Helicobacter pylori DnaB helicase can bypass *Escherichia coli* DnaC function *in vivo*

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In *Escherichia coli*, DnaC is essential for loading DnaB helicase at *ori*C (the origin of chromosomal DNA replication). The question arises as to whether this model can be generalized to other species, since many eubacterial species fail to possess *dnaC* in their genomes. Previously, we have reported the characterization of HpDnaB (*Helicobacter pylori* DnaB) both *in vitro* and *in vivo*. Interestingly, *H. pylori* does not have a DnaC homologue. Using two different *E. coli dnaC* (*EcdnaC*) temperature-sensitive mutant strains, we report here the complementation of EcDnaC function by HpDnaB *in vivo*. These observations strongly suggest that HpDnaB can bypass EcDnaC activity *in vivo*.

Key words: DNA replication, helicase, loader, *ori*C (origin of chromosomal DNA replication).

INTRODUCTION

The classical model of DNA replication initiation at *ori*C (origin of chromosomal DNA replication) in *Escherichia coli* starts with the binding of bacterial replication initiator protein DnaA to the DnaA boxes, followed by unwinding of the nearby AT-rich sequences to form an open complex [1]. Subsequently, a hexameric $(DnaB–DnaC)_6$ complex is recruited at the *ori*C, with the help of the DnaA protein [2]. However, DnaB cannot be loaded at the *ori*C alone. After loading, DnaC protein is released, with the concomitant hydrolysis of ATP [3]. Finally, DnaB exerts its $5' \rightarrow 3'$ helicase activity with the help of ATP, which results in strand separation [4]. For detailed molecular mechanisms behind helicase recruitment and loading in prokaryotes, see a recent review [5].

Although DnaB is conserved in bacteria, only HpDnaB (*Helicobacter pylori* DnaB) has been characterized both *in vitro* and *in vivo* to date [6], in addition to EcDnaB (the *E. coli* DnaB helicase). Cloning and characterization of DnaB has been reported from *Thermus aquaticus* [7], *Pseudomonas putida* and *Pseudomonas aeruginosa* [8]. However, these *dnaB* genes fail to complement *EcdnaB*^{ts} (*E. coli dnaB* temperature-sensitive) mutant strains. In contrast, *HpdnaB* can complement *EcdnaB*^{ts} strains efficiently [6].

Unlike DnaB, DnaC is not as well conserved among bacteria. Some bacteria, such as *Salmonella typhimurium, Neisseria gonorrhoeae* and *Klebsiella pneumoniae*, contain a *dnaC*-like gene [8]. A homologue of *dnaC* is absent from many other recently sequenced bacterial genomes, suggesting that DnaC function may not be universal. It is important to note that there are many examples of proteins that perform similar functions at the replication forks without sharing significant sequence homology [9]. The DnaC and λP accessory proteins having no homology can recruit DnaB helicase at the replication origins of *E. coli* and bacteriophage λ respectively [9]. In bacteriophage T4, gp59 protein serves as a helicase loader for gp41 helicase [10]. In higher eukaryotes, Cdc6 has been proposed to load MCM (mini-chromosome maintenance) complex [9]. One aspect common to these helicase loaders is that all of them contain nucleotide binding and hydrolysis domains.

It will be misleading to assume that an accessory protein is always required to load helicases, based on the *E. coli* model; no other bacterium has been studied thoroughly *in vitro* and *in vivo* in this regard. A recent study in *Pseudomonas* nicely demonstrated that, although the *E. coli* and *Pseudomonas* helicases share approx. 80 % similarity at the amino acid level, EcDnaC (*E. coli* DnaC) is not required to load *Pseudomonas* DnaB at the origin of plasmid RK2 *in vitro* [8]. However, this study failed to show any *in vivo* data.

To investigate the issue of the requirement of the loader protein, we have employed a combined biochemical and genetic approach using *H. pylori* and *E. coli* systems. Unlike *Pseudomonas dnaB*, *HpdnaB* complements a *dnaB*^{ts} strain of *E. coli* [6]. Investigation of the *H. pylori* genomic database fails to identify a DnaC homologue, or an ORF (open reading frame) with a nucleotide-binding domain of similar molecular mass to that of DnaC, suggesting that *H. pylori* might not require an accessory protein. Conditional lethal mutants of replication proteins in *H. pylori* are not available yet. We used the *E. coli* mutants for the complementation studies, and we hypothesized that, if a DnaC loader function is not required for the loading of HpDnaB, *HpdnaB* should be able to complement *EcdnaC*^{ts} (*E. coli dnaC* temperature-sensitive) strains *in vivo*.

Here we show that, although HpDnaB can functionally complement EcDnaB function *in vivo,* it does not interact physically with EcDnaC in a GST (glutathione S-transferase) pull-down experiment or in yeast two-hybrid analysis. Furthermore, HpDnaB can complement two different *EcdnaC*^{ts} strains, suggesting that HpDnaB can bypass EcDnaC function *in vivo*. The presence of an intact *HpdnaB* gene, and the expression of the protein in the *dnaC*^{ts} strains at the restrictive temperature, ruled out the possibility of any recombination effect. These results strongly suggest that a DnaC-like accessory protein may not be universally required for loading bacterial replicative helicases.

MATERIALS AND METHODS

Bacterial strains and plasmid construction

H. pylori (strain 26695) genomic DNA and specific primers (as shown in Table 1) were used for PCR in order to amplify the

Abbreviations used: DTT, dithiothreitol; *dnaB*^{ts}, *dnaB* temperature-sensitive; *dnaC*^{ts}, *dnaC* temperature-sensitive; EcDnaB/C, *Escherichia coli* DnaB/C; GST, glutathione S-transferase; HpDnaB$^{(wt/mut)}$, wild-type or mutant *Helicobacter pylori* DnaB respectively; *ori*C, origin of chromosomal DNA replication; ORF, open reading frame; PaDnaB, *Ps. aeruginosa* DnaB.

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Table 1 Primers list

FW, forward primer; RV, reverse primer.

