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# DNA replication defects in a mutant deficient in the thioredoxin homolog YbbN

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ABSTRACT

*Escherichia coli* contains two thioredoxins, Trx1 and Trx2, and a thioredoxin-like protein, YbbN, that displays both redox and chaperone properties. Since three out of the six proteins of the YbbN interactome (Butland et al., 2005) are components of DNA polymerase 3 holoenzyme (i.e. the β-clamp DnaN, the θ subunit HolE and the δ′ subunit HolB), we investigated whether the *ybbN* mutant presents DNA replication defects. We found that this mutant incorporates $^{3}$H-thymidine at higher rates than the parental strain and displays overinitiation, hypermutator and filamentation phenotypes with the occurrence of anucleated cells. Moreover, YbbN functions as a *bona fide* chaperone in the refolding of the urea-unfolded β-clamp. These results suggest that the DNA replication and cell division defects of the *ybbN* mutant might best be explained by chaperone functions of YbbN in the biogenesis of DNA polymerase 3 holoenzyme.

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## 1. Introduction

*Escherichia coli* contains two thioredoxins, Trx1 and Trx2 [1] that are equivalent for most of the *in vivo* functions tested [2,3], and a thioredoxin-like protein, YbbN, that shares homology in its 12 kDa N-terminal region with thioredoxin and has a 20 kDa C-terminal region of unknown function [4]. The thioredoxin system (thioredoxin, thioredoxin reductase, NADPH) functions as a hydrogen donor for ribonucleotide reductase, sulfate reductase and methionine sulfoxide reductase [5], and plays a key role in maintaining cytoplasmic proteins reduced [6–8]. In addition to its redox functions, Trx1 forms complexes with several enzymes such as T7 DNA polymerase to which it confers processivity [9], and displays chaperone properties [10,11].

YbbN is highly represented in microbial genomes, with an evolutionary retention index of 0.969 (the fraction of times this gene occurs in 33 different microbial genomes). It displays protein disulfide oxidoreductase and chaperone activities *in vitro* [4]. *In vivo*, its chaperone functions may be more important than its redox functions, as shown by the increased sensitivity of the *ybbN* mutant to heat stress but not to oxidative stress, by the normal redox state of cellular proteins in the *ybbN* mutant and by the decreased expression of several chaperones (DnaK, GroEL and trigger factor) and Krebs cycle enzymes (aconitase, isocitrate dehydrogenase, isocitrate lyase and malate dehydrogenase) in the mutant [12].

The YbbN interactome was determined by reverse chromatography of crude *E. coli* extracts with YbbN used as a bait [13]. YbbN interacted with three components of DNA polymerase 3 holoenzyme, the β-clamp DnaN (processivity-conferring subunit), the δ′ subunit HolB of the β-clamp loading complex and the θ subunit HolE of the polymerase 3 core (which stabilizes the ε proofreading subunit [14]). The other three proteins found in the YbbN interactome, as published in [13] were GroEL, the fructose biphosphate aldolase FbaB, and YjjK, the ATP-binding component of a putative transporter. Because of the high occurrence of polymerase 3 holoenzyme components among members of the YbbN interactome, we determined whether the *ybbN* mutant presented DNA replication defects and found that it displayed: (i) a hypermutator phenotype, (ii) an increased incorporation of tritiated thymidine, (iii) an abnormal chromosome content per cell and (iv) a filamentation phenotype accompanied by nucleoid irregularities with the occurrence of anucleated cells. We also found that YbbN increased the refolding of the urea-unfolded β-clamp.

## 2. Materials and methods

### 2.1. Bacterial strains

The *ybbN* mutant JW3112 was kindly provided by Dr. H. Mori and contains *ybbN* disrupted by λRed in the *E. coli* strain BW25113 (*lacI$^{q}$ rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1*) [15].

*Abbreviations:* Trx, thioredoxin; DAPI, 4′,6 diamidino-2-phenylindole; DTT, dithiothreitol.

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### 2.2. $^{3}$H-thymidine incorporation

Bacteria were grown at 37 °C to mid-exponential phase in M9 minimal medium [16] containing glucose (0.4%), casamino acids (50 μg/ml, each) and thymine at 5 μg/ml, and pulse-labeled for 1 min with $^{3}$H-thymidine under conditions that resulted in a linear increase in acid-insoluble counts per minute per unit time (1 ml of culture was added to 2.5 μCi of $^{3}$H-thymidine in 0.1 ml of growth medium) [17]; at different times, the pulses were stopped by the addition of trichloroacetic acid (10%) at 0 °C, and acid-precipitable radioactivity was determined after filtration of samples onto glass fiber filters (Whatman GF/C).

