Functional characterization of *Helicobacter pylori* DnaB helicase

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ABSTRACT

***Helicobacter pylori* causes gastric ulcer diseases and gastric adenocarcinoma in humans. Not much is known regarding DNA replication in *H.pylori* that is important for cell survival. Here we report the cloning, expression and characterization of *H.pylori* DnaB (HpDnaB) helicase both *in vitro* and *in vivo*. Among the DnaB homologs, only *Escherichia coli* DnaB has been studied extensively. HpDnaB showed strong 5′ to 3′ helicase and ATPase activity. Interestingly, *H.pylori* does not have an obvious DnaC homolog which is essential for DnaB loading on the *E.coli* chromosomal DNA replication origin (*ori*C). However, HpDnaB can functionally complement the *E.coli* DnaB temperature-sensitive mutant at the non-permissive temperature, confirming that HpDnaB is a true replicative helicase. *Escherichia coli* DnaC co-eluted in the same fraction with HpDnaB following gel filtration analysis suggesting that these proteins might physically interact with each other. It is possible that a functional DnaC homolog is present in *H.pylori*. The complete characterization of *H.pylori* DnaB helicase will also help the comparative analysis of DnaB helicases among bacteria.**

INTRODUCTION

Helicobacter pylori, a Gram-negative, spiral bacterial pathogen is considered to be the causative agent for the induction of chronic gastritis, gastroduodenal ulcer diseases, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (1–4). Approximately half of the world's population is infected with *H.pylori*. The bacterium was first isolated and cultured from gastric biopsy samples by Marshall and Warren in 1984 (5,6). Although a lot of effort has been made to understand the major virulence factors secreted by the bacteria and the underlying mechanisms which cause the disease, the basic biology of the bacteria is not completely understood.

DNA replication is a fundamental process that takes care of the faithful duplication of the genome in each cell division cycle. Central to this process are the events that take place at the replication origin. In bacteria, the initiator protein DnaA binds specifically to the DnaA boxes. The *Escherichia coli* *ori*C region contains four DnaA boxes which allow binding of DnaA proteins leading to the unwinding of the adjacent AT-rich region. This is followed by the entry of a helicase complex ($DnaB_6$–$DnaC_6$) at the origin, with the concomitant loading of other proteins required to form the replisome (7,8).

Thorough *in silico* analysis of the genome sequence of two unrelated isolates of *H.pylori* strains 26695 and J99 suggests that the basic mechanism of chromosomal DNA replication may likely be similar to that found in other eubacteria, although experimental evidence to support this hypothesis is not available (9,10).

There are many dissimilarities when compared with the *E.coli* genome, like the absence of the *recF* gene, the presence of the *dnaA* gene ~600 kb away from the *dnaN*–*gyrB* genes and most importantly the absence of the *dnaC* gene, which codes for DnaC protein, required to load DnaB helicase to the origin. DnaC is essential in *E.coli*. It is possible that a functional DnaC counterpart is present in *H.pylori*, though it is not very obvious from the sequence analysis. Alternatively, HpDnaB can independently be loaded on *ori*C without the presence of DnaC. Therefore, it is important to find out first whether HpDnaB is functionally a true DnaB homolog of *E.coli* DnaB.

Recently, *H.pylori* DnaA protein has been purified and characterized (11). HpDnaA contains four domains like other DnaA proteins. The C-terminal domain of HpDnaA has been shown to be responsible for DNA binding. The putative *ori*C region in *H.pylori* containing five DnaA binding sites has also been mapped to be located upstream of the *dnaA* gene (11).

DnaB is considered to be a true multifunctional enzyme since it interacts with a number of other proteins during replication of DNA. The enzymatic activities of DnaB includes, but is not limited to, helicase activity, ATP hydrolysis and DNA binding (8). *Escherichia coli* DnaB interacts with DnaG primase (12), DNA polymerase III holoenzyme (13) and the helicase loader DnaC (8). It is considered to be the major replicative helicase in most eubacteria. However, among these helicases only *E.coli* DnaB has been characterized extensively both *in vitro* and *in vivo*. Apart from *E.coli*, some of the bacterial species whose DnaB homologs have been reported so far include *Thermus*

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aquaticus (14), *Bacillus stearothermophilus* (15), *Pseudomonas putida* and *Pseudomonas aeruginosa* (16).

A putative *E.coli* DnaB homolog has been identified by *in silico* analysis of the *H.pylori* (strain 26695) genome database (9). The open reading frame (ORF) HP1362 has been found to be 32% identical and 57% similar to its *E.coli* counterpart suggesting that this could be a putative DnaB homolog in *H.pylori*. However, there is no experimental evidence to support this hypothesis.

In an effort to understand the replication initiation processes in *H.pylori*, we cloned, purified and characterized the putative DnaB homolog of *H.pylori* strain 26695 (ORF HP1362). Although *H.pylori* lacks an obvious DnaC homolog, we found that HpDnaB fulfils the characteristics of the *E.coli* DnaB both *in vivo* and *in vitro*. Unlike *E.coli* DnaB, HpDnaB can use UTP as energy source, Ca^{2+} as a co-factor and untailed substrate for the helicase activity.

MATERIALS AND METHODS

Bacterial strains

The *E.coli* strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in LB media (supplemented with 100 µg/ml ampicillin or 50 µg/ml kanamycin wherever needed) either at 37 or 30°C, as per the requirement.

DNA manipulations

Helicobacter pylori dnaB gene was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Stratagene) and *H.pylori* strain 26695 genomic DNA (obtained from ATCC™) as template with the following primers: 5′-GCGGATCCATGGATCATTTAAAGCATTTGC-3′ and 5′-GCGGATCCTCAAGTTGTAACTATATCATAA-3′.

The PCR-amplified DNA (1.5 kb) was cloned in expression vector pET28a (Novagen) at the BamHI site and subsequently sequenced. For glutathione-*S*-transferase (GST) fusion protein, the PCR product was cloned into pGEX2T (Amersham Pharmacia) at the BamHI site. *Escherichia coli dnaC* was PCR amplified using genomic DNA of *E.coli* strain K12 with the following primers: 5′-GCGGATCCATGAAAA-ACGTTGGCGACCTG-3′ and 5′-GCGGATCCATACTCTT-TACCTGTTACCCG-3′. The PCR-amplified product was cloned into the BamHI site of pET28a and subsequently sequenced.

Mutation in the ATP binding site of HpDnaB (A**R**PSMGKT) was introduced by PCR using the following mutagenic oligonucleotides: 5′-GTCATTATAGGGGCAT-

GCCCGTCTATGGGTAAA-3′ and 5′-TTTACCCATAGA-CGGGCATGCCCCTATAATGAC-3′.

Mutagenesis was carried out on a pET28aHpDnaB DNA template using a Stratagene site-directed mutagenesis kit, as directed by the vendor. This mutation introduces a cysteine (C) residue at position 204 instead of arginine (R) and created a SphI site in the DNA.

For the complementation assay, wild-type and mutated *dnaB* genes were released from respective pET28a recombinant clones by using XbaI (followed by end-filling using Klenow polymerase) and EcoRI and cloned into pBR322 (downstream of the Bla-P2 promoter) (17) at HindIII (followed by end-filling using Klenow polymerase) and EcoRI sites. *Escherichia coli* DJ58 (*dnaB*^ts) strain was transformed with the recombinant positive clones.

Purification of His-tagged HpDnaB (Wt), His-tagged HpDnaB (Mut), GST-HpDnaB (Wt) and His-tagged *E.coli* DnaC; gel filtration chromatography and western blotting

Escherichia coli strain BL21 (DE3) (Novagen) harboring pET28aHpDnaB (Wt), pET28a HpDnaB (Mut) or pET28aEcDnaC were grown at 37°C in LB media containing 50 µg/ml kanamycin. The bacterial cultures were induced for the expression of the recombinant proteins using 1 mM IPTG. His-tagged proteins were purified using Ni-NTA agarose (Qiagen) beads essentially following the protocol supplied by the vendor. Fractions containing HpDnaB (Wt) or HpDnaB (Mut) or EcDnaC were pooled, dialyzed against buffer A [50 mM Tris–Cl (pH 7.4), 1 mM EDTA, 10 mM βME, 100 µM PMSF, 10% glycerol] and loaded on a 1 ml Q–Sepharose column (Amersham) that had been previously equilibrated with buffer A with 50 mM NaCl (buffer B). The column was connected to a Duo-Flow protein purification system (Bio-Rad). After loading the protein, the column was washed with the same buffer until OD_{280} reached baseline and proteins were eluted by applying a linear gradient of 50 mM–700 mM NaCl in buffer A. All the fractions were checked for HpDnaB (~55 kDa) on 10% SDS–PAGE. Positive fractions were pooled and dialyzed against buffer A with 100 mM NaCl and stored at –80°C. GST-HpDnaB was purified using agarose immobilized GST beads (Sigma-Aldrich) using the instructions supplied by the vendor.

