Bacteriophage P1 Ban protein is a hexameric DNA helicase that interacts with and substitutes for Escherichia coli DnaB

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ABSTRACT

Since the ban gene of bacteriophage P1 suppresses a number of conditionally lethal dnaB mutations in Escherichia coli, it was assumed that Ban protein is a DNA helicase (DnaB analogue) that can substitute for DnaB in the host replication machinery. We isolated and sequenced the ban gene, purified the product, and analysed the function of Ban protein in vitro and in vivo. Ban hydrolyses ATP, unwinds DNA and forms hexamers in the presence of ATP and magnesium ions. Since all existing conditionally lethal dnaB strains bear DnaB proteins that may interfere with the protein under study, we constructed a dnaB null strain by using a genetic set-up designed to provoke the conditional loss of the entire dnaB gene from E.coli cells. This novel tool was used to show that Ban restores the viability of cells that completely lack DnaB at 30°C, but not at 42°C. Surprisingly, growth was restored by the dnaB252 mutation at a temperature that is restrictive for ban and dnaB252 taken separately. This indicates that Ban and DnaB are able to interact in vivo. Complementary to these results, we demonstrate the formation of DnaB–Ban hetero-oligomers in vitro by ion exchange chromatography. We discuss the interaction of bacterial proteins and their phage-encoded analogues to fulfil functions that are essential to phage and host growth.

INTRODUCTION

DnaB of Escherichia coli is the paradigmatic member of the replicative helicases family of hexameric ring-shaped enzymes that use the energy of nucleotide hydrolysis to unwind duplex DNA at a replication fork (1). In addition, it has been recently suggested that DnaB could participate in DNA recombination, since DnaB drives DNA branch migration in vitro (2). DnaB is not only required for E.coli chromosome replication and cell growth, but also for the replication of most plasmids and bacteriophages that use this bacteria as a host. Yet, E.coli lysogens of particular phage systems were found to survive under conditions that are restrictive for dnaB function, thus suggesting the existence of bacteriophage-encoded functional homologues of DnaB (3–6). Particularly, bacteriophage P1bac mutants were shown to constitutively express a gene, ban (for DnaB analogue), that compensates for several dnaB-defective host mutations, notably insertions and unsuppressed amber mutations (5–8). Furthermore, converging genetic and biochemical approaches suggested that Ban and DnaB could interact and form hetero-hexamers in vivo (8,9). These data supported the hypothesis that Ban protein is not only able to substitute for DnaB in E.coli cells, but also to form functional hetero-oligomeric structures. However, because the ban gene was never isolated, nor the enzymatic properties of its purified product biochemically characterised, these issues have remained as open questions for more than 20 years. Here we address these questions and further investigate the properties of the Ban protein. We have: (i) isolated the ban gene and expressed it in a strain that completely lacks DnaB; (ii) purified the Ban protein and tested its DNA unwinding and ATP hydrolysis activities; (iii) tested in vivo and in vitro the hypothesis that Ban and DnaB are able to form hetero-oligomers. Our data show that Ban is a bona fide DNA helicase, and that it can substitute for DnaB in E.coli cells that completely lack this protein. We also provide genetic and biochemical evidence that DnaB and Ban are able to interact.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Table 1 lists the E.coli strains, plasmids and bacteriophages used in this study. Mutation rpsL266 was transferred from LN2843 (a gift from F. Cornet, CNRS, Toulouse, France) to W3110 by transduction with P1vir to yield MLM329. Bacteriophage P1Cm (10) was the source for the ban gene, and single-stranded circular DNA was obtained from M13mp18. To overproduce and purify the Ban protein, strains were grown in media as described previously (11). For other
purposes, cultures were grown with aeration in Luria–Bertani (LB) broth (12), supplemented with 1.5% agar for solid medium. Antibiotics were added when appropriate to the following concentrations: ampicillin (sodium salt), 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin sulfate, 50 μg/ml; streptomycin, 200 μg/ml; tetracycline, 2.5 μg/ml.

Construction of ban overexpressing plasmids

pMLM115. Vector plasmid pFUS2 (KmR) (13), is a pMB1-derived replicon designed to allow the expression of genes under the control of the arabinose regulated ParaBAD promoter. Plasmid pMLM115, in which the ban gene is placed under the control of ParaBAD, was constructed by inserting a PCR fragment containing the ban gene amplified with primers ban5 (5′-CATACGCAATGTCCCGATC-CCCTCTT) and ban3 (5′-CCGGAGCTTAAATCTCCTTGGCCAGT), and cleaved with Ndel and HindIII into the low copy number and replication proficient plasmid PMS470Δ8 (16) to result in pTR101.

pTR101. To construct the ban overexpression plasmid pTR101, the ban gene was amplified by PCR using P1cm DNA as template and a pair of primers, each containing the recognition sequences for Ndel and HindIII, which are underlined (5′-TTTATATATATagTCGATCCCCCTCTTGAACTCC, 5′-TAATAGCTCGTATCC-GTCGAGTCCGAGC). The codons delimiting the ban reading frame are in lower case. The fragment was inserted into the Ndel–HindIII digested PMS470Δ8::Tc regulated plasmid pMS470Δ8 (16) and cloned into the EcoRI and BamHI sites of plasmid pMS470Δ8::Tc/pPLM105.