### 2.3. Analysis by flow cytometry

The DNA content of individual cells in the culture of the *ybbN* mutant and of its parental strain was analyzed by flow cytometry [18,19]. Cells were stained with DAPI (4′,6 diamidino-2-phenylindole). Rifampicin (300 μg/ml) and cephalexin (10 μg/ml) were added to a subculture that was incubated for an additional 2 h at 37 °C. This treatment inhibits the initiation of chromosomal DNA replication and cell division, but allows completion of ongoing replication.

### 2.4. Reconstitution of a RIDA (regulatory inhibition of DnaA) system coupled with clamp loading

DnaA-ATP hydrolysis was measured, in the presence of 0.5 pmol $^{32}$P-ATP-DnaA, 10 fmol clamp-loaded DNA (M13E10 replicative form I (an oriC plasmid)), 4 ng Hda, and variable amounts (0–400 ng) of YbbN in conditions described in [20]. The nucleotide-bound form of DnaA was analyzed by immunoprecipitation and polyethyleneimine-cellulose thin layer chromatography.

### 2.5. Immunoblots

Bacteria were grown in LB medium to mid-exponential phase, lysed by sonication as described in [12], and the levels of DnaA and DnaN in bacterial extracts were measured by immunoblotting with anti-DnaA and anti-DnaN antibodies [20].

### 2.6. Resolubilization of urea-unfolded DnaN (β-clamp)

DnaN was unfolded in 30 mM Tris pH 7.4, 8 M urea, 20 mM DTT for 40 min at 20 °C and then diluted 100-fold (to a concentration of 0.4 μM) in 40 mM Hepes pH 7.4, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM sodium acetate, 10 mM magnesium acetate at 20 °C, in the absence or presence of YbbN, as described in [10]. DnaN and YbbN were purified as described in [4,20].

### 2.7. Determination of mutation rates

The *ybbN* mutant and the parental strain were grown to saturation in LB medium. The total titer, and the titer of drug-resistant mutants were determined by plating samples on plates with no drug, rifampicin (100 μg/ml) or nalidixate (50 μg/ml). Colonies were counted after 1 day except for rifampicin-resistant colonies, which were counted after 2 days of incubation.

### 2.8. Microscopic observation of bacterial cells

Cells grown at 37 °C in LB medium were transferred to a microscope slide and stained using 1 μg of DAPI per ml in ethanol-$H_2O$ (1:1). Cells were visualized with combined phase contrast microscopy using a Zeiss Axiophot microscope and photographed with Kodak T-Max 400 black-and-white film.

## 3. Results

### 3.1. The YbbN interactome

The 10 subunits of DNA polymerase 3 holoenzyme are listed in Table 1, and three of these subunits belong to the YbbN interactome, as described by Butland et al. [13], listed in the IntAct protein database (www.ebi.ac.uk/intact/). These subunits are the beta clamp DnaN, the θ subunit HolE and the δ′ subunit HolB (isolated by reverse purification of a crude bacterial extract on a YbbN affinity column and characterized by mass spectrometry). In an earlier version of the YbbN interactome that comprised 21 proteins (IntAct protein database, 2008), the τ and ψ subunits of DNA polymerase 3 were given in addition to DnaN, HolE and HolB (this earlier version of the YbbN interactome also comprised the chaperones DnaK, DnaJ and GroEL, as well as ClpB, protease Lon and five ribosomal proteins. In an earlier work [12], we used reverse purification followed by SDS–PAGE analysis of proteins retained on an YbbN-affinity column to show that YbbN interacts with GroEL, DnaK, GatY and RplE. Notably, we did not detect any interaction between YbbN and any subunit of polymerase 3 holoenzyme, perhaps because most of these subunits are expressed at low levels and were not detectable on the polyacrylamide gel.