Primers	Primer sequence
HpDnaB FW	5′-GCGGATCCATGAAAAACGTTGGCGACCTG-3′
HpDnaB RV	5′-GCGGATCCTCAAGTTGTAACTATATCATAA-3′
EcDnaB FW	5′-CGGGATCCAATGGCAGGAAATAAACCCTTC-3′
EcDnaB RV	5′-CGGGATCCTCATTCGTCGTCGTACTGCGGCC-3′
EcDnaC FW	5′-GCGGATCCATGAAAAACGTTGGCGACCTG-3′
EcDnaC RV	5′-GCGGATCCATACTCTTTACCTGTTACCCG-3′

coding regions of wild-type *HpdnaB* (*HpdnaB*^{wt}; 1.5 kb). The amplified products were cloned at the BamHI site of pET28a expression vector, as described previously [6]. *HpdnaB* was also cloned into pGEX2T to produce a GST-tagged protein. The coding regions of the *EcdnaB* and *EcdnaC* genes were PCR-amplified using *E. coli* genomic DNA and specific primers (Table 1), and subsequently cloned at the BamHI site of pGEX2T or pET28a respectively. Primers for the PCR reactions and the strains and plasmids used in the present study are shown in Tables 1 and 2.

pdnaBC and *pdnaB* plasmids were kindly given by Dr Dhruba K. Chattoraj (National Institutes of Health, Bethesda, MD, U.S.A.). In brief, in *pdnaBC*, the coding regions of *EcdnaC* and *EcdnaB* were cloned under the control of bacteriophage λ promoters P_R and P_L (P_R–P_L) [11] to direct co-transcription of these genes in a synthetic operon. This plasmid construct contains a short 23 bp linker separating the ochre stop codon of the *dnaC* gene and the synthetic ribosome-binding site fused upstream of *dnaB*. *pdnaB* contains the coding region of *EcdnaB* under the same P_R–P_L promoters.

pBR-*HpdnaB*^{wt} and pBR-*HpdnaB*^{mut} (containing a point mutation in the Walker A nucleotide-binding domain of HpDnaB) [6] plasmid constructs were made by subcloning the respective genes (including the His_6-tag) from the pET28a recombinant clones into pBR322 downstream of the Bla-P2 promoter [12]. The details of the cloning strategies are described elsewhere [6].

Protein purification and helicase assay

GST and His_6-tagged proteins were purified as described previously [6]. GST-EcDnaB was purified in the presence of 2 mM ATP and 5 mM $MgCl_2$. Helicase assays were performed as described previously [6]. The substrates for the helicase assays were obtained by annealing a radiolabelled 5′-tailed 23-mer oligonucleotide (5′-CCAAAACCCAGTCACGACGTTGTAAAACG-3′) to M13mp18 DNA, followed by purification of the annealed products, as described elsewhere [6].

Gel-filtration chromatography

Gel-filtration chromatography was performed essentially following the protocol described previously [6]. Samples for gel-filtration chromatography of an equimolar mixture of His_6-HpDnaB and His_6-EcDnaC were prepared as follows: 200 μg of His_6-HpDnaB and 100 μg of His_6-EcDnaC were mixed and dialysed against buffer A [50 mM Tris/HCl (pH 7.4)/50 mM NaCl/1mM DTT (dithiothreitol)/1 mM ATP/2 mM $MgCl_2$] at 4 °C. The dialysed sample was centrifuged at 15000 ***g*** for 15 min. The supernatant was transferred to a fresh tube, and 200 μl of the supernatant (containing 40 μg of HpDnaB and 20 μg of EcDnaC) was subjected to size-exclusion chromatography on a Bio-Sil SEC 250-5 (Bio-Rad) column. His_6-HpDnaB and His_6-EcDnaC were also dialysed separately in buffer A for individual gel-filtration chromatography experiments.

Western blot analysis and antibodies

Western blot analysis was performed following standard procedures [13]. Anti-His_6 and anti-GST rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-HpDnaB polyclonal antibodies were raised in rabbits using purified HpDnaB as the antigen. Anti-EcDnaC rabbit polyclonal antibodies were obtained from Professor Kenneth Marians (Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.).

GST pull-down assay

GST pull-down assays were performed by incubating purified His_6-EcDnaC (2 μg) in the presence of either GST–HpDnaB or GST–EcDnaB beads in the binding buffer [50 mM Tris/HCl (pH 7.5)/1 mM DTT/4 % (v/v) glycerol/0.1 mg of BSA/5 mM $MgCl_2$/1 mM ATP/50 mM NaCl] at 4 °C for 1 h with gentle rotation. The beads were then washed three times with binding buffer containing 250 mM NaCl, and the bound proteins were analysed by SDS/PAGE followed by Western blot analysis.

Complementation assay

E. coli temperature-sensitive mutant strains DnaC2 and BR1701 (*dnaC*^{ts}) and DJ 58 (*dnaB*^{ts}) were transformed with pBR322, pBR-*HpdnaB*^{wt}, pBR-*HpdnaB*^{mut}, *pdnaB* and *pdnaBC*. The transformed

Table 2 Bacterial strains and plasmids used in this work

Strain/plasmid	Genotype/relevant characteristics	Reference
E. coli DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook et al. [13]
DJ58 (*EcdnaB*^{ts})	Temperature-sensitive mutant of *EcdnaB*	Gift from Dr Dhruba K. Chattoraj [6]
BR1701 (*EcdnaC*^{ts})	*dnaC2 thyA47 leu6 deoC3 str*^{r}	Gift from Dr Dhruba K. Chattoraj [19]
MG1655*dnaC2* (*EcdnaC*^{ts})	*thr*:: Tn*10 dnaT2dnaC2*(Ts)	Gift from Dr Santunu DasGupta [20]
pdnaBC	Plasmid for overproduction of EcDnaB and DnaC	Gift from Dr Dhruba K. Chattoraj
pdnaB	Plasmid for overproduction of EcDnaB	The present study
pET28a	*T7*, *his*, *kan*^{R}	Novagen
pET28a *HpdnaB* (wild-type/mutant)	pET28a derivative containing 1.5 kb of *HpdnaB*/point mutation in the Walker A motif	Soni et al. [6]
pGEX2T *EcdnaB*	pGEX2T derivative containing 1.4 kb of *EcdnaB*	This work
pGEX2T *HpdnaB*	pGEX2T derivative containing 1.5 kb of *HpdnaB*	This work
pET28a *EcdnaC*	pET28a derivative containing 753bp *EcdnaC*	This work
pBR322	*tet*^{R}, *amp*^{R}	Bolivar et al. [12]
BL21 (DE3)	*F*$^{-}$ *ompT hsdS*$_B$ (*r*$_B$$^{-}$ *m*$_B$) *gal dcm* BL21 (DE3)	Novagen
pBR-*HpdnaB* (wild-type/mutant)	pBR derivative containing 1.5 kb of *HpdnaB*/point mutation in the Walker A motif	Soni et al. [6]

cells were grown either at the permissive (30 °C) or non-permissive (37 °C for DnaC2 and BR1701 cells; 40 °C for DJ58 cells) temperature.