Construction of dnaB overexpressing plasmids

pMLM105/pMLM116. The procedures to construct plasmids pMLM105 and pMLM116 were identical, except for the dnaB alleles that were used (dnaB and dnaB252, respectively). PCR fragments containing either the dnaB or the dnaB252 genes were amplified from total genomic DNA of strains W3110 and RS162, respectively (Table 1) with primers dnaB56 (5′-GGATCCAACAGTTGCCGCTTGCATT), cleaved with EcoRI and BamHI with and cleaved with EcoRI and BamHI into the EcoRI and BglII sites of plasmid pFUS2. This places the dnaB alleles under the control of the ParaBAD promoter.

pMLM121. To transfer the ParaBAD::dnaB construct from pMLM105 into the low copy number and replication temperature-sensitive plasmid pLN135 (Table 1), the large AlwNI–HindIII fragment of pMLM105 was inserted between the BglII and HindIII sites in pLN135, yielding pMLM121.

Table 1. Strains and plasmids used in this work

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**DnaB substitution in the chromosome: construction steps and corroborations by PCR and immunodetection**

**Construction of the dnaB368::Ω-Tc integration plasmid pMLM125.** In the pMLM122 plasmid, the HpaI–BamHI chromosomal fragment (4034 bp) containing the dnaB gene and its flanking regions was inserted into the pACYC184 plasmid (17) cleaved with NruI and BamHI. A BamHI–Eco47III fragment from pMLM122 containing the entire 4034 bp dnaB region was ligated to a vector fragment obtained from cleavage with Xhol (followed by end-repair using Klenow polymerase) and BglIII of the integration-excision vector pLN135 (18), to give pMLM123. To replace the wildtype dnaB gene in pMLM123 with the dnaB368 amber mutant allele, borne by plasmid pBRdnaB368 (M. Lemmonier and R. Díaz-Orejas, unpublished results), the dnaB-containing NdeI–BstXI fragment from pMLM123 was excised and replaced with the equivalent NdeI–BstXI fragment from pBRdnaB368, to give pMLM124. Next, the bulk of the dnaB gene was removed by cutting pMLM124 with Cclal and the remaining plasmid DNA was end-repaired using Klenow polymerase and ligated to a SmaI fragment containing tet obtained from pHP45Ω–Tc (19), to give plasmid pMLM125.

**Integration of the dnaB368::Ω-Tc construct into the chromosome.** The multi-step procedure to substitute the dnaB368::Ω–Tc mutation present in pMLM125 for dnaB in the chromosome was performed as described by Cornet et al. (18). Briefly, pMLM125 was used to transform the streptomycin-resistant (SmR) strain MLM329. Integration of pMLM125 into the chromosome was selected by plating cells on chloramphenicol-containing medium at 42°C. Colonies obtained were cultured in liquid in the same medium and temperature conditions and were transformed with a pUC18 derivative carrying the dnaB and dnaC genes (pPS562) (Table 1). Transformants were then plated at 37°C on medium containing streptomycin, to select for the excision of dnaB from the chromosome (this event is tightly linked to the loss of the pMLM125 borne rpsL* allele that confers sensitivity to streptomycin), and tetracycline, to select for the presence of dnaB368::Ω–Tc in the chromosome. This procedure yielded the MLM337 strain.

**Construction of MLM368, the indicator strain for the complementation of the dnaB368::Ω–Tc null mutation.** Strain MLM337 was transformed with plasmid pMLM121. Cells were then cured from the resident pPS562 plasmid by replica plating for several rounds at 30°C in solid medium without ampicillin. Chloramphenicol was included to maintain the pMLM121 plasmid. This yielded the MLM368 strain.

**Amplification of the dnaB locus by PCR.** Total DNA was extracted from MLM337 (and from its parental dnaB+ counterpart MLM329, as a control) and was used as template in polymerase chain reactions (PCR) with primers dnaB53 (5′-CTGCTGCTTCGGTGCCTAATC), dnaB33 (5′-CGCGCAGACGCCCATAAAGAAAT) in one series of reactions, and with dnaB53 and tet1 (5′-TAGGCCGCGCCCTATACCTTGTC) in a second series. Identical conditions were used for all the reactions: 10 ng of chromosomal DNA as template, 200 pmol of each primer, DNA polymerase Pfu (Stratagene) and 25 cycles of 95°C for 45 s, 62°C for 1 min, 72°C for 7.5 min followed by 72°C for 10 min.