### 3.2. Increased mutation rates of the ybbN mutant

Mutator strains of *E. coli* are deficient in genes involved in DNA replication or repair including the *dnaE, dnaQ, polA, mutHLMRST,*

**Table 1**
DNA polymerase 3 subunits and the YbbN interactome. The membership of DNA polymerase 3 subunits to the YbbN interactome (indicated 'yes' in the fourth column) is taken from the study of Butland et al. [13], and is listed in the IntAct YbbN interactome (http://www.ebi.ac.uk/intact/main.xhtml). Two other polymerase 3 subunits (indicated '(yes)' in the fourth column), in addition to HolE, HolB and DnaN, were listed in the YbbN interactome published in an earlier version (2008) of IntAct (in this version the YbbN interactome comprised 21 proteins, instead of six in the current version). Subunits θ and γ are the products of the same gene, *dnaX*. τ is the full-length product, while γ results from early termination within the dnaX reading frame by α (−1) ribosomal frameshift over codons 428–430.

| Subunit | Function | Groupings | YbbN interactome (intact) |
|---|---|---|---|
| α (DnaE) | 5′-3′ polymerase | Core enzyme, elongates | |
| ε (DnaQ) | 3′-5′ exonuclease | polynucleotide chain and proofreads | |
| θ (HolE) | Core assembly | | Yes |
| γ (DnaX) | β-Clamp loader | γ Complex | |
| δ (HolA) | | | |
| δ′ (HolB) | | | Yes |
| χ (HolC) | | | |
| ψ (HolD) | | | (Yes) |
| τ (DnaX) | | | (Yes) |
| β (DnaN) | Processivity factor | Sliding clamp | Yes |

*uvrD, lig, ssB* and *recA* genes. The mutation rates of the *ybbN* mutant and of its parental strain to rifampicin or nalidixic acid resistances were measured on stationary phase cells at 37 °C. Mutation frequencies were 4-fold higher in the *ybbN* mutant than in the parental strain, either for rifampicin or nalidixic acid resistance (Table 2). Since YbbN is a heat shock protein, we investigated the mutation frequencies of the *ybbN* mutant at 42 °C, but we observed similar mutator effects of the *ybbN* mutation at 37 and 42 °C (not shown). This mutator phenotype of the *ybbN* strain is much weaker than that of a *dnaQ* mutant deficient in the 3′5′ proofreading exonuclease activity of DNA polymerase 3 (that displays mutation frequencies several 100-fold higher than wild-type strains) [21]. It is, however, similar to that of *holE* mutants deficient in the DNA polymerase 3 θ subunit (that displays mutation frequencies 2–4-fold higher than wild-type strains) [14] or of *dnaX* mutants deficient in the polymerase 3 holoenzyme τ subunit (that displays mutation frequencies 2–6-fold higher than wild-type strains) [22]. Thus, like mutants deficient in DNA replication or repair enzymes, the *ybbN* mutant displays a hypermutator phenotype.

### 3.3. Increased $^{3}$H-thymidine incorporation rates in the ybbN mutant

The relative rate of $^{3}$H-thymidine incorporation was measured in bacteria in mid-exponential phase, as described in Section 2. This rate was 1.8-fold higher in the *ybbN*-deficient strain than in the control strain (Fig. 1), although both strains had the same doubling time (not shown). Since both the elongation of replication forks and the frequency of initiation contribute to the rate of DNA synthesis, we also measured the relative number of replication origins per cell.

### 3.4. Flow cytometry experiments

Using flow cytometry, we analyzed the DNA content of individual cells in the cultures of the *ybbN* mutant and the parental strain [19]. Bacteria were grown in LB medium to mid-exponential phase. For wild type bacteria (Fig. 2A), most cells were found in two distinct peaks, representing populations of cells with two (36% of the cells) and four (45% of the cells) chromosomes, and 19% of the cells (with DNA synthesis underway) were found between these two peaks. For the *ybbN* mutant (Fig. 2C), the first peak (representing cells in the pre-replicative state) was much smaller than in the control strain (14% vs. 36%), the second peak was similar (41% vs. 45%) and a much higher number of cells was found between the two peaks (41% vs. 19%). The higher number of cells in which DNA synthesis was underway and the increased DNA synthesis rates observed in the *ybbN* mutant both correlate with an increase in the number of replication initiation events in the mutant.