The molecular masses of the native wild-type or mutated HpDnaB were determined by subjecting them to size-exclusion chromatography on a Bio-Sil SEC 250-5 column (7.8 × 300 mm; Bio-Rad, Hercules, CA) in buffer B supplemented with 2 mM $MgCl_2$ and 2 mM ATP. The column

Table 1. Bacterial strains and plasmids used in this work

Strain/plasmid	Genotype/relevant characteristics	Reference
Escherichia coli DH5α	*supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	Molecular cloning, Sambrook *et al.* (18)
DJ58 (*E.coli dnaB*^ts)	Temperature-sensitive mutant of *E.coli*	Gift from Dr Dhruba K. Chattoraj
pET28a	*T7, his, kan*^R	Novagen
pET28a HpDnaB (wild-type and mutant)	pET28a derivative containing 1.5 kb of *H.pylori* DnaB	This work
pBR322	*tet*^R, *amp*^R	Bolivar *et al.* (17)
BL21 (DE3)	*F⁻ ompT hsdS_B (r_B⁻ m_B) gal dcm* (DE3)	Novagen
pdnaBC	Plasmid expressing *E.coli* DnaB and DnaC (wild type)	Gift from Dr Dhruba K. Chattoraj
Helicobacter pylori 26695		ATCC™ 700392D

was previously calibrated against Bio-Rad molecular weight standards.

Protein concentrations were determined by the Bradford method (Bio-Rad kit) as per the instructions of the vendor with BSA as a standard.

Western blot analysis was carried out following standard procedures (18).

Helicase activity

The following oligonucleotides were used for the helicase activity: 5′-TCGGTACCCGGGGATCCT-3′ (18mer); 5′-CCCAGTCACGACGTTGTAAAACG-3′ (23mer); 5′-TCG-AGCTCGGTACCCGGGGATCCTCTAGAG-3′ (30mer); 5′-AATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTC-G-3′ (36mer); 5′-CCAAAACCCAGTCACGACGTTGTAA-AACG-3′ (5′ tailed 23mer); and 5′-CCCAGTCACGAC-GTTGTAAAACGTGCCGG-3′ (3′ tailed 23mer). The oligonucleotides were radiolabeled at the 5′ end and were allowed to anneal to either M13mp18 or M13mp19 as described earlier (19), with minor modifications. Briefly, 100 ng of the respective labeled oligonucleotides were annealed to 1 µg of M13mp19 or M13mp18 single-stranded DNA (ssDNA) in 40 mM Tris–HCl (pH 7.5), 10 mM MgCl$_2$, 50 mM NaCl and 1 mM DTT and purified by passing through a 1 ml Sepharose CL-4B (Amersham) spin column.

For determination of polarity of unwinding (3′-5′ or 5′-3′), 5′-end-labeled 36mer oligonucleotides (5′-AATTCGAGCTC-GGTACCCGGGGATCCTCTAGAGTCG-3′) were annealed to M13mp19 DNA, digested with SmaI and purified by passing through a 1 ml Sepharose CL-4B spin column. For 5′-3′ polarity determination, 36mer oligonucleotides were annealed to M13mp19 DNA followed by 3′-end-labeling by Klenow polymerase, digested with SmaI, purified as before and used for the helicase assay.

The helicase assay was carried out in 10 µl of reaction buffer containing 20 mM Tris–HCl (pH 7.5), 8 mM DTT, 1.5 mM MgCl$_2$, 2 mM ATP, 80 µg/ml BSA, 10 mM KCl, 4% (w/v) sucrose, ~2000 c.p.m. of labeled partial duplex DNA and purified HpDnaB (Wt) or HpDnaB (Mut) (1.8 pmol or as indicated in the Figure legends) at 37°C for 30 min. Reactions were stopped by adding 0.25% SDS, 15 mM EDTA, 5% glycerol and products were separated on 10% non-denaturing PAGE. The gel was dried and analyzed by a phosphorimager (Fujifilm-BAS 1800) or autoradiographed.

ATP hydrolysis assay

The ATPase activity of HpDnaB was measured in a reaction mixture (10 µl) containing 20 mM Tris–HCl (pH 8.0), 1 mM MgCl$_2$, 100 mM KCl, 8 mM DTT, 4% sucrose, 80 µg/ml BSA, 1 mM ATP, 3.4 fmol of [γ-^{32}P]ATP and the required amount of DnaB, as indicated in the Figure legends. The reaction mixtures were incubated at 37°C for 30 min and the reactions were stopped by putting the

tubes on ice. Released inorganic Pi was separated by thin-layer chromatography (TLC) on a polyethylenemine cellulose strip (Sigma-Aldrich) in 0.5 M LiCl and 1 M formic acid at room temperature for 1 h. The TLC plate was dried, autoradiographed and analyzed by a phosphorimager (Fujifilm-BAS-1800) for quantitation.

Nucleotide binding assay

UV-mediated cross-linking of [α-^{32}P]dATP. The cross-linking mixtures, in a final volume of 20 µl, contained 20 mM HEPES buffer (pH 7.5), 10% glycerol, 0.1 mM DTT, 10 µCi of [α-^{32}P]dATP (3000 Ci/mmol) and 1 µg of wild-type or mutated HpDnaB. The mixtures were incubated for 10 min on ice, followed by UV (254 nm) irradiation at a distance of 5 cm (Stratagene) for 30 min at 4°C. After termination of UV exposure, 0.8 µl of 100 mM dATP and 20 µg of BSA were added to reaction mixtures. Proteins were precipitated by trichloroacetic acid, washed with acetone containing 0.5% HCl and then twice with acetone. Proteins were separated by SDS–PAGE and the labeled protein was visualized by a phosphoimager.

Complementation assay

Complementarity of HpDnaB with its *E.coli* counterpart was tested by transforming *E.coli* strain DJ58 (*dnaB*^{ts}), a conditional lethal mutant for the *E.coli dnaB* allele, with recombinant plasmids containing HpDnaB, *E.coli* DnaB and DnaC (wild type) or pBR322 alone, followed by checking the survival of *E.coli* colonies at the non-permissive temperature in the presence of ampicillin. *Escherichia coli* plasmid pdnaBC was used as a positive control for complementation analysis.

RESULTS

Cloning and purification

In the *H.pylori* genomic database, an ORF HP1362 was annotated as the putative *H.pylori* DnaB homolog. When the amino acid sequence of the gene was aligned with its *E.coli* and *Campylobacter jejuni* counterparts, it was found that *H.pylori* DnaB is ~32% identical and 57% homologous to *E.coli* DnaB (Fig. 1A). Expression of the *dnaB* gene in *H.pylori* was confirmed by western blotting using anti-DnaB (*E.coli*) antibody (Fig. 1B).

In order to clone the *H.pylori dnaB* gene (HP1362; strain 26695) (9), the 1.5 kb DNA fragment representing the ORF was amplified from *H.pylori* strain 26695 genomic DNA by PCR. The amplified product was subsequently cloned into *E.coli* expression vector pET28a (Novagen) and sequenced. The cloned gene when expressed in the *E.coli* strain BL21 (DE3) produced a ~55 kDa polypeptide that matches with the calculated molecular weight of the His-tagged gene product (Fig. 2A). The protein was subsequently purified using an

Figure 1. Primary sequence analysis of HpDnaB and *in vivo* expression. (**A**) Protein sequence alignment of homologous DnaB proteins. Comparison of the *H.pylori*, *C.jejuni* and *E.coli* DnaB primary structures. The amino acid sequences of the respective proteins were aligned by CLUSTALW multiple alignment program. '*' indicates the residues which are identical, ':' indicates the residues which are strongly similar and '.' indicates the residues which are weakly similar. Nucleotide binding (Walker A) and hydrolysis (Walker B) motifs are also shown. (**B**) *In vivo* expression of DnaB in *H.pylori*. Western blot of *H.pylori* and *E.coli* cell extract using a polyclonal antisera against DnaB (*E.coli*). Lane 1 contains 1.0 µg of purified *E.coli* DnaB, lanes 2 and 3 contain cell extract of *H.pylori* (5.0 and 10.0 µg, respectively), lanes 4 and 5 contain cell extract of *E.coli* (5.0 and 10.0 µg, respectively) and lane 6 contains 100 ng of purified GST-tagged DnaB of *H.pylori*.