**Immunoblot analysis using anti-DnaB serum.** Cells were grown exponentially at 30°C in medium containing kanamycin. At an A600 of ~0.3, cells were induced or not with arabinose (0.2% final concentration). After 15 min, samples were collected and resuspended in SDS–mercaptoethanol buffer (20) and subjected to electrophoresis in 15% SDS–polyacrylamide gels. Proteins were transferred to Sequi-Blot PVDF membranes (Biorad) in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol) using a Trans-Blot Semi-dry transfer cell (Biorad) as recommended by the manufacturer. Membranes were treated with polyclonal anti-DnaB serum from sheep (1:2500 dilution) and bound antibodies were detected using an ECL Western Blotting detection kit (Amersham Pharmacia Biotech) as recommended by the manufacturer.

**Purification of the Ban protein**

A 4.8 l culture of SCS1(pTR101) was grown at 37°C with shaking. At an A600 of 0.5, isopropyl β-D-thiogalactopyranoside was added to 1 mM. Shaking was continued for 5 h. Cells were centrifuged at 4000 g for 10 min, resuspended in 1 mM spermidine tris(hydrochloride), 200 mM NaCl and 2 mM EDTA (pH 7.5) (1 g of wet cells in 5 ml) and frozen in liquid nitrogen. All the following steps were performed at 0–4°C. Frozen cells were thawed (15 g in 75 ml) and adjusted to 40 mM Tris–HCl pH 7.6, 4% sucrose, 0.13% Brij-58, 50 mM NaCl, 2.5 mM diethiothreitol (DTT) and 0.3 mg/ml lysozyme. Following incubation for 1 h, the highly viscous lysate was centrifuged at 100 000 g for 60 min. The pellet was washed with 1 M NaCl in buffer A (20 mM Tris–HCl pH 7.6, 2.5 mM EDTA and 10% (wt/vol) glycerol) containing 6 M urea and 100 mM NaCl to dissolve Ban. The supernatant of both urea steps were combined, and the proteins were precipitated with ammonium sulphate at 55% saturation. The pellet was solubilised in buffer B [20 mM Tris–HCl pH 7.6, 1 mM DTT, 0.1 mM EDTA and 10% (wt/vol) glycerol] containing 6 M urea and 100 mM NaCl. Ban was renatured by dialysis against buffer B containing 100 mM NaCl (fraction I, 107 ml). Fraction I was applied at 80 ml/h to a DEAE Sephacel column (2.6 × 20 cm), equilibrated with buffer B containing 100 mM NaCl. The column was washed with 250 ml of buffer B 100 mM NaCl. Proteins were eluted with an 800 ml linear gradient of 0.1 to 0.8 M NaCl in buffer B. Ban eluted at 500 mM NaCl. Peak fractions were pooled (fraction II, 95 ml). A 20 ml portion of fraction II was adjusted to 5 mM MgCl2 and loaded at 10 ml/h onto an ATP agarose column (0.9 × 7 cm) equilibrated with buffer B containing 450 mM NaCl and 5 mM MgCl2. The column was washed first with 15 ml of the same buffer, and then with 15 ml 10 mM AMP and 10 mM MgCl2 in buffer B without glycerol. Ban was eluted by 10 mM sodium pyrophosphate and 5 mM MgCl2 in buffer B without glycerol. The protein was concentrated by dialysis against 20% (wt/vol) polyethylene glycol 20 000 and dialysed against 50% (wt/vol) glycerol in buffer B containing 5 mM MgCl2, and stored at –20°C for at least 2 years without loss of activity.
Ban protein was also purified from the dnaB null strain MLM371 under native conditions. Based on the procedure described above, the potential inclusion bodies were extensively extracted in the presence of 1 M NaCl that eluted part of the Ban protein. Phenyl-Sepharose was used instead of ATP-agarose to obtain nearly homogenous protein. The protein was eluted with a linear gradient of ethylene glycol (0–80%). The yield is ~30% of that of the urea procedure.

Helicase and ATPase assays

A forked helicase substrate based on that of Crute et al. (21) was used. To viral M13mp18 DNA, a 5'-32P-labeled 53mer oligonucleotide was annealed, resulting in a double-stranded segment of 31 bp and 22 unpaired nucleotides at the 3' end. Helicase reactions were performed essentially as described at 30°C for 20 min in 20 μl of buffer containing 20 mM Tris–HCl pH 7.6, 10 mM MgCl2, 1 mM DTT, 2 mM ATP and 50 μg/ml bovine serum albumin (BSA) (22). To study the pH dependency of the reaction, Tris–HCl was replaced by 40 mM MES–NaOH pH 5.5, or 40 mM HEPES–NaOH pH 9.0; 45 fmol of helicase substrate was used per assay. The products were separated by electrophoresis on 10% polyacrylamide gels in 89 mM Tris–borate pH 8.3, containing 1 mM EDTA at 8 V/cm. The radioactivity present in the substrate and the displaced oligonucleotide was visualised using phosphor storage technology and quantified with the ImageQuant software version 5.0 (Amersham Pharmacia Biotech). The values plotted are averaged from at least two independent experiments.

