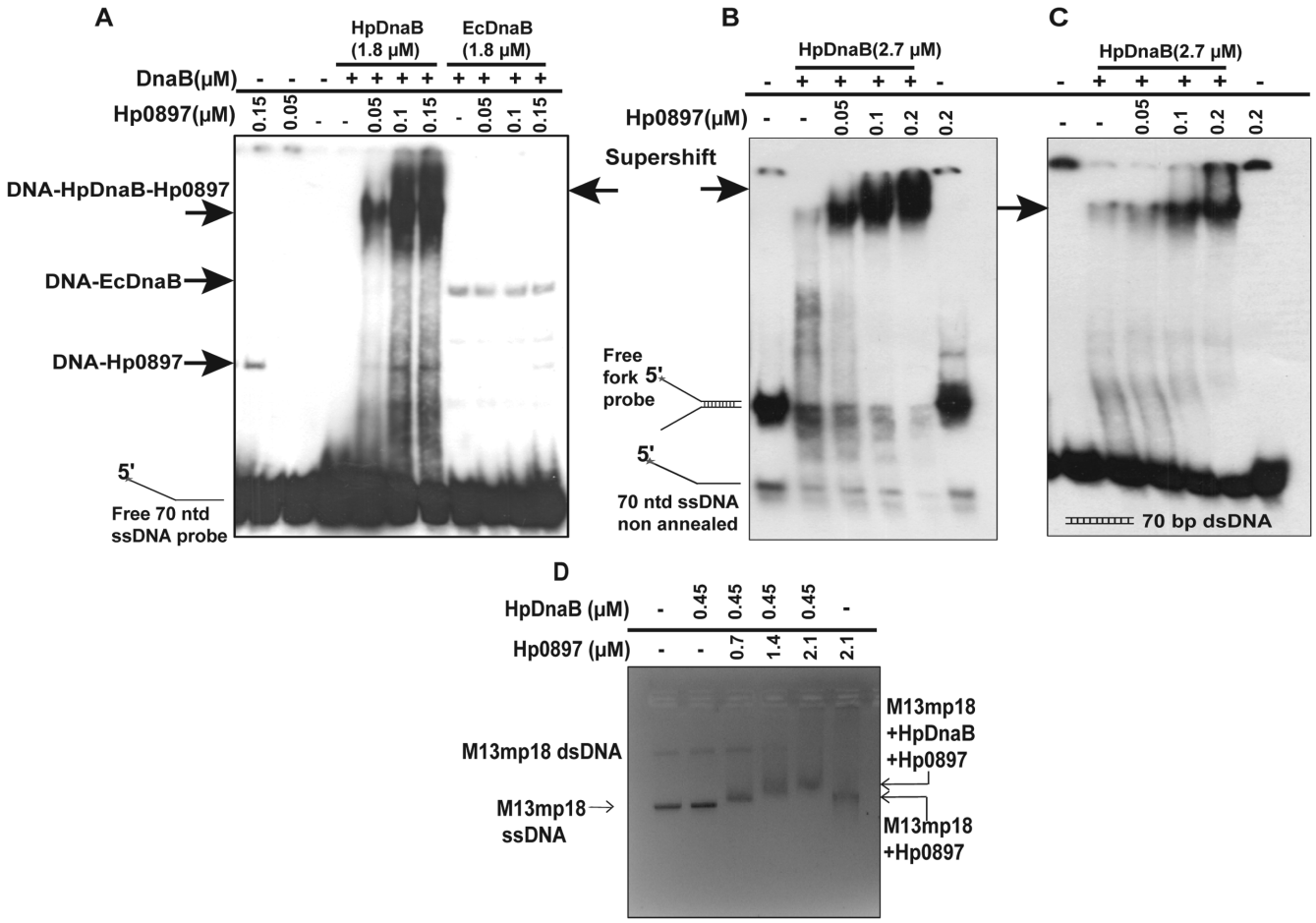

Figure 6. Hp0897 stimulates the DNA binding property of HpDnaB. (**A**) ^{32}P-labelled 70-nt long ssDNA was incubated for 1 h at 4°C with constant amount (1.8 μM each) of HpDnaB or EcDnaB and various concentrations of Hp0897 as indicated in the figure. The position of DNA–protein complex and super-shifted bands are shown by arrowheads. (**B**) Stimulation in the fork structure DNA binding property of HpDnaB (2.7 μM) in the presence of different concentration of Hp0897 as shown in the figure. ^{32}P-labelled 70-nt long ssDNA was annealed with 70-nt ssDNA that created a fork structure with 30 nt complementary region. (**C**) Stimulation of dsDNA binding property of HpDnaB (2.7 μM) in the presence of different concentration of Hp0897 as shown in figure. ^{32}P-labelled 70-bp long dsDNA probe was used for this purpose. (**D**) M13mp18 ssDNA (400 ng) was incubated with constant amount of HpDnaB (0.45 μM) and various amount of Hp0897 as indicated in figure. The band corresponding to the M13 ssDNA was retarded significantly in the presence of HpDnaB and Hp0897 suggesting that HpDnaB–Hp0897 complex shows higher affinity towards ssDNA. The bands corresponding to DNA complexed with Hp0897 and HpDnaB are also shown by arrowheads.

action mixture onto PAGE for separation of DNA–protein complex from the unbound DNA.