A

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H. pylori    --------------------MDHLKHLQQLQNIERIVLSGIVLANHKIEEVHSVLEPSDF 40
C. jejuni    -----------------------MQQEYYDLDLERAILSSCIMSEEAYASIAGDISPKDF 37
E. coli      MAGNKPFNKQQAEPRERDPQVAGLKVPPHSIEAEQSVLGGLMLDNERWDDVAERVVADDF 60
                              ::      : *: :*.. :: :.   .:   : ..**


H. pylori    YYPPNGLFFEIALKLHEEDCPIDENFIRQKMPKDKQIKEE-----DLVAIFAASPIDNIE 95
C. jejuni    SLKAHQDIFKAVIACSNNKEPISVSFLR----KHHKKIDEQ-----ILAEILATPSMIDLP 88
E. coli      YTRPHRHIFTEMARLQESGSPIDLITLAESLERQGQLDSVGGFAYLAELSKNTPSAANIS 120
                 .: :*       :. **. :     :. ::..         :.. ::


H. pylori    AYVEEIKNASIKRKLFGLANTIREQALES-AQKSSDILGAVEREVYALLNG--STIEGFR 152
C. jejuni    AYVNELHEKSVKRQLLSFAHLLPTRINE--DRAVSEIADEIGKEIFSITNR--VNSRDIK 144
E. coli      SYADIVRERAVVREMISVANEIAEAGFDPQGRTSEDLLDLAESRVFKIAESRANKDEGPK 180
             :*.: ::: :: *:::..*: :      :   :  .: .   .::  : :    ... :


                                              WALKER A
H. pylori    NIKEVLESAMDLITENQRKGSLEVTGIPTGFVQLDNYTSGFNKGSLVIIGARPSMGKTSL 212
C. jejuni    DVDMVISELFEEFKKQKTLENKGIIGLDTGFEGLNKMTKGFKGGELIIIAARPGMGKTTL 204
E. coli      NIADVLDATVARIEQLFQQPHDGVTGVNTGYDDLNKKTAGLQPSDLIIVAARPSMGKTTF 240
             ::  *:.  .  : :        : *: **:  *:: * *::  ..*:*:.***.****:: 


H. pylori    MMNMVL-SALNDDRGVAVFSLEMSAEQLALRALSDLTSINMHDLESGRLDDDQWENLAKC 271
C. jejuni    CLNFID-KVLRQKKGVALFSLEMPATQIMQRMLSSKTSIPLQKILTADLNDDEWERLGDA 263
E. coli      AMNLVENAAMLQDKPVLIFSLEMPSEQIMMRSLASLSRVDQTKIRTGQLDDEDWARISGT 300
              :*::.    .: :.: * :*****.: *:  * *:. : :    .: : :. *:*::* .:.


                                              WALKER B
H. pylori    FDHLSQKK-LFFYDKSYVRIEQIRLQLRKLKSQHKELGIAFIDYLQLMSGSKATKERHEQ 330
C. jejuni    CNDYSQKK-LYIYDSGYATIADVRAILRRLKSQDESIGLCVIDYIGLMMSNSNFNDRHLQ 322
E. coli      MGILLEKRNIYIDDSSGLTPTEVRSRARRIAREHGGIGLIMIDYLQLMRVPALSDNRTLE 360
              .   :*: ::: *..     ::*    *:: :.  :*: .***: **      .:*  :


H. pylori    IAEISRELKTLARELEIPIIALVQLNRSLENRDDKRPILSDIKDSGGIEQDADIVLFLYR 390
C. jejuni    VSEISRGLKLLARELDMPIIALSQLNRGLEQRANKRPLMSDLRESGAIEQDADAILFVYR 382
E. coli      IAEISRSLKALAKELNVPVVALSQLNRSLEQRADKRPVNSDLRESGSIEQDADLIMFIYR 420
             ::**** ** **:**::*::** ****.**:* :***: **::**.****** ::*:*:**


H. pylori    GYIYQMRAEDNKIDKLKKEGKIEEAQELYLKVNEERRIHKQNGSIEEAEIIVAKNRNGAT 450
C. jejuni    DEVYREQEEKERENKAKAEG------------KAYQRLFIPNPMQENAEIIVGKNRNGPV 430
E. coli      DEVYHENSD--------------------------------LKGIAEIIIGKQRNGPI 446
             . :*: . :                                ****:.*:***.


H. pylori    GTVYTRFNAPFTRYEDMPIDSHLEEGQETKVDYDIVTT 488
C. jejuni    GTIEVVFLKEKSCFVDKPIGYETTEFTG---------- 458
E. coli      GTVRLTFNGQWSRFDNYAGPQYDDE------------- 471
             **:  *   : : : .       *
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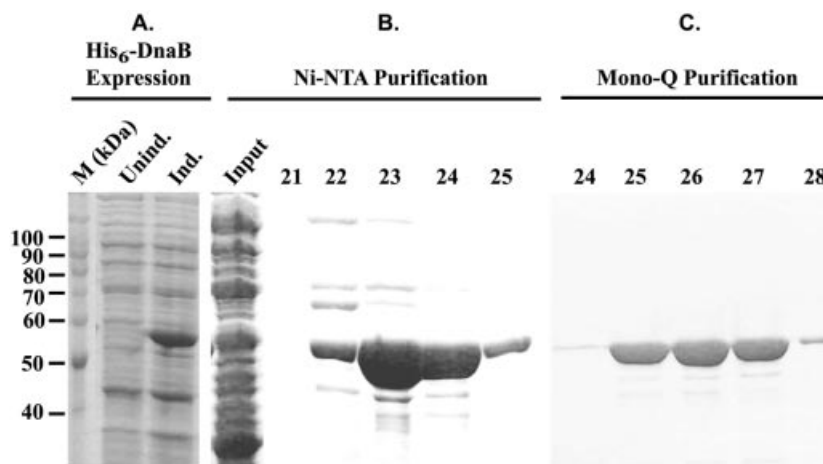*, identical; :, strongly similar; ., weakly similar

B
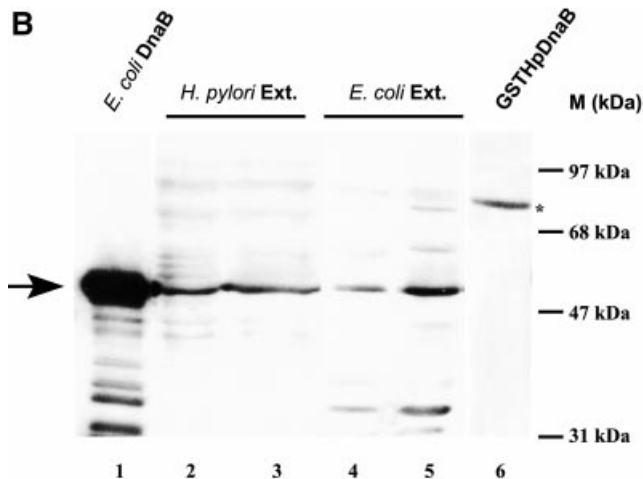
Figure 2. Purification of HpDnaB. (**A**) Induction of expression of His₆-HpDnaB protein in *E.coli* strain BL21(DE3). (**B**) Purification of His₆-HpDnaB by Ni-NTA resin. The fractions which contain the purified proteins are indicated on the top. (**C**) Purification of His₆-HpDnaB using an ion-exchange Mono-Q column. The fractions containing DnaB after Ni-NTA purification were pooled and passed through the Mono-Q column. The fraction numbers are indicated on the top.