ATP hydrolysis reactions were performed for 15 min at 30°C as previously described (22) in 20 μl of helicase buffer (see above) containing 0.2 mM ATP, 100 nCi [γ-32P]ATP, 1 μg viral M13mp18 DNA and 50 μg/ml BSA. Products were separated by thin layer chromatography and quantified as described above.

DEAE-chromatography of Ban/DnaB mixtures

The chromatography was performed at 4°C. Equimolar amounts of purified Ban and DnaB were mixed in buffer B containing 100 mM NaCl, 5 mM ATP and 5 mM MgCl2 and incubated at 30°C for 2 h. Then the mixture was loaded onto a DEAE-Sephacel column (0.9 x 7 cm) equilibrated in buffer B containing 100 mM NaCl, 5 mM ATP and 5 mM MgCl2. The column was washed with the same buffer. Proteins were eluted with a linear gradient of 0.1 to 1 M NaCl in buffer B containing 1 mM ATP and 5 mM MgCl2. Fractions were collected and analysed on denaturing polyacrylamide gels. After staining the protein bands with Serva Blue R, the gel was scanned with a densitometer and the signal strength produced by Ban and by DnaB within each fraction was determined using the ImageQuant software version 5.0 (Amersham Pharmacia Biotech). The signal strength corresponds to the amount of protein present in a protein band. The ratio Ban/DnaB was determined by dividing the signal obtained for Ban by the signal for DnaB.

RESULTS

Nucleotide sequence of ban and the deduced primary structure of the protein

Both strands of ban were sequenced by cycle sequencing using appropriate primers. The complete ban sequence is published in the GenBank database under the accession number AJ011592. Ban encodes a product of 453 residues resulting in a calculated mass of 50.3 kDa and an isoelectric point of 4.83. The comparison of the Ban and the DnaB amino acid sequences deduced by the respective nucleotide sequences revealed 84% similarity and 78% identity. Even more highly conserved are the C-terminal thirds of both proteins, where identity reaches ~85% (Fig. 1). Especially conserved are the five helicase motifs of the DnaB family of helicases. The high degree of conservation is followed by cross-reactivity of Ban with anti DnaB serum (23). The difference in length between DnaB and Ban (471 versus 453 residues) is mainly due to a stretch of 19 N-terminal residues of DnaB that are absent in Ban (Fig. 1). The genes are less conserved than the proteins (69% identity) and did not hybridise (A. Jakschik and E. Lanka, unpublished observations).

Purified Ban is essentially free of DnaB and hexamerises

To purify the Ban protein, we wanted to ensure that the ban reading frame we had defined encoded an active protein. Therefore, the ban overexpression plasmid pTR101 (see Materials and Methods) was introduced into the dnaB70-ts strain WM0331 (24). Spotted on solid medium, WM0331 (pTR101) grew at 42°C, whereas WM0331 containing the...
vector plasmid was not viable (data not shown). This demonstrates that the encoded Ban protein suppressed the dnaB mutation and therefore is functionally active. Thus, pTR101 was used to overproduce and purify the protein (Materials and Methods; Fig. 2A). The N-terminus of purified Ban was determined to be SASPLESMP, demonstrating that the initial formyl-methionine is cleaved off post-translationally. Crucial in purification was to separate Ban from the accompanying DnaB, that might co-purify due to the high overall similarity of both proteins (see above), and therefore result in misleading data on Ban activity. The difference in molecular mass of 2 kDa between Ban and DnaB allowed for a distinction to be made in denaturing polyacrylamide gel electrophoresis (50.3 versus 52.3 kDa, corresponding to 453 and 471 residues). Since polyclonal anti DnaB serum cross-reacts with Ban (23), the Ban preparation was analysed by immunoblotting for the presence of minute DnaB impurities that were not detectable by staining procedures. Two samples of purified Ban (35 and 350 ng) were analysed and compared to an extract of SCS1 cells that carried the vector plasmid. As expected, treatment with 6 M urea and gel filtration in the presence of urea yielded monomeric molecules eluting at the size of BSA (data not shown).