Incubation of the proteins together for a longer time (∼30 min) stimulated the DNA binding activity and more complex was formed than after a short incubation period (∼5 min) (Figure 7B and C). These results suggest that pre-complex formation between Hp0897 and HpDnaB was required for the enhancement of ssDNA binding activity. In order to validate the above experiment further, we pre-incubated Hp0897 in the presence of pre-immune or immune sera against Hp0897 followed by addition of HpDnaB in the mixture for complex formation. Pre-incubation of recombinant Hp0897 protein with antibodies against Hp0897 followed by addition of HpDnaB, completely abrogated the stimulation of HpDnaB's DNA binding activity (Figure 7D). However, incubation of Hp0897 with pre-immune sera did not affect the stimulation of HpDnaB DNA binding activity. These results suggest that the prior complex formation between Hp0897 and HpDnaB is a prerequisite for the stimulation of DNA binding activity of HpDnaB.

DISCUSSION

Bacterial helicases are hexameric closed ring structures and their loading onto DNA comprises threading of a DNA single strand through the central gap. This poses a structural problem common for all species and there are some common aspects shared by the process of helicase loading, although the details of the acquisition of helicase function by the replisome may differ (see the reviews: 33,43). The mechanisms of helicase loading at replication origins are best understood in model bacteria *E. coli* and *B. subtilis*; *Saccharomyces cerevisae* and *Xenopus laevis* are the eukaryotes with somewhat detailed understanding of the events of helicase loading. Since *H. pylori* is a gram-negative bacterium, the model system *E. coli* would be used for comparison. As mentioned above and in earlier works, the major differ-