Ni-NTA agarose (Qiagen) affinity matrix (Fig. 2B). The peak fractions were found to be >90% pure. The protein was further purified on a Mono-Q ion-exchange column to near homogeneity as shown in Figure 2C.

In vitro helicase activity of *H.pylori* DnaB

DnaB, a hexameric helicase is essential for replication of the bacterial chromosome, some bacterial phage genomes (like bacteriophage lambda and bacteriophage P2) and plasmid DNA (8,20,21). In order to test helicase activity of the purified HpDnaB, unwinding of a partial duplex DNA by the protein was monitored as described earlier (22). As shown in Figure 3A and B, the peak fractions from the Mono-Q column exhibited the protein concentration and ATP-dependent helicase activity, converting >80% of the partial duplex substrate to product. We also checked the effect of ATP concentration on helicase activity (Fig. 3B). HpDnaB helicase activity was stimulated at low ATP concentrations, reached a peak at 2 mM ATP concentration, followed by a decrease in the activity at higher concentrations. Since the Mg²⁺ ion concentration was not compensated with increasing ATP concentration, it is possible that Mg²⁺ ions become limiting with increasing ATP concentration, affecting both ATP hydrolysis and helicase activity.

The *in vitro* helicase activity was also dependent on the time of incubation. In the presence of 1.8 pmol (~100 ng) protein ~50% of the substrate was converted to product in 10 min (Fig. 3C). We also tested the effect of KCl concentration on helicase activity. Helicase activity was not affected up to 25 mM concentration. However, increasing the KCl concentration beyond that point inhibited the helicase activity (Fig. 3D).

HpDnaB unwinding activity ranges from ~45% to nearly 100% in different sets of experiments (Fig. 3A–D) under the same experimental conditions. This discrepancy in enzyme activity in different sets of experiments could be due to the variation in the enzyme activity in the different batches of enzyme purification.

Like other DnaB from different bacterial sources, HpDnaB was also found to be dependent on nucleoside triphosphate for helicase activity and ATP, GTP and UTP were found to be preferred compared with other NTPs and dNTPs (Fig. 4A). However, when ADP and ATPγS were included in the reaction mixture instead of ATP, no helicase activity was detected (Fig. 4B), suggesting nucleoside triphosphate hydrolysis is required.

Mutation in the ATP binding motif of DnaB affects helicase activity

To further test that the unwinding activity shown by HpDnaB was not due to the presence of any other minor contaminating proteins and the activity required NTP binding, a mutation was introduced in the Walker A motif (R→C at position 204) and the mutated protein was analyzed for helicase activity.

His-tagged mutant HpDnaB was purified following the wild-type HpDnaB purification protocol (Fig. 5A) and tested for helicase activity using the same 23mer partial duplex substrate.

No DNA unwinding activity was detected even at the highest protein amount tested, 3.6 pmol (~200 ng), whereas 1.8 pmol (~100 ng) of wild-type HpDnaB showed a strong helicase activity (Fig. 5B). This indicates that the helicase activity shown here is solely due to HpDnaB and not due to the presence of any other contaminating protein and also reconfirms that the helicase activity is dependent on ATP. In order to test the difference in nucleotide binding of wild-type and mutated HpDnaB, both wild-type and mutated HpDnaB proteins were incubated with [α³²-P]dATP and subjected to UV cross-linking analysis. Unlike wild-type protein, no ATP binding was detected in the case of mutated HpDnaB (Fig. 5C and D).

In solution, HpDnaB exists as a hexamer

Wild-type and mutated HpDnaB protein were subjected to size-exclusion chromatography on a calibrated Bio-Sil SEC 250-5 column. In each case, a single protein peak (Fig. 6A) was observed. Molecular mass standards were also subjected to gel filtration chromatography under the same conditions. A standard curve was obtained by plotting the molecular mass of the standards (in logarithmic scale) against the fraction
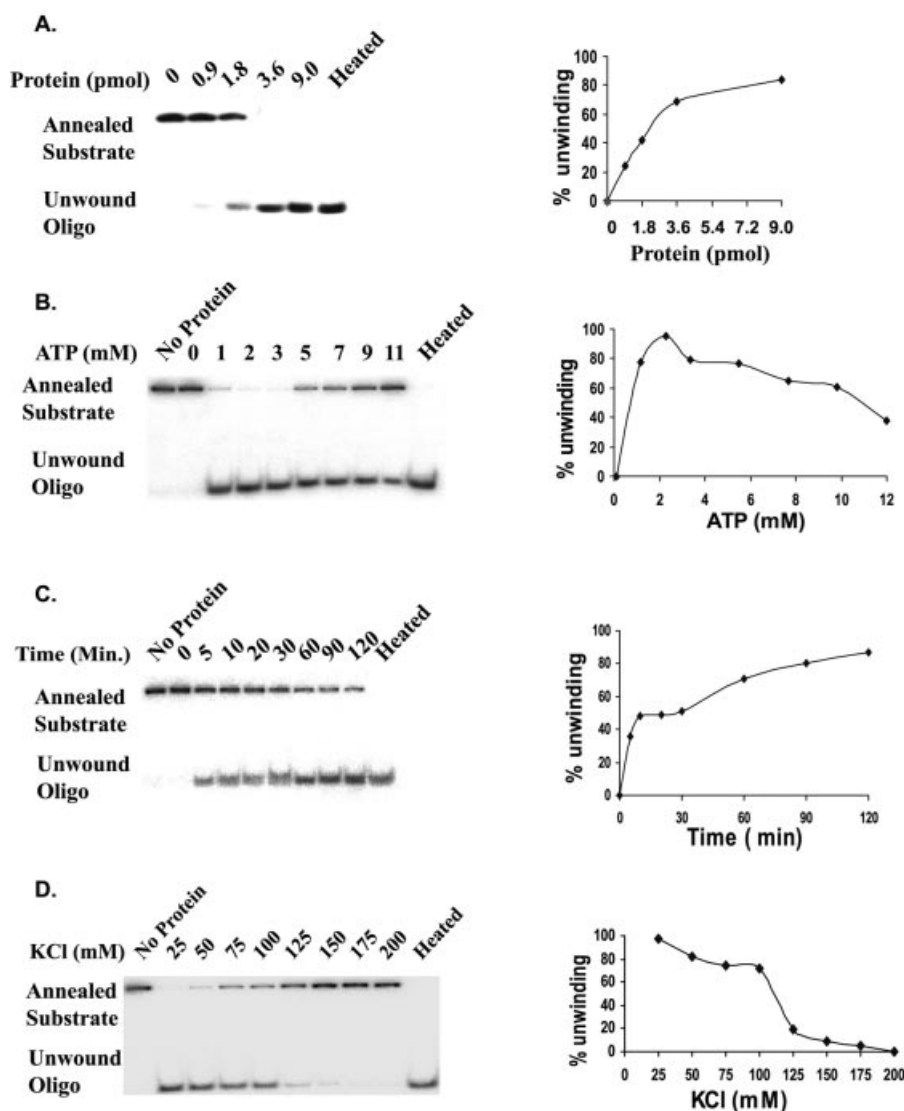
Figure 3. Effect of protein amount, ATP, time and KCl on DnaB helicase activity. Annealed substrates (18mer radiolabeled oligo annealed to M13mp19 ssDNA, as described in the Materials and Methods) were used in all four cases. (**A**) The helicase reaction was carried out using increasing amounts of purified protein (0.9 pmol to 9.0 pmol). Annealed and unwound oligos were separated by non-denaturing PAGE. (**B**) The helicase activity of HpDnaB was further characterized by using 1.8 pmol (~100 ng) of purified HpDnaB in the presence of increasing concentrations of ATP. (**C**) HpDnaB helicase activity varying the time of incubation of reaction or (**D**) increasing KCl concentration. Percent unwinding activity of HpDnaB for each reaction was also plotted.

number. From this plot, the native molecular mass of HpDnaB (Wt) and HpDnaB (Mut) was estimated to be ~360 kDa (Fig. 6B). SDS–PAGE followed by silver staining of the peak fractions revealed the presence of a ~55 kDa protein band that corresponds to His-tagged HpDnaB (Wt) and HpDnaB (Mut) (Fig. 6C).