Ban is a DNA helicase

Since Ban was able to hydrolyse ATP (Fig. 4A), we also tested the protein for DNA unwinding. The helicase substrate was viral M13mp18 DNA with a double-stranded segment of 31 bp and 22 unpaired nucleotides at the 3’ end resembling a replication fork. In the presence of 5 pmol Ban (as monomers) in the reaction mixture corresponding to a concentration of 250 nM, >50% of the oligonucleotide was displaced from the substrate (Fig. 4B and C). At 500 nM Ban, unwinding reached a plateau. As expected, no activity was observed in the absence of nucleotide triphosphates. Ban exerts its highest activity at pH 7.6 (Fig. 4A and B). Reactions still took place at pH 9.0, at pH 5.5 the turnover was negligible. The ATPase activity of Ban was identical in the presence/absence of ssDNA indicating that there is no stimulation of the nucleotide hydrolysing activity. Helicase activity has been determined in the presence of each of the eight nucleotides. Ribonucleoside

Figure 2. Purification of Ban protein. (A) Gel electrophoresis of Ban. Aliquots of Ban fractions (see Materials and Methods) were electrophoresed on a 15% polyacrylamide gel containing 0.1% SDS and stained with Serva Blue R after electrophoresis. Lane a, crude cell extract; lane b, fraction I; lane c, fraction II; lane d, fraction III; lane e, molecular mass standards. (B) Immunoblot analysis of purified Ban protein. After gel electrophoresis the proteins were electroblotted onto a nitrocellulose membrane, followed by reaction with anti DnaB sheep serum and dichlorotriazinylamino-fluorescein-conjugated goat anti sheep IgGs. Lane a, 35 ng Ban; lane b, 350 ng Ban; lane c, 7.5 μl of crude extract of SCS1 cells without plasmid; lane d, molecular mass standards.
triphosphates proved to be the best low molecular weight substrates with ATP and GTP as the most efficient cofactors for unwinding. Hydrolysis of UTP and CTP was ~2-fold lower. dNTPs were not suitable to fuel Ban, since unwinding occurred at a negligible low rate (data not shown). The results obtained with Ban prepared from inclusion bodies solubilised in 6 M urea and with the soluble Ban fraction were almost identical. Since the denaturation and renaturation procedure applied for solubilisation of the inclusion bodies yielded an enzymatically active protein, the functional Ban conformation was recovered reasonably well in the renaturation process.

Ban helicase substitutes for E.coli dnaB

Since existing strains still encode N-terminal DnaB remnants of more than 10 amino-acid (aa) residues that may have the ability to interact with the protein under study, we aimed to construct a strain in which DnaB would be completely absent, in order to demonstrate that Ban may substitute for DnaB in E.coli. Therefore, the dnaB gene was replaced by the dnaB368::Ω-Tc construct in the E.coli chromosome (Materials and Methods; Fig. 5A). In this construct, 690 bp of the dnaB sequence contained between the two ClAI sites were removed and replaced by an Ω fragment containing the tetracycline resistance gene, tet (19). This means that ~50% of the dnaB coding capacity is missing, including the coding sequences for the H1 and H1a helicase motifs. In addition, we introduced the dnaB368 mutation that changes the 10th codon of the dnaB gene to an amber stop codon (M. Lemonnier and R. Díaz-Orejas, unpublished results). This additional precaution was taken to avoid the synthesis of an 81 aa N-terminal fragment of DnaB encoded by the remaining 5′ sequences of the dnaB open reading frame that could constitute a source of interference.

The dnaB368::Ω-Tc construct was made on a plasmid and was crossed into the E.coli chromosome to yield the MLM337 strain (Materials and Methods). In this strain, the essential dnaB product was provided by a multicopy plasmid, pPS562.

To confirm the structure of the dnaB368::Ω-Tc locus in the chromosome, total DNA was extracted from MLM337 and was used as template in a series of PCR reactions using oligonucleotides dnaB53, dnaB33 and tet1 (Materials and Methods; Fig. 5A). The PCR product obtained with the couple of primers dnaB53/dnaB33 was a 3866 bp fragment when MLM337 DNA was used as template, which clearly differed from the 2405 bp fragment obtained with DNA from the parental dnaB+ strain W3110 (Fig. 5B). With the couple of primers dnaB53/tet1, PCR made on MLM337 DNA yielded a 1023 bp fragment, while the same reaction on W3110 yielded no product, as expected because the tet1 oligonucleotide hybridises to DNA corresponding to the tet gene, absent in W3110. Note that none of the primers used here hybridises to sequences of the dnaB gene contained in plasmid pPS562 present in MLM337. Nucleotide sequences of the obtained PCR products were determined, which confirmed their identity (data not shown). The structure of the dnaB368::Ω-Tc mutation in MLM337 was also established in Southern blot experiments using a probe specific to dnaB (data not shown). These results confirmed the substitution of dnaB368::Ω-Tc for dnaB in MLM337.