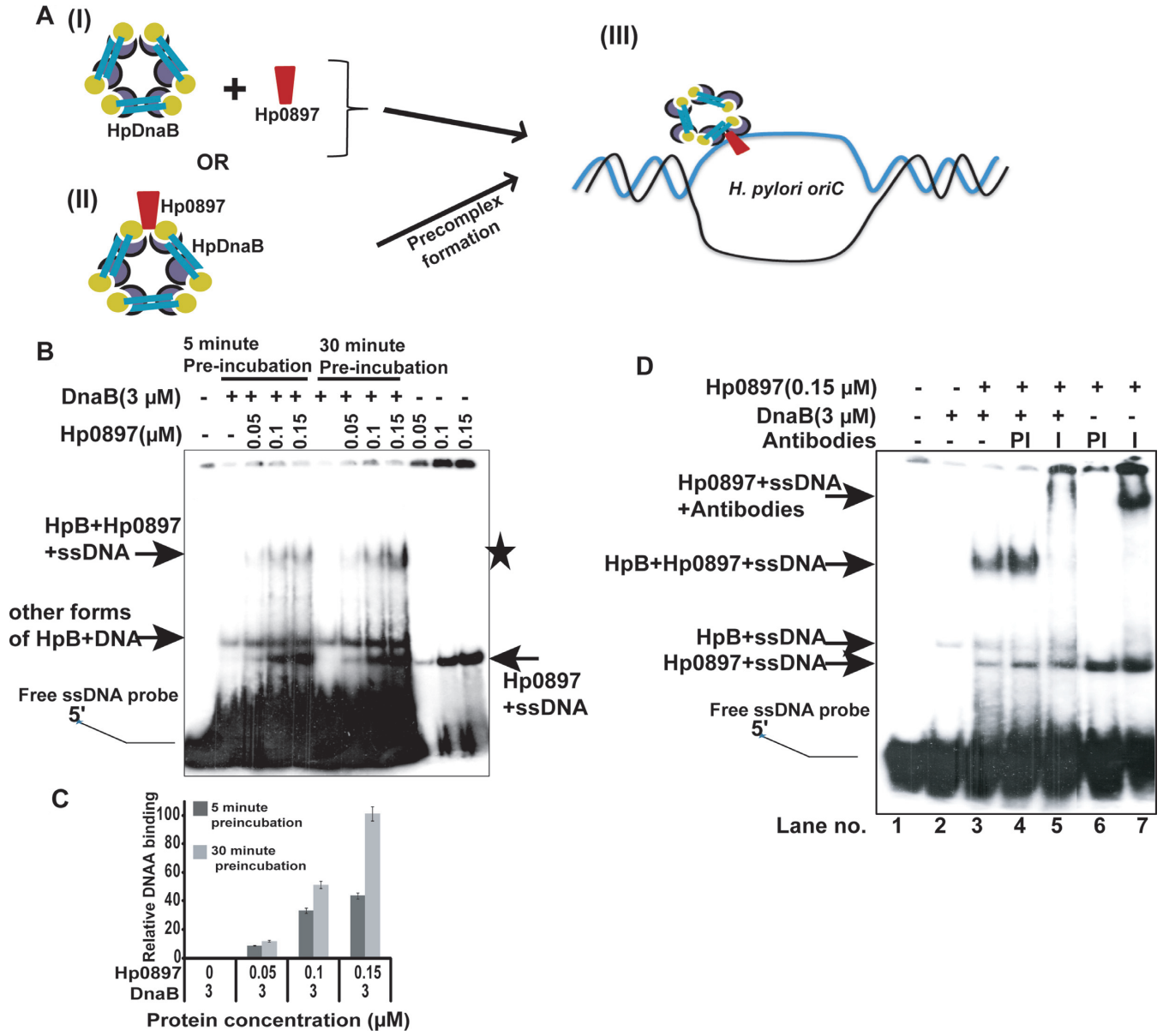

Figure 7. Pre-complex formation of HpDnaB–Hp0897 enhances the DNA binding activity. (**A**) Schematic representation of possible way of interacting with DNA. Whether these proteins individually bind to DNA (I) or pre-complex formation (II) takes place prior to DNA binding. (III) After loading and encircling the DNA, the Hp0897 disociates following weak DNA binding of HpDnaB that may be responsible for helicase translocation. (**B**) Both the proteins (HpDnaB and Hp0897) were incubated for different time periods (5 and 30 min) using different amount as shown in the figure. After incubation, ^{32}P-labelled 70-nt long ssDNA probes were added and immediately loaded on 5% native gel. The protein complex obtained from longer incubation period shows higher ssDNA binding activity (upper band corresponds to hexameric form of HpDnaB; marked with asterick) compared to protein complex obtained from shorter incubation period. The bands corresponding to Hp0897 binding to ssDNA as well as other oligomeric forms of HpDnaB (monomer/dimer) binding to ssDNA are also indicated. (**C**) Shows the quantitative analysis of the upper DNA–protein complex mark by star (*) of (**B**). The intensity of the relevant bands was quantified in triplicates by ImagesJ software. Error bars indicate standard deviation from the mean (Mean ± SD). There are few lower migrating DNA–protein complex which may represent lower oligomeric forms of HpDnaB binding to DNA. (**D**) EMSA in the presence of anti-Hp0897 antibodies against Hp0897 affects the ternary complex (ssDNA–HpDnaB–Hp0897). The formation of the ternary complex is affected in the presence of antibodies against Hp0897 (lane 5) but not in the presence of pre-immune sera (lane 4). The specificity of antibodies against Hp0897 is further shown by supershift of the binary complex (ssDNA–Hp0897) in the presence of immune sera (lane 7) but not in the presence of pre-immune sera (lane 6).