Yeast two-hybrid system

The GAL4 Two-hybrid Phagemid System (Stratagene) was used to perform the yeast two-hybrid experiments. The *EcdnaC* gene, encoding EcDnaC, was subcloned into the plasmid encoding the transcription activation domain (pAD) of GAL4, and the *EcdnaB* and *HpdnaB* genes were subcloned into the DNA-binding domain (pBD) of GAL4 at the BamHI site, generating plasmids pAD*EcdnaC*, pBD*EcdnaB* and pBD*HpdnaB*. Recombinant clones were confirmed by restriction digestion and sequencing.

Yeast strain AH109 was co-transformed with 500 ng of plasmids (either a combination of pAD*EcdnaC* and pBD*EcdnaB*, or pAD*EcdnaC* and pBD*HpdnaB*, using the lithium acetate method [14]). The transformed yeast cells were then plated on SD agar medium containing 2 % (w/v) glucose and either lacking tryptophan and leucine (SD−Trp, −Leu; 2D plates) or lacking leucine, tryptophan, histidine and adenine (SD−Leu, −Trp, −His, −Ade; 4D plates), followed by incubation at 30 °C for 3 days, as described in the GAL4 Two-hybrid Phagemid manual (Stratagene).

To confirm protein–protein interactions, β-galactosidase colony lift filter assays were performed using the yeast β-galactosidase assay kit, as described by the manufacturer (Stratagene). Briefly, colonies were lifted from the 4D plates (see above) using a pre-soaked sterile Whatman #5 filter, by placing it in 2.5–5 ml of β-galactosidase assay buffer [110 mM Na_2HPO_4/35 mM NaH_2PO_4 (pH 7.0)/10 mM KCl/1 mM $MgSO_4$,7H_2O/20 mM 2-mercaptoethanol)] solution containing X-gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside; 20 mg/ml) in a clean 150 mm plate. The filter paper was then frozen completely using liquid nitrogen to permeabilize the cells, followed by incubation of the filter paper at 30 °C for 30 min for the appearance of the blue colour.

RESULTS

Primary structure comparison of HpDnaB with the *E. coli* and PaDnaB (*Ps. aeruginosa* DnaB) proteins

In vitro, PaDnaB was reported to be loaded at the plasmid RK2 *ori* in the absence of DnaC, but it failed to complement the EcDnaB function in a temperature-sensitive strain [8]. In contrast, HpDnaB, without having an obvious DnaC homologue in the *H. pylori* genome, can complement *EcdnaB*^{ts} strains. In order to investigate whether HpDnaB has some unique features, the amino acid sequences of HpDnaB, EcDnaB and PaDnaB were aligned (Figure 1). HpDnaB shows 32 % identity and 53 % similarity with EcDnaB, whereas PaDnaB shows 61 % identity and approx. 80 % similarity with EcDnaB. The similarity in amino acid residues between EcDnaB and PaDnaB is spread from the N-terminus through to the C-terminus, whereas the C-terminus of HpDnaB shares more homology with EcDnaB than does the N-terminus. The C-terminus of EcDnaB contains important features such as the ATPase domain, DNA-binding domain and leucine zipper motif, which are all conserved in HpDnaB [15].

It has been shown previously that the overexpression of *EcdnaC* can overcome the temperature-sensitive phenotype of the mutant *dnaB252 in vivo* [16]. Mutation of residue 299 from glycine to aspartic acid was found to be responsible for this effect, suggesting that this residue would be important for DnaB–DnaC interaction [17]. Comparison of this region among the three different DnaB sequences reveals a high degree of divergence, failing to identify

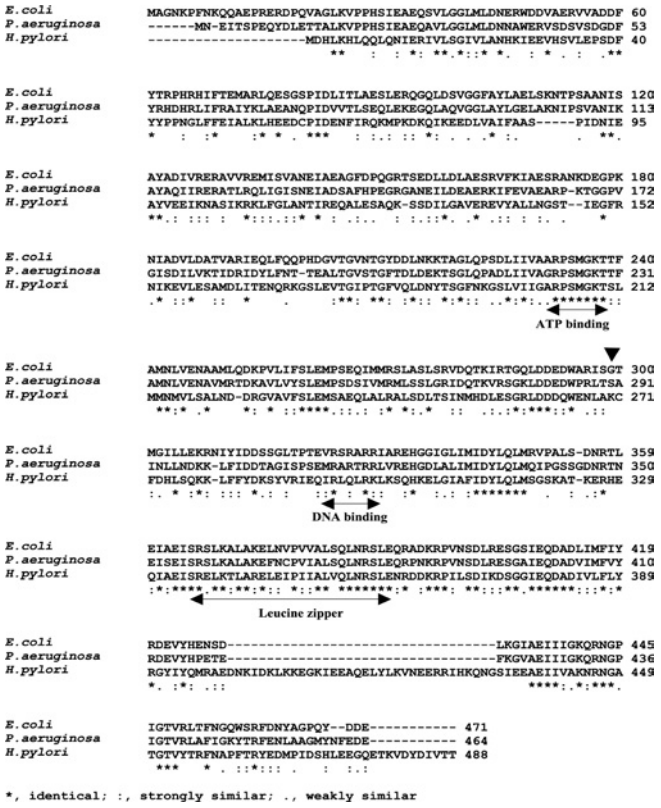

Figure 1 Primary sequence analysis of HpDnaB

Alignment of the *H. pylori*, *Ps. aeruginosa* and *E. coli* DnaB primary structures using the CLUSTALW multiple alignment programme. The ATP binding, DNA binding and leucine zipper domains are also shown. '▼' indicates the position of the 299th amino acid residue (glycine) of EcDnaB.

any conserved residue that might be essential for DnaB–DnaC interaction. Although the C-terminus of HpDnaB is highly similar to that of EcDnaB, there is a unique insertion of 34 amino acid residues (residues 400–433) that is not present in any DnaB known so far. These differences could make HpDnaB unique and different from EcDnaB.