Strain MLM337 was transformed with a replication temperature-sensitive plasmid, pMLM121, which bears the dnaB gene and the dominant rpsL* allele that confers sensitivity to streptomycin, and was cured of the pPS562 plasmid, to yield strain MLM368 (Materials and Methods). This novel strain was designed to select directly for the capacity of any plasmid-borne gene to complement the absence of dnaB. This can be performed by (i) a transformation of the MLM368 strain with a candidate plasmid; and (ii) a selection for the loss of the resident pMLM121 plasmid, by plating on streptomycin-containing medium either at 42°C, a temperature that prevents pMLM121 replication, or even at 30°C, because pMLM121 is unstable at this temperature due to its low copy number, and probably to leakage of the repA-ts mutation. Therefore, transformations of strain MLM368 were...
lacking the pMLM121 plasmid (MLM370 cells) at a given frequency: ~50% of the cells which were plated did not contain pMLM121 (Fig. 6A, column 1), whereas colonies containing the pFUS2 vector produced streptomycin-resistant cells, if any, at a frequency below our detection level (<10^-5; Fig. 6A, column 2). This indicates that dnaB368::Ω-Tc cells are not viable unless a dnaB copy is provided in trans. Moreover, growth rates of MLM370 and of the parental MLM329 (dnaB+ chromosome) cells were comparable both at 30°C (doubling times of 44 and 43.2 min, respectively) and 42°C (30 and 28.9 min; data not shown). Thus, normal growth of dnaB368::Ω-Tc cells depends solely on the presence of the pMLM105 dnaB copy. Hence, the dnaB368::Ω-Tc construct does not exert any polar interference on the expression of neighbouring chromosomal genes that could significantly contribute to growth inhibition.

The same analysis performed on colonies containing the ban plasmid pMLM115 showed that cells that had lost pMLM121 (MLM369 cells, genotype dnaB+ ban+) accumulated at a frequency of ~10^-2 (Fig. 6A, column 3). This difference, of about one order of magnitude compared to the pMLM105 situation (Fig. 6A, columns 1 and 3), can be attributed to the reduced growth rate of cells under the control of the Ban helicase (see next section), although the unlikely possibility that ban enhances the stability of the pMLM121 plasmid, and therefore delays its loss, cannot be completely ruled out. Nevertheless, these results indicated that the Ban
impaired at 42°C. The reported observations of suppression of toxic at high temperatures in the absence of DnaB. Therefore, this result shows that Ban protein was either not functional or by at least 1000-fold compared to MLM370 (data not shown). (Fig. 7B), and 4 h after the shift the viability had dropped significantly reduced at 30°C (Fig. 7A) and dramatically impaired at 42°C (Fig. 7B), compared to its dnaB* equivalent strain MLM370. Immediately after shifting cultures of MLM369 to 42°C increase in cell mass was blocked (Fig. 7B), and 4 h after the shift the viability had dropped by at least 1000-fold compared to MLM370 (data not shown). This result shows that Ban protein was either not functional or toxic at high temperatures in the absence of DnaB. Therefore, the reported observations of suppression of dnaB252 by ban helicase encoded by pMLM115 was able to support growth of E.coli cells in the absence of DnaB.

Loss of pMLM121 that carries the cat gene was confirmed by the sensitivity of MLM369 cells to chloramphenicol, and by purification and analysis of total cellular plasmid DNA. Additional evidence was provided by immunoblot experiments using anti-DnaB serum on crude protein extracts from MLM369 cells (Fig. 6B). Whereas DnaB protein was not detected (lanes 3–4), a lower molecular weight protein corresponding to Ban was observed. Moreover, the concentration of this protein increased in extracts of MLM369 in which ban expression from pMLM115 was induced by adding arabinose to the medium (compare lanes 3 and 4), confirming that this protein is Ban. Thus, the viability of the MLM369 strain demonstrates that, under the experimental conditions used, the Ban helicase is able to support growth of E.coli cells in the complete absence of the DnaB protein.