ence in the processes of helicase loading between *E. coli* and *H. pylori* is the absence of any obvious DnaC-like AAA^+ protein to direct helicase loading at the *HporiC*. However, absence of specialized helicase loaders is not uncommon; self-loading of helicase using an accessory primase domain (bacteriophage T7 gp4 and mitochondrial helicase-primase TWINKLE) or a specialized DNA-binding domain (SV40 large T antigen and Papilloma E1 helicase) have been recently characterized (44–47). Eukaryotes and archaea have been found to form double hexameric helicase dodecamers demonstrating a completely different mechanism for DNA-loading (48–50). Among the bacteria, *Pseudomonas aeruginosa* and *putida* can load replicative helicase at the plasmid RK2 origin *oriV* using the host initiator DnaA without requiring a helicase loader (51).

Since *H. pylori* does not code for an obvious DnaC homologue essential for loading of EcDnaB at the *oriC* in *E. coli*, there has been a debate about whether HpDnaB is loaded at the *HporiC* by itself or if it needs a yet-unknown helicase loader (11). Modelling of HpDnaB by docking crystal structure onto EM-generated electron density maps revealed a dodecameric head-to-head ring hexamer with relatively large side gaps that might allow the threading of ssDNA into the central ring without the need for a ring-breaking helicase loader (23,29). Dodecameric HpDnaB with head-to-head NTD might seem inimical to the HpDnaB(NTD)-HpDnaG(CTD) interaction demonstrated earlier (52–53) but after loading, the dodecamer might split into oppositely oriented hexamers on the separated ssDNA strands with their NTD accessible for interaction with HpDnaG(CTD) (54). However, there might still be additional proteins that improve the affinity of HpDnaB for ssDNA at the unwound region near *oriC*. Here, we have functionally characterized Hp0897 protein that not only interacts with HpDnaB, but also modulates its various biochemical activities significantly, including DNA-binding, suggesting a probable role for Hp0897 as an accessory factor to the replicative helicase HpDnaB.

Hp0897 is not found in other bacterial species whereas it is present in the *Helicobacter* strains sequenced so far suggesting it is conserved in *H. pylori* strains (Figure 1 and Supplementary Figure S1) although the level of expression may differ from strain to strain (Figure 2D). Despite several attempts, we were unable to knock out *Hp0897* from the bacterial chromosome while we could delete the non-essential genes like *HpcagN* and *Hpcagδ* (39,40) under similar experimental conditions (Supplementary Figure S3). Therefore, Hp0897 might be essential for viability and growth. Hp0897 shows high pI (10.7) similar to other helicase loader proteins and its molecular weight (∼26 kDa) is in the range of other helicase loaders. The high pI or positive charge of helicase loaders may facilitate their interaction with DnaB helicase of low pI, as well as with negatively charged DNA. Electrostatic analyses of DnaB reveal that negative charges are distributed on the outer surface of the folded protein while the basic potential groove extends from the NTD to the CTD almost parallel to the central axis (55).

His_6-Hp0897 is present as multimers (dimer/trimer) in solution as shown from size-exclusion chromatography (Figure 2B and C; and see Supplementary Figure S2A) and glutaraldehyde crosslinking experiments (Supplementary Figure S2B). Oligomerization is one important feature of replicative helicases and helicase loaders as formation of oligomers often produces increased stability with improved function for the multi-enzyme complexes. Helicase loaders T4 gp59 and EcDnaC form pentamers or hexamers and ladders of multimers, respectively (17,56–57).