Flow cytometry experiments were also performed upon treatment of cells with rifampicin and cephalexin after which time the cells were allowed to complete ongoing rounds of replication (this procedure inhibits both the initiation of chromosomal replication and cell division, but allows the completion of ongoing replication). The DNA histograms of the control strain (Fig. 2B) and of the *ybbN* mutant (Fig. 2D) both displayed cells containing either four origins (new born cells at the time of antibiotic addition) or eight origins (cells that had already initiated replication at the time of antibiotic addition). However, the relative fractions of the two peaks were different for the two strains. The *ybbN* mutant showed a decreased fraction of cells in the first peak (four origins) (28% vs. 38%) and an increased fraction of cells in the second peak (65% vs. 54%), suggesting that it enters in the replicative state more quickly than the control strain. The *ybbN* mutant did not display an uncoordinated phenotype of chromosome initiation such as that observed in several *dnaA* mutants, which results in the appearance of significant additional peaks at three, five, six and seven fully replicated chromosomes [23].

**Table 2**
Rates of mutation of the *ybbN* mutant to resistance to rifampicin or nalidixic acid. The number of antibiotic resistance mutants were corrected for each strain by the number of colonies counted on drug-free plates. Mutation frequencies or the *ybbN* mutant are normalized to the mutation frequencies of the parental strain.

| Genotype | Rifampicin | Nalidixic acid |
|---|---|---|
| *wt* | 1 | 1 |
| *ybbN* | 4.3 | 3.8 |

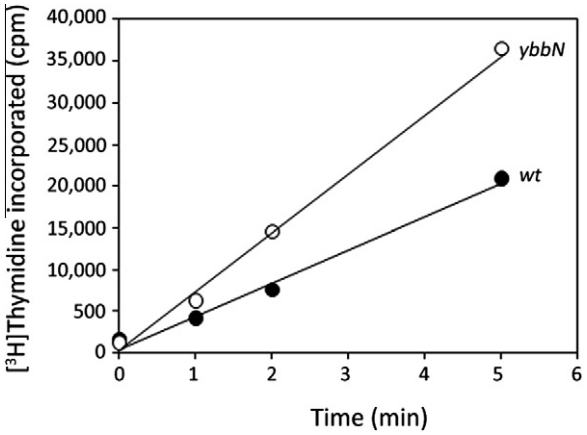


**Fig. 1.** DNA synthesis in the *ybbN* mutant. Incorporation of $^{3}$H-thymidine in the *ybbN* mutant JW3112 (empty symbols) and in its parental strain BM BW25113 (filled symbols). Cells were grown at 37 °C until mid-exponential phase, and DNA synthesis was measured by pulse-labeling.

### 3.5. Microscopic observation of the ybbN mutant

The microscopic appearance of the DAPI-stained *ybbN* mutant is shown in Fig. 3. The *ybbN* mutant was filamented, and showed defective nucleoid segregation; in addition, anucleated cells were apparent, and several of them appeared as minicells, suggesting that cell division defects occur in the *ybbN* mutant. Interestingly, filamentation, defective nucleoid segregation and anucleated cells have been observed in a *dnaQ* mutant deficient in the ε proofreading subunit of DNA polymerase 3 [21].

### 3.6. Measure of the regulatory inactivation of DnaA in the presence of YbbN

Since the *ybbN* mutant displays an overinitiation phenotype, we determined whether YbbN affects the RIDA system (regulatory inactivation of DnaA) [24]. This system promotes hydrolysis of DnaA-ATP (the initiator of DNA replication at *oriC* to produce the inactivated form DnaA-ADP in a manner that depends on the Hda protein and the DNA-loaded form of the β-sliding clamp DnaN. DnaA-ATP hydrolysis was measured as described in [20] in the presence of $^{32}$P-ATP-DnaA, β-clamp-loaded DNA, Hda, and variable amounts of YbbN, but there was no effect of YbbN on the kinetics of DnaA-ATP hydrolysis (not shown). Thus, YbbN does not affect the *in vitro* inactivation of the DnaA replication initiator.

We also measured by immunoblotting the levels of DnaA and DnaN in the *ybbN* mutant and its parental strain: the DnaN (β-clamp) pool was lower in the *ybbN* mutant than in the parental strain (ratio = 0.76) whereas the DnaA pool was higher (ratio = 1.35) (Fig. 4A). The higher pool of DnaA in the *ybbN* mutant might explain, in part, its overinitiation phenotype since DnaA levels have been reported to affect DNA replication initiation [25].