HpDnaB does not interact with EcDnaC *in vitro*

HpdnaB complements *EcdnaB*^{ts} strain at the restrictive temperature [6]. It would be interesting to know whether EcDnaC mediates this rescue event. The non-availability of a *dnaC*-like gene in the *H. pylori* genome already suggests that EcDnaC might not be a partner of HpDnaB. To test whether HpDnaB interacts with EcDnaC, we performed GST pull-down experiments, which are often used for the demonstration of direct protein–protein interactions. For this purpose, GST–EcDnaB and GST–HpDnaB proteins were purified through binding to GST beads, and His_6-EcDnaC was purified as a soluble protein (Figure 2A). His_6-EcDnaC was incubated in the presence of either the GST–EcDnaB or GST–HpDnaB beads. After washing the beads carefully, proteins were released by boiling them in loading buffer, and analysed by SDS/PAGE followed by Western blotting analysis using anti-His_6 antibodies. We found that the binding of EcDnaC to GST–EcDnaB beads was very specific compared with the control GST or GST–HpDnaB beads. The equal loading of GST–HpDnaB and GST–EcDnaB was shown by probing the same blot with anti-GST antibodies (Figure 2B).

The question arises as to whether the inability of GST–HpDnaB to interact with EcDnaC in the pull-down experiments is due to

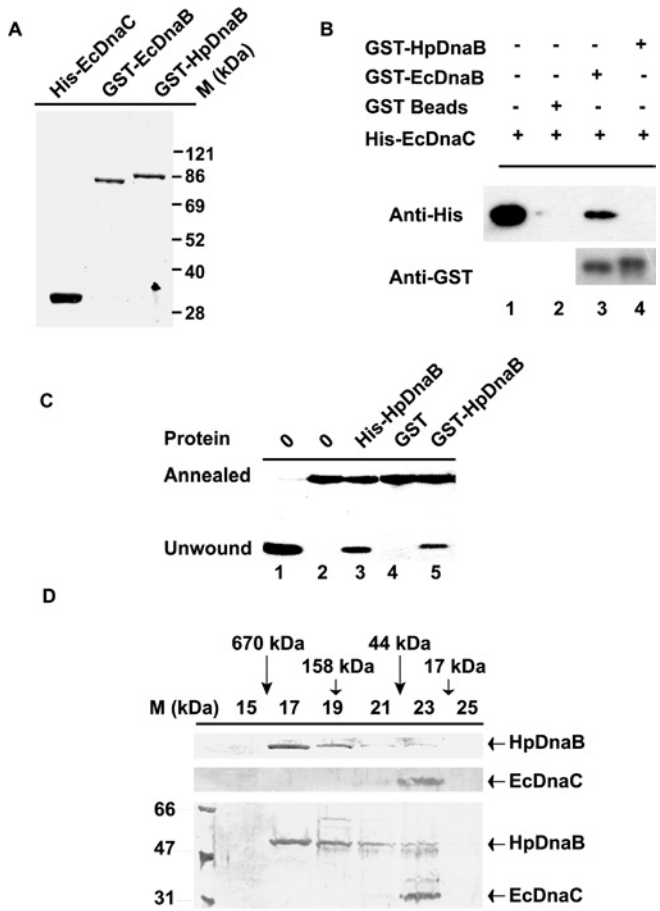

Figure 2 HpDnaB does not interact with *E. coli* DnaC *in vitro*

(**A**) Purification of GST–EcDnaB, GST–HpDnaB and His_6–EcDnaC. EcDnaB and HpDnaB were purified as GST fusion proteins, and EcDnaC was purified as a His_6 fusion protein. The purity of the proteins was checked by SDS/PAGE analysis. (**B**) GST pull-down experiment: GST (lane 2) or GST–EcDnaB (lane 3) or GST–HpDnaB beads (lane 4) were incubated in the presence of purified His_6-EcDnaC. Proteins bound to the beads were subjected to SDS/PAGE, followed by Western blot analysis using anti-His_6 and anti-GST antibodies. GST beads were used as a control. Lane 1 contains purified His_6-EcDnaC protein as the input (5 %). (**C**) Helicase activity of the purified proteins, as indicated on the top of the panel. The details of the substrates used for the helicase assay have been described in the materials and methods section. All the proteins were tested for helicase activity following the protocol described previously [6]. Lane 1 contains heated sample to show the complete unwinding of the radiolabelled oligonucleotides. Lanes 1–2 do not contain protein; lanes 3–5 contain His_6-HpDnaB, GST and GST–HpDnaB respectively. (**D**) Gel-filtration chromatography of His_6-HpDnaB, His_6-EcDnaC and an equimolar mixture of His_6-HpDnaB and His_6-EcDnaC. The proteins were passed through a Bio-Sil SEC 250-5 column, and 0.5 ml fractions were collected in each case, followed by SDS/PAGE and silver staining of the gel containing alternate fractions (15–25), as shown along the top. The positions of the gel-filtration standards are shown along the top as follows: thyroglobulin (670 kDa; fraction 16), bovine γ-globulin (158 kDa; fraction 19), chicken ovalbumin (44 kDa; fraction 22) and horse myoglobin (17 kDa; fraction 24).

the differential effect on the HpDnaB–EcDnaC interaction due to the addition of GST tags at the N-terminus. In order to find out whether GST–HpDnaB is functionally active, we checked the helicase activity of GST–HpDnaB. GST–HpDnaB showed helicase activity with no activity in the GST control lane, suggesting that the GST tag at the N-terminus did not affect the conformation related to the *in vitro* activity of the protein (Figure 2C). It is unlikely that the addition of the GST tag has specifically protected the EcDnaC-interacting domain of HpDnaB, but not that of EcDnaB, while these proteins have been treated the same way. It is important to note in this regard that His_6-HpDnaB was used for both the *in vitro* helicase assay (Figure 2C) and *in vivo* rescue experiments (see Figures 4A and 4B), and His_6-HpDnaB could rescue the temperature-sensitive phenotypes, effectively suggesting that the addition of the tags did not affect the *in vivo* activity of these proteins.