HpDnaB shows ATPase activity

All the helicases tested so far, have been shown to require NTPase activity for helicase action (23,24). It has already been shown that a non-hydrolysable ATP analog could not support unwinding activity (Fig. 4B). Therefore, HpDnaB was tested for associated ATPase activity. To test ATPase activity, increasing amounts of HpDnaB were incubated with [γ-^{32}P]ATP and released free phosphate from labeled ATP was monitored following TLC.

Wild-type HpDnaB showed strong ATPase activity as evidenced by the release of more free phosphate with increasing amounts of HpDnaB (Fig. 7A). However, mutated HpDnaB failed to show any ATPase activity, even at the highest amount of protein tested (Fig. 7A), suggesting that mutation in the Walker A motif also affects ATP hydrolysis, which is expected since mutated HpDnaB cannot bind ATP. The HpDnaB-associated ATPase activity was also observed to be stimulated by ssDNA (Fig. 7B), suggesting that HpDnaB has DNA-dependent helicase activity.

Hydrolysis of NTPs also depends on divalent cations. We first titrated the Mg^{2+} ion concentration in helicase reactions and 2 mM Mg^{2+} was found to be optimal for the helicase activity (data not shown). Later, we used several divalent cations to check their effect on helicase activity. At 2 mM concentration of all the divalent cations used, Mg^{2+} was found
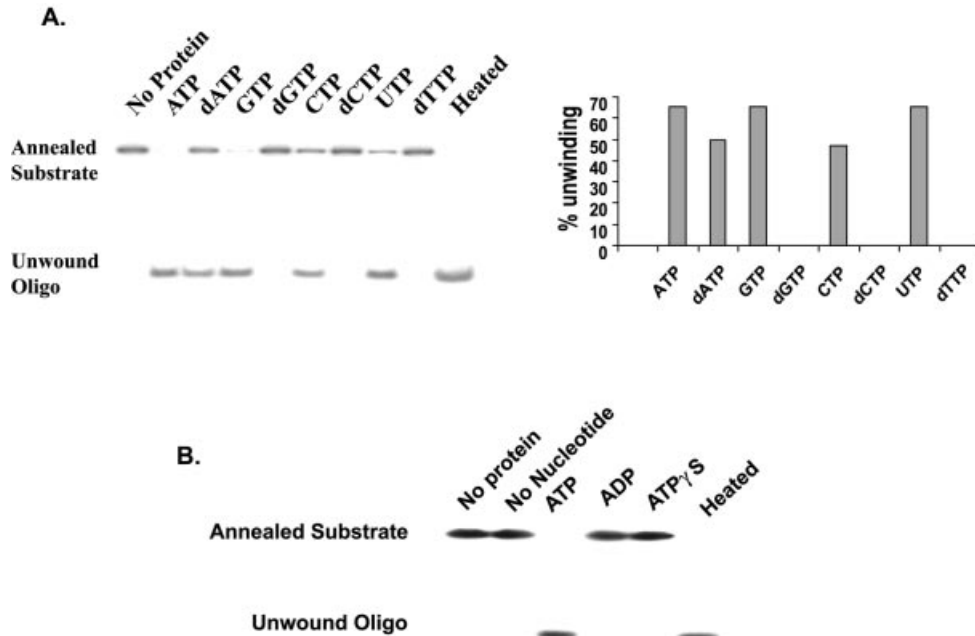
Figure 4. Effect of nucleotides and divalent cations on DnaB helicase activity. (**A**) The helicase reaction was carried out using 1.8 pmol (~100 ng) of purified protein in the presence of a 5 mM concentration of various nucleotides, as indicated on the top. (**B**) The effect of ATP analogs was tested on HpDnaB helicase activity. The substrates used in both cases were 18mer radiolabeled oligos (as described in the Materials and Methods) annealed to M13mp19 ssDNA.
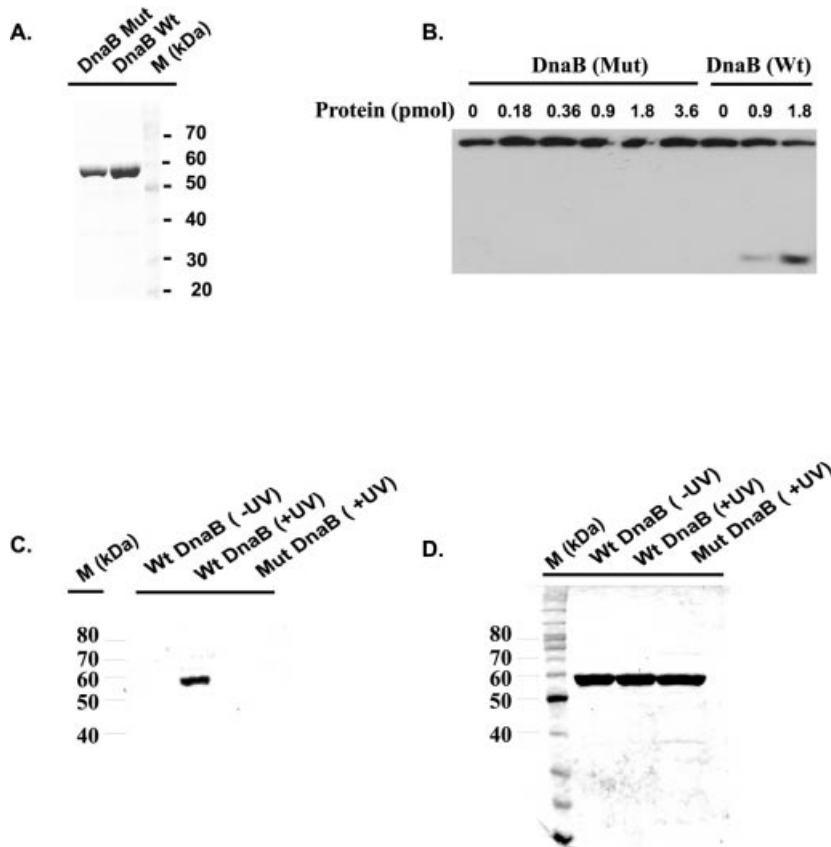