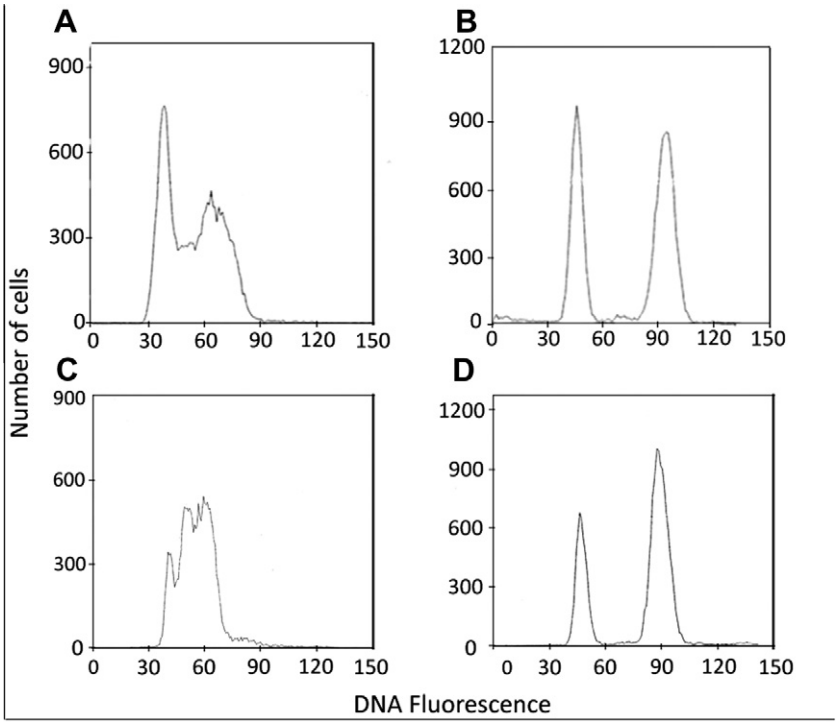


**Fig. 2.** Flow cytometry analysis of the *ybbN* mutant. Cultures of control cells (A) and of the *ybbN* mutant (C), grown at 37 °C in LB medium to mid-exponential phase, were fixed and stained with DAPI, and analyzed using a Becton Dickinson flow cytometer. The panels are density plots that show the relative cell number plotted against DAPI DNA fluorescence intensity. Cultures of control cells (B) and of the *ybbN* mutant (D), grown at 37 °C in LB medium to mid-exponential phase were treated with rifampicin (300 μg/ml) and cephalexin (10 μg/ml) and incubated for 2 h, fixed and stained with DAPI, and analyzed as described.

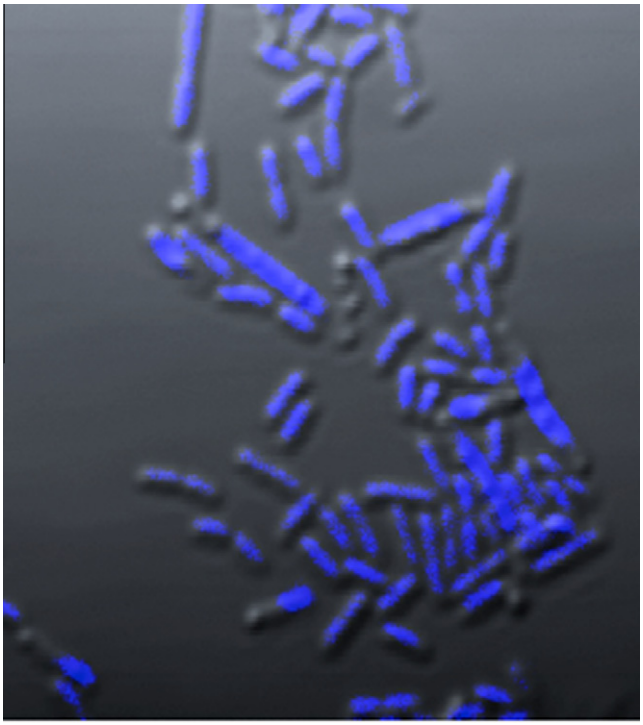

**Fig. 3.** Production of DNA-free bacteria in the *ybbN* mutant. The *E. coli ybbN*-mutant culture was treated as described in the text, and cells were visualized using phase-contrast and fluorescence microscopy. DNA was stained with DAPI.