Previously, we claimed that HpDnaB might have an affinity towards EcDnaC, since His_6-HpDnaB and His_6-EcDnaC co-eluted in a fraction following gel-filtration chromatography [6]. The co-elution of HpDnaB and EcDnaC was detected by Western blot analysis using anti-His_6 antibodies. However, following repeated gel-filtration chromatography and critical evaluation of the results, we found that the amount of EcDnaC present in the same fraction containing HpDnaB was not stoichiometric. We have already shown that HpDnaB exists as a hexamer in solution [6]. In general, six monomers of EcDnaC bind to the hexameric EcDnaB to form a $(EcDnaB–EcDnaC)_6$ complex. In the present study, by mixing His_6-HpDnaB and His_6-EcDnaC in an equimolar ratio, followed by gel-filtration and silver staining of the gel containing alternate fractions from the gel-filtration chromatography, we found that the peak fractions of HpDnaB and EcDnaC do not co-elute (Figure 2D, bottom panel). Although we see the presence of both HpDnaB and EcDnaC in fractions 21–23, we believe that this is not due to the formation of a stoichiometric HpDnaB–EcDnaC complex, since the peak fractions of hexameric HpDnaB (fraction 17) and monomeric EcDnaC (fraction 23) are far apart. When His_6-HpDnaB and His_6-EcDnaC were subjected to gel-filtration chromatography separately, the majority of these proteins were found to be present in fractions 17 and 23 respectively (Figure 2D, upper two panels). It has been reported previously [8] that the peak fractions of EcDnaB and EcDnaC overlap with each other when a mixture of EcDnaB and EcDnaC is subjected to gel-filtration chromatography under the same experimental conditions as those described here. These results taken together suggest that HpDnaB does not interact with EcDnaC *in vitro*.

EcDnaB, but not HpDnaB, interacts specifically with EcDnaC in a yeast two-hybrid system

To investigate the potential interactions between EcDnaB and HpDnaB with EcDnaC in an *in vivo* environment, we used a yeast two-hybrid system (the GAL4 Two-hybrid Phagemid ssytem; Stratagene). In this system, the yeast GAL4 transcription activator has been divided into the following two separate functional domains: (i) the transcription activation domain (AD) present on plasmid pAD-GAL4-2.1 (pAD), which encodes the *LEU2* gene as a selectable marker; and (ii) the DNA-binding domain (BD) present on the plasmid pBD-GAL4-Cam (pBD), which encodes the *TRP1* gene as a selectable marker [18]. If two fusion proteins interact in this system, they will bring into close proximity the GAL4-transcription activation domain and the GAL4 DNA-binding domain to the GAL4 promoters, which in turn will initiate the transcription of the *HIS3*, *ADE2* and *lacZ* reporter genes. Protein–protein interactions are then detected by the ability of the co-transformed yeast cells to grow in selective medium lacking leucine, tryptophan, adenine and histidine (SD−Leu, −Trp, −His and −Ade), and by production of β-galactosidase activity.

In order to test whether interactions between EcDnaB, HpDnaB with EcDnaC could be detected in this system, we first subcloned *EcdnaC* at the BamHI site of pAD. *EcdnaB* and *HpdnaB* genes were cloned at the BamHI site of pBD. The yeast reporter strain AH109 was subsequently co-transformed either with: (case a) pAD*EcdnaC* and pBD*EcdnaB*, or (case b) pAD*EcdnaC* and pBD*HpdnaB*, followed by plating them on 2D plates (see the Materials and methods section). Growth of the co-transformed yeast cells on these plates confirmed that the transformation had been successful, and provided an indication of the relative number