Figure 5. Helicase and nucleotide binding activity of HpDnaB (Wt) and HpDnaB (Mut). (**A**) Purification of wild-type and mutated HpDnaB proteins. (**B**) The effect of wild-type and mutated HpDnaB on helicase activity. (**C**) HpDnaB (Wt) but not HpDnaB (Mut) binds to radiolabeled nucleotide in the presence of UV light. One microgram of the wild-type or mutated DnaB was incubated with [α-^{32}P]dATP and was further cross-linked using UV, as described in Materials and Methods. (**D**) Gel showing the presence of proteins in all the lanes of Figure 6C.
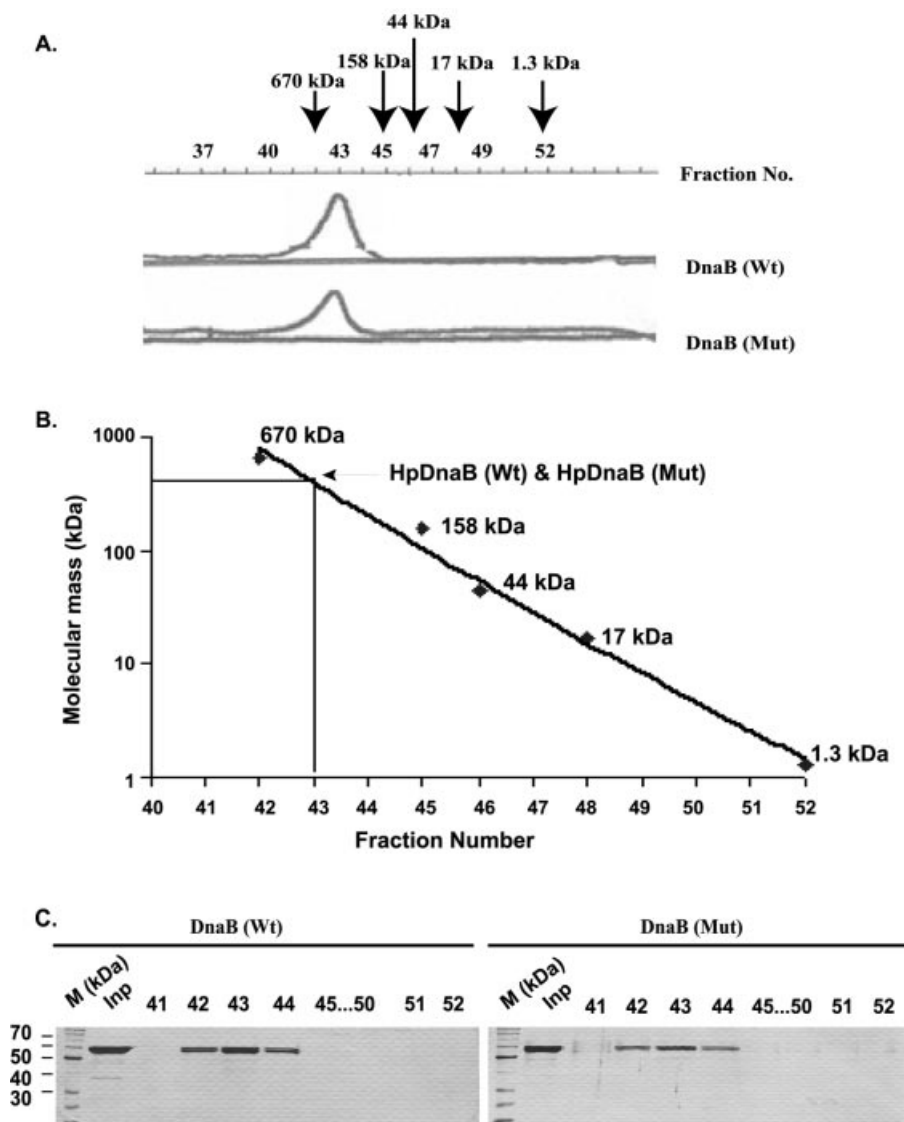
Figure 6. Size-exclusion chromatography of wild-type and mutated DnaB. (**A**) Wild-type or mutated DnaB was passed through a Bio-Sil SEC 250-5 column and 0.2 ml fractions were collected in each case. The fraction numbers and the elution pattern of the proteins are shown. The elution pattern of the gel filtration standards is marked on the top as followed: thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B-12 (1.35 kDa). (**B**) Estimation of molecular masses of HpDnaB (Wt) and HpDnaB (Mut). Molecular masses of the standards were plotted in logarithmic scale against the fraction numbers. Molecular masses of HpDnaB (Wt) and HpDnaB (Mut) were deduced from the plot. (**C**) SDS–PAGE followed by Coomassie stain of the gel filtration fractions.

to be the most effective for helicase activity (Fig. 7C). It has been shown earlier that DnaB helicase exists as a stable hexamer in a large protein concentration range and this form is specifically stabilized by magnesium (25). The order of divalent cation preference on helicase activity is as follows: $Mg^{2+} > Mn^{2+} > Ca^{2+} > Co^{2+} > Zn^{2+}$. No activity was measured in the absence of divalent cations.

HpDnaB has 5′ to 3′ polarity

The direction of unwinding by a helicase is defined by the strand to which the enzyme binds and moves along that strand. All the DNA helicases characterized so far exhibited either 5′ to 3′ or 3′ to 5′ polarity. *Escherichia coli* DnaB protein unwinds DNA in a 5′ to 3′ direction (26). In order to test the

polarity of HpDnaB helicase action, two radiolabeled (at 5′ or 3′) partial duplex substrates were linearized by digestion with SmaI restriction enzyme to generate a duplex DNA at both ends of a long linear ssDNA molecule (26). In the presence of HpDnaB, the displacement of a specific labeled fragment (5′ or 3′ end-labeled) was followed to determine directionality of action.

Experimental results as shown in Figure 8A, exhibited only displacement of the 3′-end-labeled ssDNA fragment, indicating that HpDnaB possesses a 5′ to 3′ polarity. The partial duplex substrates used in this set of experiments did not have any short 5′ or 3′ tail attached to them, however, in subsequent experiments substrates containing 5′ or 3′ tails were also tested. Under the same experimental conditions, oligos having
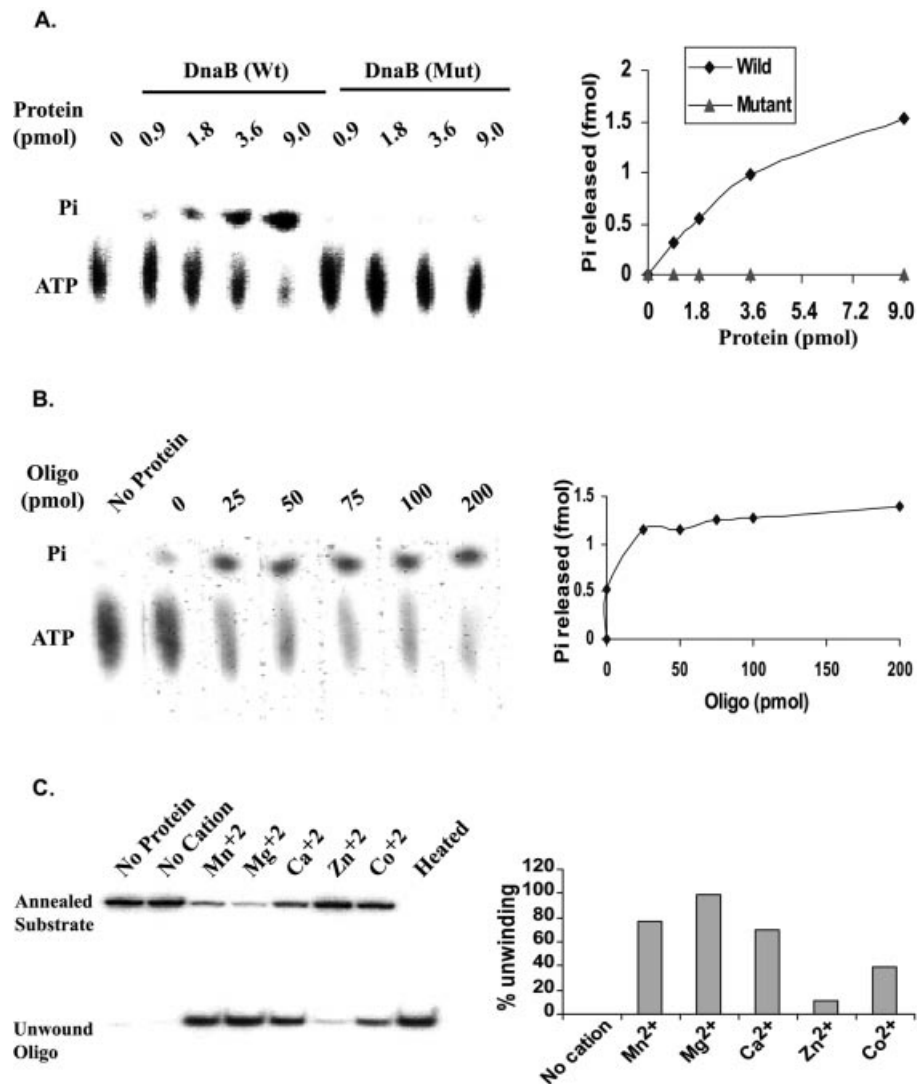
Figure 7. Characterization of ATPase activity of HpDnaB (Wt) and HpDnaB (Mut). (**A**) ATPase activity is enhanced with increasing amounts of HpDnaB (Wt). HpDnaB (Mut) did not show any ATPase activity. Release of radiolabeled inorganic phosphate (Pi) is also marked. (**B**) Analysis of DNA-dependent ATPase activity of HpDnaB. Helicase activity was measured in the presence of 0.9 pmol of HpDnaB and increasing amounts of 36mer oligonucleotides. Release of inorganic phosphate was quantitated and plotted against protein or oligo concentration. (**C**) Effect of divalent cations on helicase activity. Helicase assays were performed in the presence of various divalent cations (2 mM each) as indicated. Percent unwinding activity in the presence of nucleotides and divalent cations is also shown. 18mer oligos annealed to M13mp19 ssDNA were used as substrate.

a 5′ single-stranded tail were released more efficiently than oligos having no tail or having a 3′ single-stranded tail (Fig. 8B).