Pull down (Figure 3A and B) and gel filtration chromatography experiments (Supplementary Figure S4) show direct interaction between HpDnaB and Hp0897 which can modulate the enzymatic function of HpDnaB. Co-immunoprecipitation and immunofluorescence results confirm that Hp0897 interacts with HpDnaB *in vivo* (Figures 3C and 4C) suggesting important role(s) for Hp0897 in *H. pylori* DNA replication. Both ATPase and helicase activities of HpDnaB are modulated in parallel by Hp0897, an initial stimulation followed by inhibition at high concentration (Figure 5A and B). This is reminiscent of the modulation of EcDnaB activities by EcDnaC (41). Hp0897 considerably reduces the DNA-dependent ATPase activity of HpDnaB (Supplementary Figure S6B and C). The inhibitory effects of Hp0897 could be attributed to either non-specific protein–protein (Hp0897–HpDnaB) or protein–DNA (Hp0897–ssDNA) interactions. Non-specific protein binding to ssDNA could inhibit DnaB activities as has been shown for SSB in *E. coli* (58). The dual action by Hp0897 of stimulation of DnaB function at lower and inhibition at higher concentrations suggests a feedback control mechanism designed to maintain an optimal stoichiometry between the two proteins whose interactions are critical for bacterial growth and its control. A precise ratio of Hp0897 and HpDnaB might be optimal for helicase function as has been reported for EcDnaB and EcDnaC. *Ec*DnaB and EcDnaC proteins can be isolated as a tight complex in the presence of ATP (12,14–15,41) and the ratio of *Ec*DnaB to EcDnaC (1:0.8) is critical for achieving optimal activity of EcDnaB and to avoid inhibition by EcDnaC (59–60). At this point, we do not know the optimal ratio between HpDnaB and Hp0897 needed to form a stable complex. The stoichiometry of HpDnaB–Hp0897 *in vivo* remains to be estimated.

At initiation, the replisome assembles at *HporiC* near one of the cell poles and the two forks move together towards midcell with progress of elongation (38). Polar colocalization of Hp0897 with HpDnaB in majority of the mononucleoid cells (∼92%; 176 out of 191 cells; Figure 4, Table 2) suggests the presence of Hp0897 during the assembly of polar replication machinery at initiation. While the HpDnaB focus moved with the forks towards mid-cell region during elongation, Hp0897 remained at the pole in cells with non-colocalized foci of the two proteins (∼35%; 103 out of 294 cells; Figure 4, Table 2). It is established that the forks do not progress unimpeded but are often stalled, paused, causing collapse and disassembly of the replisome (61) requiring replication restart that involves re-assembly of the replisome. Replisome dissociation and helicase reloading occurs with surprising frequency in bacteria (62). Approximately 20% of *E. coli* chromosomes require DnaC-assisted helicase re-loading in order to complete their round of replication (63). The repair and restart of collapsed replication forks in *E. coli* has been worked out in some details revealing several sets of recombination-repair systems and helicase load-

ing mechanism comprising PriA-C, DnaB, DnaC, DnaT, DnaG and the Rep helicase (64–65). *H. pylori* is missing several components of the restart machinery such as PriB, PriC, DnaT and DnaC. However, the restart pathways in *E. coli* are many and redundant; PriA might be adequate for replication restart from blocked forks and recombination intermediates. The replication restart machinery for *H. pylori* needs to be worked out further. Since Hp0897 and HpDnaB foci do not move together from pole proximal to the mid-cell region (Figure 4D, Table 2 and Supplementary Figure S5-I, II and III) it is tempting to speculate that Hp0897 is needed only at initiation for loading of the helicase at *oriC* and may not be required further for continuation of helicase function for restarting the stalled replication forks. Since the restart proteins: RecO, RecG and PriA did not seem to travel with replication forks during elongation of *E. coli* chromosome (66), significance of above result is not clear.