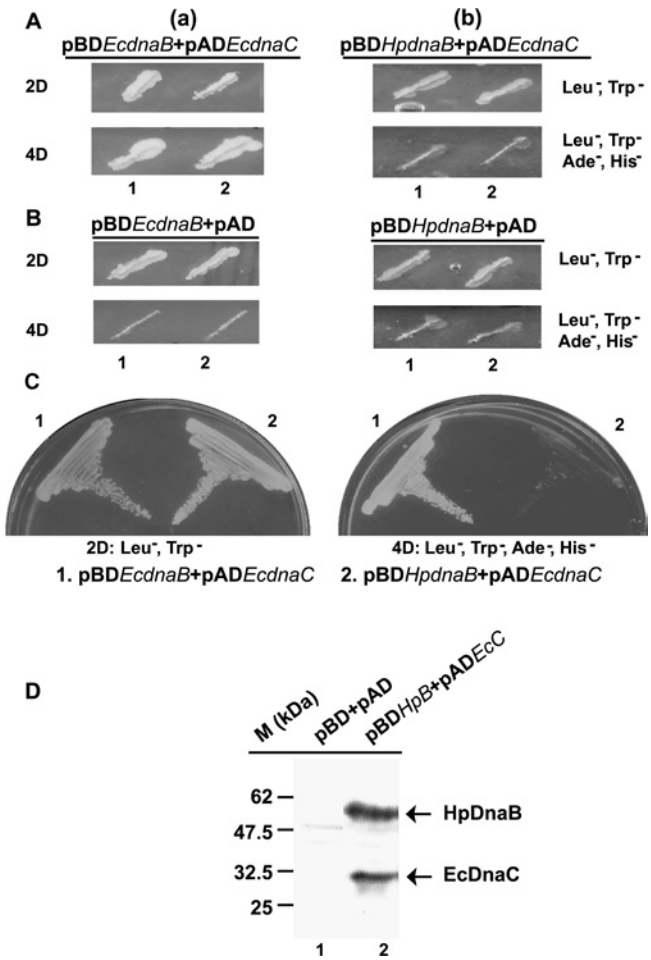

Figure 3 EcDnaB but not HpDnaB interacts with EcDnaC

(**A**) Yeast reporter strain AH 109 was co-transformed with either (**a**) pAD*EcdnaC* and pBD*EcdnaB* or (**b**) pAD*EcdnaC* and pBD*HpdnaB*, and the transformed cells were plated in an SD agar minimal-media plate (2D plate). Two representative colonies (the numbers are indicated along the bottom of the respective Figure parts) in each case were streaked further in SD minimal 2D or 4D plates, followed by incubation at 30 °C for 2–3 days. All the colonies grew on 2D plates. Both the colonies grew on 4D plates in the case of (**a**), whereas none grew on 4D plates in the case of (**b**). (**B**) Yeast reporter strain AH 109 was co-transformed with either pAD (empty vector) and pBD*EcdnaB*, or pAD and pBD*HpdnaB*, and two colonies (the numbers are indicated along the bottom of the respective Figure parts) grown on 2D plates were streaked further in 2D or 4D plates. All of them grew on 2D plates, but none grew on 4D plates. (**C**) In order to confirm the results, colony 2 from 2D plates in **A**(**a**) and **A**(**b**) was re-streaked in 2D and 4D plates. While both grew on 2D plates, only one (**a**) grew on the 4D plate. (**D**) Western blot analysis of the cell lysate obtained from yeast strain AH 109 co-transformed either with empty vectors pAD and pBD (lane 1) or pAD*EcdnaC* (pAD*EcC*) and pBD*HpdnaB* (pBD*HpB*) (lane 2) using anti-HpDnaB and anti-EcDnaC antibodies.

of co-transformed yeast cells. Furthermore, 20 colonies in each case (either a or b) were re-streaked on 2D plates or 4D plates (i.e. SD agar medium lacking leucine, tryptophan, histidine and adenine; see the Materials and methods section for further details). In the case of (a), all the colonies grew on the 2D as well as the 4D plates. On the other hand, in the case of (b), all the colonies grew on 2D plates, but none grew on 4D plates. A representation of two colonies streaked on such plates is shown in Figure 3(A). These observations strongly suggest that EcDnaC interacts with EcDnaB, but not with HpDnaB. As a control, yeast strains were co-transformed either with pBD*EcdnaB* and pAD (empty vector) or with pBD*HpdnaB* and pAD, followed by plating them on 2D and 4D plates (Figure 3B). As expected, all grew on 2D plates, but none were found to grow on 4D plates, suggesting that the yeast cells growing in the 4D plates [Figure 3A(a)] are the outcome of the real interaction between EcDnaB and EcDnaC. In order to consolidate the results from Figure 3(A), yeast cells [shown in Figures 3A(a) and 3A(b), '2D' panels, lanes 2] were re-streaked further on to 2D and 4D plates (Figure 3C).

The EcDnaB–EcDnaC interaction observed in co-transformed yeast cells was confirmed further by analysis of β-galactosidase activity, which reflects the activation of the *lacZ* reporter gene (results not shown). The expression of HpDnaB and EcDnaC from the respective plasmids pBD*HpdnaB* and pAD*EcdnaC* was also confirmed by SDS/PAGE followed by Western blot analysis of the yeast cell lysate [Figure 3A(b), '2D' panel] using anti-HpDnaB and anti-EcDnaC antibodies (Figure 3D). These results strongly suggest that, even though HpDnaB and EcDnaC were expressed in the yeast reporter strain AH109, the reporter genes were not activated due to the failure of their interaction.

HpdnaB rescues the temperature-sensitive phenotype of the *EcdnaC*^{ts} strains

Absence of the DnaC homologue in the *H. pylori* genome, and the failure of HpDnaB to interact with EcDnaC *in vitro* and in the yeast two-hybrid assay, raised the issue of whether HpDnaB needs a loader. We reasoned that, if the above results were truly reflected *in vivo*, HpDnaB would be able to perform its function in the absence of a functional DnaC protein. This hypothesis was experimentally proved by complementing two different *EcdnaC*^{ts} strains with HpDnaB at the non-permissive temperature.

The *E. coli* system allows us the opportunity to study gene function due to the availability of conditional lethal mutants. To carry out the complementation analysis, a His_6-tagged *HpdnaB* gene was subcloned into the low-copy pBR322 plasmid under the control of the Bla-P2 promoter [12], and *E. coli* strain BR1701 *dnaC*^{ts} [19] was transformed with the recombinant plasmids. *HpdnaB* was found to complement the defective *dnaC* gene in *E. coli* at 37 °C, whereas pBR322 failed to do so (Figure 4A). *pdnaBC* containing wild-type *dnaC* gene under the control of the bacteriophage λ promoters (P_R–P_L) also rescued the defective gene function. These results suggest that HpDnaB is capable of functioning as a helicase *in vivo* where DnaC is not functional. It is possible that the defect in the *E. coli* strain BR1701 *dnaC*^{ts} will allow HpDnaB to still interact with EcDnaC, and rescue the temperature-sensitive phenotype. However, in order to rule out the possibility of any allele specificity in the BR1701 *dnaC*^{ts} strain, we performed the rescue experiment in another *dnaC*^{ts} strain, MG1655*dnaC2* [20] using a wide range of plasmids. It is very unlikely that HpDnaB would rescue two different *dnaC*^{ts} strains, having mutations in two different places in the *dnaC* gene, purely by chance, whereas the wild-type EcDnaB cannot rescue either of these strains. pBR-*HpdnaB*^{wt}, pBR-*HpdnaB*^{mut}, *pdnaB* (containing the *EcdnaB* gene) and *pdnaBC* (containing the wild-type *EcdnaC* gene) were used for complementation. Only *HpdnaB*^{wt} and *pdnaBC* could rescue the temperature-sensitive phenotype of the DnaC2 cells (Figure 4B). These results clearly suggest that HpDnaB can bypass the EcDnaC function *in vivo*. The failure of wild-type EcDnaB to complement the *dnaC*^{ts} strain strengthens the notion that a functional DnaC is absolutely essential for EcDnaB to be loaded at the *E. coli ori*C.