HpDnaB rescues the temperature-sensitive phenotype of *E.coli dnaB*[ts]

The comparison of primary sequences of *E.coli* and *H.pylori* DnaB (Fig. 1A) shows ~32% identity and 57% similarity between the two proteins. *In vitro* characterization of the HpDnaB also indicated that, like its *E.coli* counterpart, it is an ATP-dependent helicase, possesses ATPase activity and exists in solution as a hexamer. We were interested to test whether HpDnaB can rescue a temperature-sensitive DnaB function in *E.coli*. To carry out complementation analysis, a His-tagged *dnaB* gene was subcloned into pBR322 under the control of Bla-P2 promoter (see Materials and Methods) and *E.coli* strain

DJ58 (*dnaB*[ts]) was transformed with the recombinant plasmid. HpDnaB was found to complement the defective *dnaB* gene in *E.coli* at 40°C, whereas pBR322 and HpDnaB (Mut) failed to do so (Fig. 9A) at the same temperature. When the same *E.coli* strain was transformed with a plasmid containing wild-type *E.coli dnaB* (pdnaBC), it also complemented the defective function (Fig. 9A, sector 4) at 40°C. We further tested HpDnaB expression in *E.coli* strain DJ58 at 40°C (Fig. 9B) by western blotting using anti-His antibody. These results taken together suggest that, in fact, the ORF HP1362 encodes a functional homolog of replicative DnaB helicase.

In *E.coli*, DnaC is essential to load DnaB on *ori*C. The complementation of *E.coli dnaB*[ts] with HpDnaB raises the issue as to whether HpDnaB interacts with DnaC to facilitate the loading of HpDnaB. In order to address this issue, we performed gel filtration analysis using either purified HpDnaB
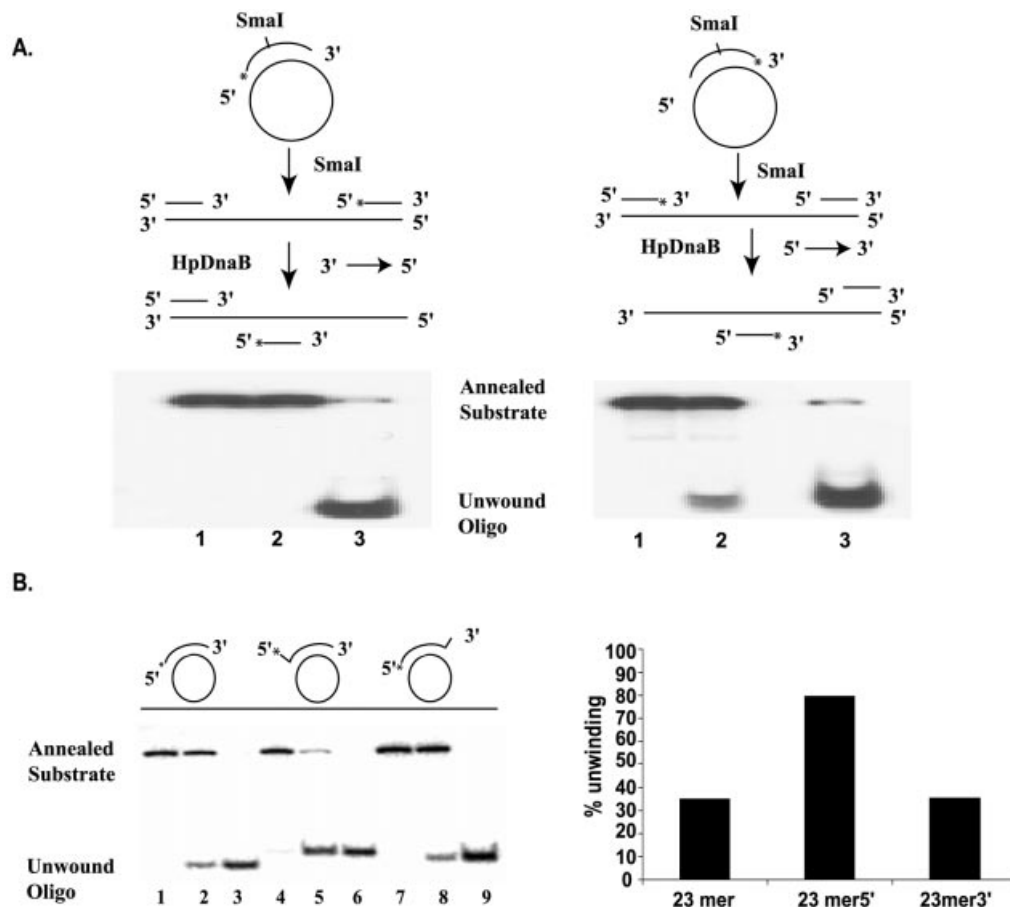
Figure 8. Effect of various substrates on DnaB helicase activity. (**A**) Polarity of DnaB helicase. Helicase reaction was carried out either using, 5′-end-labeled 18mer synthetic oligo substrate or 3′-end-labeled 18mer synthetic oligo substrate (generated by SmaI digestion of 36mer-labeled DNA annealed to M13mp19 ssDNA, M13mp19). All the oligos are described in the Materials and Methods. Lane 1 contains no protein, lane 2 contains 0.9 pmol of HpDnaB and lane 3 contains heated substrate. (**B**) Influence of 5′ and 3′ tail on helicase activity. Substrates containing no tail (lanes 1–3), 5′ tail (lanes 4–6) or 3′ tail (lanes 7–9) were used for the helicase assay.

or *E.coli* DnaC (EcDnaC) alone or the mixture of HpDnaB and EcDnaC, followed by western blot analysis of the peak fractions of gel filtration eluates using anti-His antisera in each case. Purified EcDnaC runs as a monomer when compared with the molecular mass marker (data not shown). We have already shown that HpDnaB runs as a multimer following gel filtration (Fig. 6A). When HpDnaB and EcDnaC were mixed together and subjected to gel filtration chromatography, both proteins were co-eluted in the same fraction, suggesting that these proteins may physically interact with each other (Fig. 9C). Therefore, the loading of HpDnaB on *ori*C could be mediated through EcDnaC.

DISCUSSION

In this work, we report the identification and characterization of an important *H.pylori* DNA replication protein, DnaB helicase. Unlike *E.coli*, *H.pylori* lacks an obvious helicase loader DnaC homolog from the genome sequence. Therefore, the characterization of HpDnaB homolog might help in understanding the basic mechanism of helicase loading in this organism.

This report shows that HpDnaB is a helicase and it is active in the presence of a number of hydrolysable NTPs. ATP, GTP or UTP are equally active as co-factors whereas CTP is somewhat less active. In contrast, *E.coli* DnaB was shown to be inactive in the presence of UTP (26). Among the four dNTPs, only dATP supported helicase activity of HpDnaB, whereas both dATP and dCTP were shown to support the helicase activity of *E.coli* DnaB. Like *E.coli*, HpDnaB requires Mg^{2+} for the optimum activity (26). Mn^{2+} and Ca^{2+} are also well tolerated by HpDnaB for the helicase activity, although it has been reported in the literature that for several helicases, Ca^{2+} could not be a substitute for Mg^{2+} (27,28). Like other helicases, HpDnaB showed the optimal helicase activity in the presence of 2 mM ATP. Monovalent cations (K^+) inhibited the helicase activity at a concentration higher than 100 mM. This phenomenon is also common for many other helicases.