**Ban and DnaB252 functionally interact in vivo**

Next, we tested the old hypothesis that Ban and DnaB are able to interact and form mixed Ban:DnaB oligomers. Former experiments showed that P1bac mutants suppress the dnaB252-ts mutation (8,25). Furthermore, DnaB and Ban proteins were co-purified within a dnaB complementing fraction obtained from P1bac lysogens of dnaB mutants, among which was dnaB252 (9,25). This suggested that, at high temperatures, Ban could stabilise DnaB252 protomers, probably within a functional hetero-hexamer. Paradoxically, we found that growth of the MLM369 strain (dnaB+/ban*) was significantly reduced at 30°C (Fig. 7A) and dramatically impaired at 42°C (Fig. 7B), compared to its dnaB* equivalent strain MLM370. Immediately after shifting cultures of MLM369 to 42°C increase in cell mass was blocked (Fig. 7B), and 4 h after the shift the viability had dropped by at least 1000-fold compared to MLM370 (data not shown). This result shows that Ban protein was either not functional or toxic at high temperatures in the absence of DnaB. Therefore, the reported observations of suppression of dnaB252 by ban could actually reflect the mutual stabilisation of both partner proteins through functional interaction. To test this hypothesis, dnaB252 and ban genes were placed under the control of distinct promoters and were expressed in dnaB368::Ω-Tc cells, alone or in combination. Cells that expressed dnaB252 or ban individually showed a marked decrease in viability (at least 1000-fold) at 42°C compared to cells expressing the wild-type dnaB gene (Fig. 8). In contrast, when both plasmids expressing dnaB252 and ban were present in the cells, viability was virtually restored. In the absence of IPTG, which induces ban expression, partial recovery of viability is observed (Fig. 8). This may reflect the production of a
protein. The results demonstrate that, in vitro compared to the non-denatured mixture and each single protein eluted within a narrow range of salt concentration, elution was constant at ~1.1 (Fig. 9C), and the majority of the values for each protein alone. The ratio Ban/DnaB during protein eluting at 480 mM NaCl (Fig. 9C). This is in-between the profile of Ban and of DnaB was now identical with the bulk of dialysis. Then the mixture was treated as above. The elution mixture were denatured by adding urea, and renatured by incubation at 30 °C for 2 h to allow for potential exchange of monomers, and then subjected to DEAE chromatography (see Materials and Methods). The elution profile of the Ban±DnaB mixture was similar to that of each single protein (Fig. 9B), and the ratio Ban/DnaB present in each fraction increased during elution, indicating that no prominent exchange of monomers between the Ban and DnaB oligomers occurred. Therefore, in a second attempt, the proteins of the equimolar mixture were denatured by adding urea, and renatured by dialysis. Then the mixture was treated as above. The elution profile of Ban and of DnaB was now identical with the bulk of protein eluting at 480 mM NaCl (Fig. 9C). This is in-between the values for each protein alone. The ratio Ban/DnaB during elution was constant at ~1.1 (Fig. 9C), and the majority of protein eluted within a narrow range of salt concentration compared to the non-denatured mixture and each single protein. The results demonstrate that, in vitro, Ban and DnaB are able to interact and that within the complex the molar ratio of both polypeptides is ~1.

DISCUSSION

The bacteriophage P1 ban gene product shares 84% similarity and 78% identity with the DnaB protein of E.coli. The purified Ban protein hydrolyses ATP, forms hexamers and unwinds duplex DNA in the presence of ATP and Mg2+ ions. Furthermore, Ban is able to sustain growth of an E.coli dnaB null strain. Altogether, these data demonstrate that Ban is a DNA helicase that substitutes for DnaB in the host replication machinery.

Only two further phage-encoded helicases, the Ban protein of phage P7 and the gene 12 protein of phage P22, have been shown to complement dnaB deficiencies in E.coli (3,4). While P1 and P7 Ban proteins share 98% identity (Malgorzata Lobocka, Polish Academy of Sciences, Warsaw, personal communication), P22 gene 12 protein exhibits <35% identity and 55% similarity with DnaB or Ban proteins (4; our unpublished observations). A similar situation is observed in the case of the IncI plasmid-encoded conjugal primases, which efficiently suppress E.coli dnaG mutations despite a low similarity in primary structure (26). Therefore, sequence conservation between Ban and DnaB might not be as crucial as the ability of Ban to properly interact with other replication proteins. With respect to this, the dnaB null strain MLM368 described in this work is an ideal tool to directly assay further bacterial replicative helicases, either phage or chromosome-encoded, for their ability to substitute for DnaB.

Our results show that Ban only partially complements the dnaB null mutation, as indicated by the decreased growth rate at 30°C and the complete growth block observed at 42°C. This suggests that Ban fits into the host replication machinery at low temperatures, but that the formation of Ban hexamers, or their interactions with host replication factors, are thermo-labile. On the other hand, the recently proposed involvement of DnaB in DNA recombination in vivo raises the
possibility that Ban protein either could be deficient in recombination, or escape an essential regulatory control, thus leading to cell death under certain circumstances (2). Exposure of dnaB-ts mutants to non-permissive temperatures results in the formation of double-strand breaks (27). Furthermore, overexpression of dnaB leads to increased illegitimate recombination levels in E.coli, a phenomenon suggested to be linked to DnaB recombination functions (2,28). Whether the Ban-dependent death at high temperatures that is observed in the absence of DnaB is a reflection of deficient interaction with other replication proteins or a consequence of massive formation of double-strand breaks is an issue that remains to be addressed. Certainly, a future search for potential suppressors of MLM369 thermo-sensitive growth could lead to the isolation of mutations, either in the ban gene or in the host chromosome, which would enlighten the nature of Ban interactions in vivo.