It is possible that the complementation event could be the outcome of recombination of the *HpdnaB* gene with the *E. coli* genome. To resolve this issue, plasmid DNA was isolated from the transformed DnaC2 cells (grown at 37 °C), followed by restriction enzyme digestion using BamHI. A 1.5 kb *HpdnaB* DNA fragment was observed in the *HpdnaB*-transformed DnaC2 cells (Figure 4C). The expression of HpDnaB was also confirmed

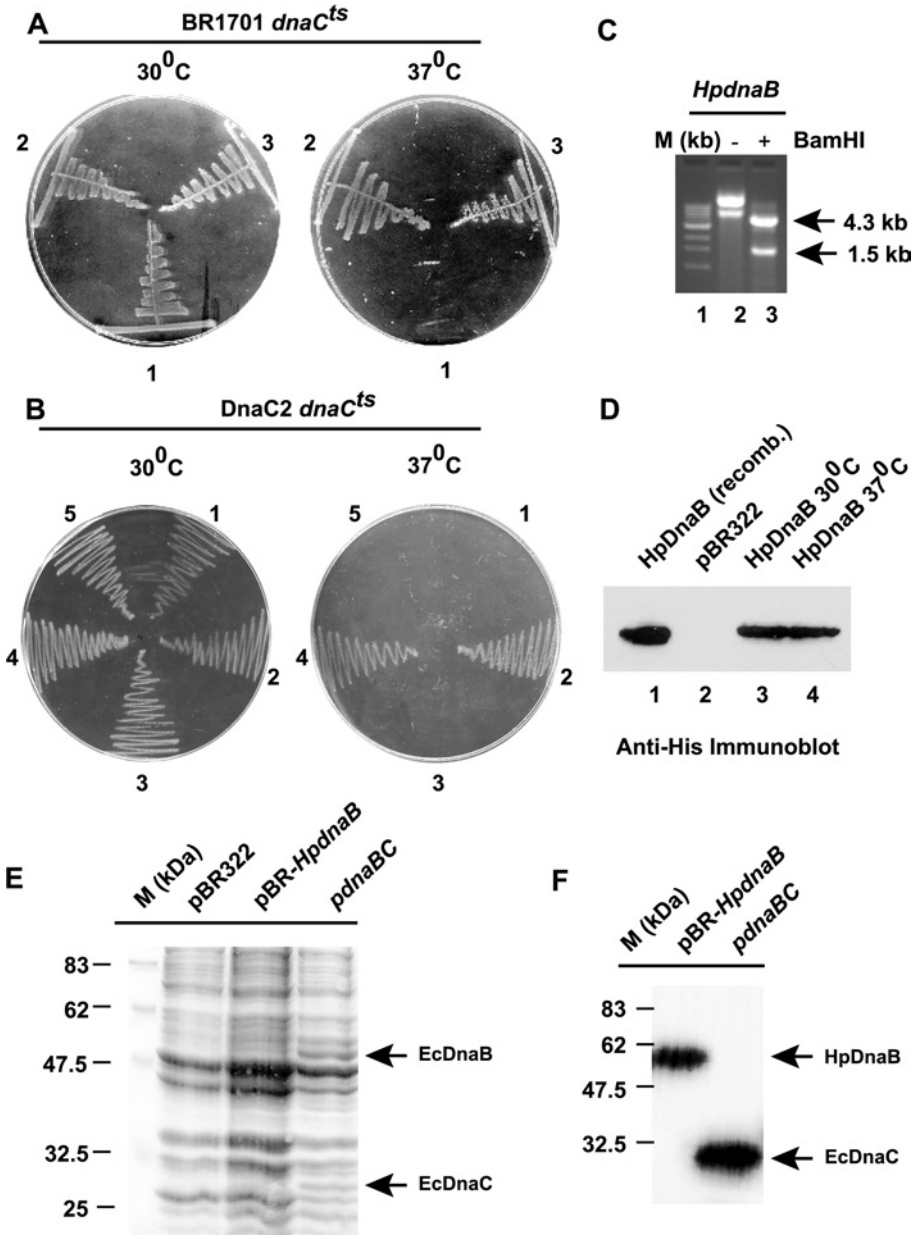

Figure 4 Complementation of *E. coli dnaC^ts* strains

(**A**) Complementation of BR1701 *E. coli* strain *dnaC^ts*. BR1701 *dnaC^ts* cells were transformed with either pBR322 (1), or *pdnaBC* (*E. coli*) (2) or pBR-*HpdnaB^wt* (3). Cells were plated on to LB agar plates and incubated either at the permissive temperature (30 °C) or at a non-permissive temperature (37 °C). (**B**) Complementation of the DnaC2 mutant strain. *E. coli* DnaC2 mutant cells were transformed with either pBR322 (1) or *pdnaBC* (2) or *pdnaB* (*E. coli*) (3), or pBR-*HpdnaB^wt* (4) or pBR-*HpdnaB^mut* (5). Cells were grown either at 30 °C or at 37 °C. (**C**) Plasmid DNA isolated from DnaC2 cells grown at 37 °C was subjected to BamHI restriction enzyme digestion, followed by agarose gel-electrophoresis analysis. Lane 1 (M) shows a 1 kb ladder used as a marker; lane 2 shows undigested plasmid DNA and lane 3 indicates DNA digested with BamHI. (**D**) Detection of HpDnaB expression in DnaC2 cells by immunoblotting using anti-His_6 antibody. Lane 1, purified HpDnaB; lanes 2 and 3, extract from pBR322- and *HpdnaB*-transformed cells respectively, grown at 30 °C; lane 4, extract from *HpdnaB*-transformed cells grown at 37 °C. (**E**) SDS/PAGE analysis of bacterial lysate (100 μg each lane) from DnaC2 temperature-sensitive cells transformed with pBR322 (grown at 30 °C) or pBR-*HpdnaB* or *pdnaBC* (grown at 37 °C). The gel was subsequently stained with Coomassie Brilliant Blue to visualize the proteins. Molecular mass (M) markers are also shown. (**F**) Detection of EcDnaC and HpDnaB expression in DnaC2 cells transformed with either pBR-*HpdnaB* or *pdnaBC* (grown at 37 °C), followed by SDS/PAGE analysis and immunoblotting using rabbit polyclonal anti-EcDnaC and anti-HpDnaB antibodies.

by Western blotting using anti-His_6 antibodies in *HpdnaB*-transformed DnaC2 cells at 37 °C (Figure 4D). The presence of an approx. 55 kDa band in the Western blot rules out the possibility of the recombination of *HpdnaB* gene with the *E. coli* genome.