Helicases are known to bind to the single-stranded region and then proceed either in a 5′ to 3′ or 3′ to 5′ direction depending on their polarity and thereby unwinding the duplex using NTPs as an energy source (23). A similar type of polarity has also been observed for a number of proteins of the DnaB family (26,29–32). It has been shown earlier that the 3′
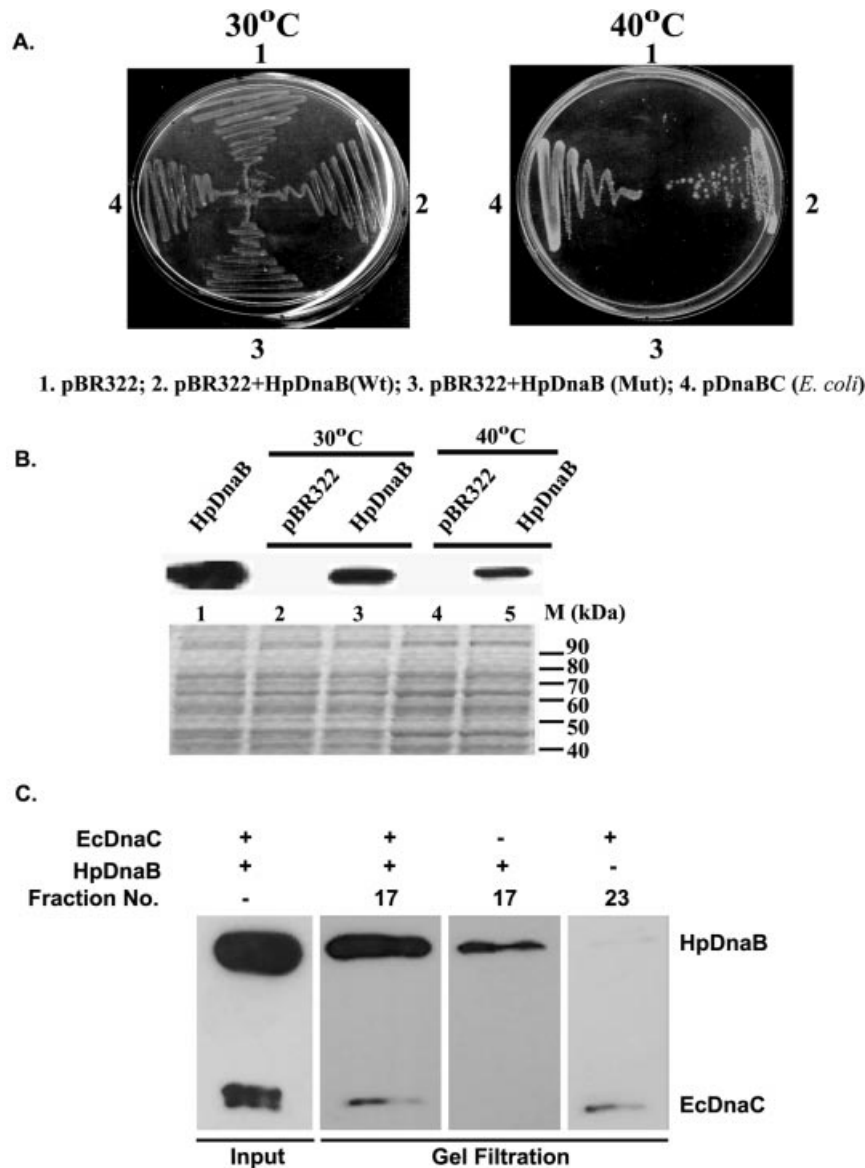
Figure 9. Complementation analysis. (**A**) Complementation of *E.coli* DJ58[ts] with plasmids expressing wild-type or mutated HpDnaB. *Escherichia coli* DJ58[ts] cells were transformed with either pBR322 (1), pBR322+HpDnaB (Wt) (2), pBR322+HpDnaB (Mut) or pdnaBC (*E.coli*) (4). Cells were plated on LB agar plates and incubated either at the permissive temperature (30°C) or at the non-permissive temperature (40°C). (**B**) Detection of HpDnaB expression in *E.coli* DJ58[ts] by immuno-blotting using anti-His antibody. Lane 1, purified HpDnaB; lanes 2 and 3, extract from pBR322 and HpDnaB transformed cells at the permissive temperature (30°C); lanes 4 and 5, extract from pBR322 and HpDnaB transformed cells at the non-permissive temperature (40°C). (**C**) Gel filtration analysis of His-EcDnaC, His-HpDnaB or a mixture of both. The above proteins were subjected to gel filtration chromatography using a Bio-Sil SEC 250-5 column. Fractions of 0.5 ml were collected in each case. The peak fraction in each case was subjected to SDS–PAGE followed by western blot analysis using anti-His antibodies. Fraction numbers are shown on the top. Input lane shows the presence of both the proteins used for the gel filtration analysis.

tail of a forked duplex DNA substrate stimulates unwinding in a length-dependent manner in the presence of the DnaB family of helicases (33). According to a recent model proposed by Kaplan (34), a forked structure containing a short 3′ tail allows DnaB to pass through the central channel without showing any helicase activity. The length of the 3′ tail determines whether one or two DNA strands will pass through the central channel of hexameric DnaB. In contrast, we observed unwinding activity in all the partial duplexes tested with or without a short 3′ tail. This discrepancy could be due to the differences in the substrate structure used. The possibility of non-specific

activity of HpDnaB can be ruled out since the mutant protein with a mutation in the NTP binding motif fails to show helicase activity. Interestingly, we observed stimulation in helicase activity in the presence of a short 5′ tail. HpDnaB can be loaded simultaneously on 5′ tail and ssDNA in the partial duplex substrate followed by subsequent movement in the 5′ to 3′ direction.

HpDnaB is 32% identical and 57% similar to *E.coli* DnaB. The C-terminal half of the protein shares more homology with *E.coli* DnaB than the N-terminal half. In general, the C-terminal half of hexameric helicases contains all the motifs

required for the helicase activity (35). Therefore, the similarity found in *in vitro* biochemical functions of HpDnaB with its *E.coli* counterpart could be due to the similarity in the C-terminal half. *Escherichia coli* DnaB has been dissected into three domains (35) and the first 150 amino acids have been assigned to energy transduction and protein–protein interaction. Thus, the less conserved N-terminal domain of *H.pylori* DnaB may functionally play some unique role in the initiation of DNA replication. Further dissection of the N-terminal half of the HpDnaB will be required to address these issues.

Unlike some enterobacteria such as *Salmonella typhimurium* (36) and *Shigella flexneri* (37), *H.pylori* lacks the *E.coli dnaC* homolog. The absence of a *dnaC*-like gene in many other bacteria whose genome sequences are available now, raises the question as to whether DnaC is essential for replication as a helicase loader. However, there are many examples of proteins both in prokaryotes and in eukaryotes that are used as helicase loaders without sharing much homology with DnaC. The best examples are DnaI in *Bacillus* (38–40) and Cdc6 in mammals which load Mcm helicases (41).

It is still not clear whether DnaB can be loaded at origin in the absence of DnaC.

Recently, it has been shown that *P.putida* and *P.aeruginosa* DnaB helicases could be loaded on plasmid RK2 *ori*V in the absence of DnaC-like ATPases (16). Whether the independent loading of DnaB is possible at the chromosomal origin of *Pseudomonas* is still not known.

The temperature-sensitive phenotype of the *E.coli* mutant DnaB252 containing a mutation in the amino acid residue 299 can be rescued by over-expression of DnaC, suggesting that this region could be important for DnaB–DnaC interaction (42). Interestingly, the residue 299 and surrounding amino acids are highly divergent between *E.coli* and *H.pylori* DnaB counterparts. Therefore, apparently HpDnaB may not interact with *E.coli* DnaC. However, we have shown clearly that HpDnaB can functionally complement the *E.coli* DnaB temperature-sensitive mutant. Since the loading of *E.coli* DnaB is dependent on DnaC, it is possible that HpDnaB also interacts with EcDnaC to facilitate the loading of HpDnaB. By gel filtration analysis we showed that HpDnaB and EcDnaC co-eluted in the same fraction. This clearly suggests that HpDnaB has an affinity towards EcDnaC. These findings strengthen the hypothesis that there could be a functional homolog of DnaC in *H.pylori* which helps loading of HpDnaB on an *ori*C equivalent of *H.pylori*. However, it is also not unlikely that HpDnaB can be loaded on *ori*C by itself, independent of DnaC. Further careful studies are required to address these issues.

Recently, using a high-throughput yeast two-hybrid assay, 261 *H.pylori* proteins were screened against a highly complex library of genome-encoded polypeptides (43). According to this study, two ORFs (HP0897 and HP0340) with unknown functions were reported to be interacting partners of HpDnaB. It will be interesting to see whether these proteins physically interact with HpDnaB and thereby facilitate its loading on the chromosomal origin of replication. Considering the high prevalence of drug resistance in *H.pylori*, identification of such candidates will be useful for screening novel drug targets.

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