Recent structural studies have revealed a major role of *oriC*-bound DnaA in the helicase loading at the *oriC* while DnaC binding trapped the spontaneously opened ring of DnaBCTD and allosterically modified the NTD controlling its access to the primase (6,23,43). Thus, even in *E. coli*, DnaC is rather more of a check point regulating primase binding to DnaB and DnaB activation into functional ATPase and helicase in the replisome complex than helicase loader (33). It may control the transition of the replisome from an initiation complex to elongation complex rather than a helicase loader which is the responsibility of DnaA (67). HpDnaB can complement both DnaB and DnaC activities in *E. coli in vivo*. Thus, EcDnaA recognizes HpDnaB well enough to load it at *E. coli oriC* unassisted by EcDnaC in correct orientation so that EcDnaG could bind and activate the ATPase and helicase functions without any assistance from Hp0897. The bacteria with heterologous helicase and inactivated DnaC are viable implying that replication restart might not be efficient but adequate. Further experiments are needed to clarify the role(s) of Hp0897 in initiation of chromosomal replication and its sustenance through a full round from origin to terminus in *H. pylori*.

What then, is/are the possible role(s) of Hp0897 as an accessory factor for helicase function in *H. pylori*? The experiments described in this work show that the DNA binding activity of HpDnaB is stimulated robustly in the presence of Hp0897 (Figure 6) and that this stimulation is a combined effect of HpDnaB and Hp0897 since Hp0897 alone cannot produce a super-shifted, high-molecular weight, DNA–protein complex as obtained in the presence of both proteins. The amino acid sequence of Hp0897 does not reveal any putative DNA binding motif except for the presence of some positively charged amino acid residues that need to be characterized further in order to understand their putative role(s) in DNA binding activity. Hp0897 also stimulates the DNA binding activity of HpDnaB in the presence of a fork structure DNA (Figure 6B). Interaction with forked DNA structure is more relevant as it mimics the binding of HpDnaB at the open *oriC* region *in vivo*. Furthermore, pre-complex formation of HpDnaB and Hp0897 seems necessary as pre-incubation of these two proteins enhances the affinity towards ssDNA (Figure 7B) and blocking the interaction between Hp0897 and HpDnaB using specific antibodies against Hp0897 abrogates the stimulation of HpDnaB DNA binding activity.

Identification of two DUE sites, capable of binding DnaA (*oriC1* and *oriC2* upstream and downstream of *dnaA* gene) of which only one (*oriC2*) can function as active replication origin (27,28) might provide some structural clues as to the architectural requirements of DnaA–DNA complex for proper loading of replicative helicase necessary for recruiting of replication proteins into a functional replisome. How HpDnaB is exclusively targeted towards *oriC1*/*oriC2* region in the genome still remains unknown.

Hp0897 might be a regulatory protein that facilitates the loading of HpDnaB helicase at the chromosomal origin of replication and thereby qualify as a functional homologue of EcDnaC protein. We cannot exclude the possibility of other regulatory proteins which may help Hp0897 in the loading of HpDnaB on chromosome. Finally, although overexpression of HpDnaB functionally complemented two temperature sensitive mutant strains of *E. coli* DnaC (8) raising the possibility of a loader independent function of HpDnaB at least in a heterologous system, it does not reflect the functional mode of HpDnaB in the *H. pylori* itself. Indeed, it may need a protein like Hp0897 to fulfil its *in vivo* helicase function.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We acknowledge Prof S. Gourinath, SLS, JNU, New Delhi for his constructive suggestions. We thank Dr Mohd Shariq, Ms Rajesh Kumari and Prof Gauranga Mukhopadhyay, SCMM, JNU, New Delhi for thier help to perform the knock out experiments and sharing reagents used for this work.

FUNDING

Department of Science and Technology, Government of India [EMR/2014/000405]; DST-PURSE, UGC-SAP, UP-EII (JNU); ICMR-CAR core funding; Indian Council of Medical Research (ICMR) (to V.V.); Dr D.S. Kothari Postdoctoral Fellowship, UGC, India (to V.V.); UGC, India (to A.K.).
Conflict of interest statement. None declared.

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