Furthermore, we wanted to compare experimentally the efficiency of the Bla-P2 promoter in the pBR-*HpdnaB* plasmid construct with the bacteriophage λ P_R–P_L promoters in *pdnaBC* or *pdnaB* plasmids, since the level of expression of proteins is an important issue for the complementation experiments. To compare the efficiency of these promoters, DnaC2 cells transformed with either pBR-*HpdnaB* or *pdnaBC* were grown at 37 °C (Figure 4B), and the bacterial cell extracts were subjected to SDS/PAGE analysis, followed by Coomassie staining of the gel to visualise the proteins. Bacterial cell extract from pBR322-transformed DnaC2 cells (grown at 30 °C) was used as control. The expression of EcDnaB and EcDnaC could be detected at

the restrictive temperature easily, but the expression of HpDnaB was not very obvious by this method (Figure 4E), suggesting that P_R–P_L promoters are stronger than the Bla-P2 promoter. The expression levels of HpDnaB and EcDnaC were confirmed further by Western blot analysis using rabbit polyclonal antibodies against HpDnaB and EcDnaC. Anti-HpDnaB antibodies do not cross-react with EcDnaC, and vice versa (results not shown). We observed that both HpDnaB and EcDnaC were expressed in the DnaC2 cells at the non-permissive temperature (Figure 4F). Owing to the unavailability of anti-EcDnaB antibodies, we could not check the expression of EcDnaB by Western blot analysis. *In vivo*, both *pdnaBC* and *pdnaB* plasmid constructs complemented the *EcdnaB*^{ts} strain DJ58 [6] at the non-permissive temperature efficiently (results not shown), suggesting that EcDnaB was indeed expressed from these plasmid constructs.

DISCUSSION

Analysis of the genome sequences of *H. pylori* reveals many unique features related to DNA replication. The typical conserved eubacteria gene cluster *rnpA-rmpH-dnaA-dnaN-recF-gyrB* is absent, and the *dnaA* gene is located approx. 600 kb away from the *dnaN-gyrB* genes [21]. The helicase loader DnaC is also absent. Here, we have shown that HpDnaB can complement two different *EcdnaC*^{ts} strains, citing the first evidence of a bypassing DnaC function *in vivo*. Recently, *in vitro*, a DnaC-independent mechanism for *Ps. aeruginosa* replicative helicase loading at the broad host range plasmid RK2 *ori* was reported [8]. Our finding is important and relevant, since several eubacterial species, including *Ps. aeruginosa* and *H. pylori*, lack a DnaC homologue, suggesting that an accessory-protein-independent loading of helicases might be possible in some bacteria.

How does HpDnaB work at the *E. coli ori*C in the absence of a functional EcDnaC? We believe that DNA replication initiation at the *ori*C in the *dnaC*^{ts} strains at the non-permissive temperature is due to the HpDnaB activity only, and does not require a helicase loader. However, functional EcDnaB is present in the *dnaC*^{ts} strains. Therefore it cannot be ruled out that the rescue phenomenon is the result of a DnaC-like activity of HpDnaB that may assist the loading of EcDnaB at the *ori*C in the *dnaC*^{ts} strains.

In the present study, we have shown that HpDnaB does not require a functional DnaC-like protein in a heterologous system. The question arises of whether HpDnaB needs a helicase loader in *H. pylori* itself. It is possible that a functional homologue of DnaC, without sharing any sequence homology with EcDnaC, is present in *H. pylori*. In fact, according to the *H. pylori* protein–protein interaction map [22], two unknown ORFs (Hp0897 and Hp0340) were reported to be interacting with HpDnaB. Interestingly, these ORFs do not contain nucleotide binding and hydrolysis domains, and Hp0897 does not have a counterpart in a similar *H. pylori* strain, J99. These observations strongly argue against their conserved DnaC-like role in *H. pylori*. Additional experiments are required to unveil their functional roles in *H. pylori*. Furthermore, according to this protein–protein interaction map, HpDnaB does not interact with HpDnaA, in contrast with EcDnaB, which needs EcDnaA to be loaded at the *ori*C. These observations suggest that HpDnaB might have some unique features, compared with its *E. coli* counterpart.

It has been reported that the overexpression of *dnaC* can rescue the temperature sensitivity of the *E. coli dnaB252* allele [16]. Mutation of residue 299 of EcDnaB, from glycine to aspartic acid, was found to be responsible for this defect [17]. DnaB252 protein showed identical ATPase, helicase and single-strand DNA binding activities, both at the permissive and restrictive temperature, suggesting that the defect in the *dnaB252* gene does not affect the gene function as such. However, this mutation must affect the interaction between EcDnaB and EcDnaC, since overexpression of *dnaC* can abrogate this defect. It has been strongly suggested that the glycine residue at position 299 of DnaB is critical for the DnaB–DnaC interaction. We chose to examine the status of this residue in the DnaB helicases shown in Figure 1, and we found that the glycine residue is absent in both *H. pylori* and *Ps. aeruginosa*. Moreover, the amino acid residues immediately adjacent to the residue at position 299 are also divergent in both the cases. It is important to note in this regard that, although PaDnaB shows 80 % overall homology with EcDnaB at the amino acid level, it lacks a DnaC homologue. We believe that these observations may shed some light on to our efforts to find a region in DnaB that might be important for the presence or absence of DnaC. However, further detailed studies are needed before we can assert that this region is an absolute determinant of the presence or absence of DnaC.

Another interesting feature of HpDnaB is the presence of a unique insertion of 34 residues at its C-terminus (residues 400–433). This region contains as many as ten lysine and arginine residues, with an overall positive charge. Further detailed mutational analysis of these residues will be required to understand their biological function.

The unavailability of the *dnaC* gene in the *H. pylori* genome, the inability of HpDnaB to interact with EcDnaC, and finally the rescue of two different *dnaC* temperature-sensitive strains using HpDnaB, clearly suggest that HpDnaB can bypass *E. coli* DnaC function *in vivo* efficiently.

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