Our results confirm the suppression of the dnaB252 thermo-sensitive mutation by Ban, reported earlier (8,25). In addition, we observed that the growth defect of Ban+/DnaB- cells at high temperatures is compensated by the overproduction of the DnaB252 mutant protein. The DnaB252 mutation, a G299E amino-acid substitution (Fig. 1), is unique in several features: (i) it is the only dnaB mutation described so far that affects initiation of replication and not elongation (25,29); (ii) the biochemical properties of purified DnaB252 protein, i.e. in vitro helicase, ATPase, ssDNA binding activities, are indistinguishable from those of wild-type DnaB, even at 42°C (24); and (iii) the dnaB252 mutation does not influence the effect of DnaB on UV-dependent illegitimate recombination (30). Moreover, overproduction of the E.coli helicase loader DnaC completely suppresses the dnaB252 mutation (31). Altogether, these data suggest that the DnaB252 protein is, from an enzymatical point of view, a fully proficient DnaB helicase. Therefore, the in vivo phenotype of dnaB252 could reflect altered DnaB–DnaC interactions and, as a consequence, deficient loading of the helicase into the replication initiation complex. Furthermore, Ban was shown to suppress several E.coli dnaC mutations (32). In light of these observations and of our results reported here, we propose that Ban and DnaB252 form functional hetero-hexamers in vivo in which Ban promoters provide an efficient interface of interaction with the DnaC loader. Further support to this hypothesis is supplied by: (i) Ban conserves the glycine residue that is changed in DnaB252; (ii) this residue is located in the C-terminal region, especially conserved between Ban and DnaB; (iii) this region corresponds to the C-terminal domain of DnaB, the likely interface of interaction with DnaC, as shown by cryo-electron microscopy studies (33).

Although our results are the first report of a mutual compensation of Ban and DnaB deficiencies in vivo, negative and positive functional interactions between Ban and several dnaB alleles have been reported (5–8; this work). Hence, there is no reason to assume that the Ban/DnaB252 interaction is allele specific. Associated wild-type DnaB and Ban proteins have been purified from P1 lysogen extracts (9). Furthermore, in this study we have shown that upon denaturation and renaturation of a mixture of both purified proteins, Ban and DnaB co-elute from a DEAE column at salt concentrations inbetween those observed for each protein alone, thus indicating formation of hetero-oligomers. Within these complexes, we found that the Ban/DnaB ratio was ~1:1. What could be the nature of these hetero-oligomers? One possibility is that they form single hetero-oligomers. The observed Ban/DnaB ratio indicates an overall 1:1 ratio (3 Ban + 3 DnaB in the hexamer), although the whole range of possibilities leading to a 1:1 ratio might occur (Banα–DnaBα, Ban1–DnaB1, ..., Banα–DnaBα). From our results we cannot conclude whether hetero-dimers are formed in a first step, which then assemble to hexamers, or whether heteromerisation takes place via other mechanisms. Alternatively, dimers of hexamers could be formed, i.e. dimers of one Ban homo-hexamer plus one DnaB homo-hexamer, or even dimers of hetero-hexamers, as long as the overall Ban/DnaB ratio is 1:1. However, the existence of such double-hexamers is highly unlikely, since in the size exclusion chromatography no material of a molecular mass higher than 320 kDa was observed. We assume that the steps of denaturation and renaturation of the mixture required to detect hetero-oligomer formation probably reflect the in vivo situation in so far that each monomer released from the ribosome has to assemble into an oligomer.

Concerning the rather puzzling issue of the biological significance of an exchange of subunits between Ban and DnaB, we need to consider the possibility that our experimental set-ups, and those of former studies, make use of artificial overproduction of Ban and DnaB proteins. Furthermore, the ban gene is normally repressed by the P1 phage C1 repressor in the prophage state, and therefore wild-type P1 lysogens are not able to compensate for dnaB deficiencies (5,34). P1 also encodes analogues to the E.coli SSB, UmuD and Tau proteins, but their precise biological role in the life cycle of the prophage is uncertain (35,36; Hansjörg Lehnherr, Ernst-Moritz-Arndt University, Greifswald, personal communication). However, recent studies on the P1 encoded single-stranded DNA-binding protein (SSB-P1), that shares 66% similarity with E.coli SSB, have provided some clues as to how P1 genes are expressed and interact with their E.coli functional homologues (35). These studies showed that: (i) like many plasmid-encoded SSB proteins, the phage-encoded SSB-P1 is able to substitute for the essential SSB protein of E.coli; (ii) P1 SSB gene is transcribed exclusively during stationary-phase growth in E.coli; and (iii) although P1 ssb is dispensable for phage growth during exponential-phase growth, it provides P1 with a selective advantage when exposed to stationary-phase host cells (35). Moreover, P1 sbb and ban genes are controlled by the same P1 regulator, Bof, suggesting that both genes could share a common regulatory pathway, albeit with distinct effects (Bof positively regulates ban and negatively regulates sbb; 34). Therefore, it is possible that under certain physiological conditions that could down-regulate dnaB expression and trigger ban expression, Ban could interact with the available DnaB molecules, and ultimately replace them to form functional hexamers for the mutual benefit of phage and host growth. Certainly, addressing this issue will require a new experimental focus on how dnaB and ban genes are expressed during the growth of bacterial P1 lysogens.

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