### 3.7. Resolubilization of urea-unfolded β-clamp (DnaN) in the presence of YbbN

We reported previously that YbbN displays chaperone properties towards the classical chaperone substrates citrate synthase and α-glucosidase [4]. We investigated whether YbbN displays chaperone properties towards the β-clamp. The β-clamp was unfolded in the presence of 8 M urea, and then diluted in buffer to a final concentration of 0.4 μM, either in the absence or in the presence of YbbN. Samples were centrifuged at 15,000*g* and the amounts of soluble and aggregated β-clamp were estimated by SDS–PAGE. 36% of the β-clamp was found in aggregates when renaturation was performed in the absence of YbbN. The amount of β-clamp in aggregates decreased to low levels (down to 8%) when renaturation was done in the presence of increasing YbbN concen-

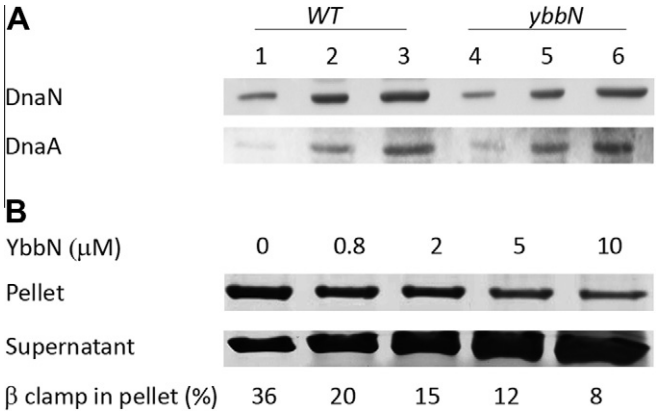


**Fig. 4.** (A) Immunodetection of DnaA and DnaN in cell lysates. Bacterial cells were lysed by sonication, and 1, 2 and 4 μg of the 30,000*g* supernatants from the parental strain and the *ybbN* mutant were probed with anti-DnaA and anti-DnaN antibodies after SDS–PAGE and elecrotransfer onto nitrocellulose sheets. Immunoblots were quantified using ImageJ software. (B) Resolubilisation of the β-clamp (DnaN) in the presence of YbbN. DnaN was denatured in urea and subsequently diluted to a concentration of 0.4 μM in buffer containing YbbN at the indicated concentrations. Samples were incubated for 20 min at 20 °C, centrifuged for 10 min at 15,000*g*, and supernatants and pellets were analyzed by SDS–PAGE. β-clamp in pellets and supernatants (silver stained) is shown.

trations (Fig. 4B). Half-maximal increase in β-clamp solubilization occurred at around 0.8 μM YbbN; this concentration is in the same range as that of its substrate (0.4 μM) and is similar to that of other chaperones in refolding experiments [4,10]. The amount of aggregated β-clamp remained high (38% and 30%, respectively) when refolding was performed in the presence of 10 μM ovalbumin or 10 μM serum albumin, instead of YbbN (not shown). Thus, YbbN functions as a *bona fide* chaperone towards the β-clamp (DnaN).

## 4. Discussion

We characterized previously YbbN as a weak protein disulfide oxidoreductase and as a molecular chaperone [4,12]. The present work was prompted by the presence of several DNA polymerase 3 subunits in the YbbN interactome [13]. We found that, indeed, *ybbN* mutants display several DNA replication defects.

The mutation frequencies of the *ybbN* mutant to rifampicin and nalidixic acid resistance are 4-fold higher than those of the parental strain. These mutation frequencies are lower than those of strong hypermutators such as *mutS* or *dnaQ* mutants, but are similar to those of *dnaX* or *holE* mutants [14,21,22]. An *ybbN* defect might affect the biogenesis of polymerase 3 holoenzyme and explain the mutator phenotype of the *ybbN* mutant, especially since deficiencies in several of the subunits that interact with YbbN lead to a mutator phenotype.

The *ybbN* mutant incorporates $^{3}$H-thymidine at a higher rates than its parental strain. During the cell division cycle, this mutant also displays an increased fraction of cells in the process of DNA synthesis, suggesting that it presents an overinitiation phenotype.

We could not detect any effect of YbbN *in vitro* on the activity of the Hda-dependent DnaA inactivation system, suggesting that YbbN is not a dedicated regulator of this system. However, since the β-clamp is a component of the Hda-dependent DnaA inactivation system, putative alterations of the pool or the activity of the β-clamp in the *ybbN* mutant might affect DNA initiation rates. We found, indeed, that DnaN was moderately underexpressed in the *ybbN* mutant, and that its folding efficiency was increased by YbbN *in vitro*, suggesting that there might be some DnaN defects in the *ybbN* mutant. Alterations in the levels of DnaA (which is moderately overexpressed in the *ybbN* mutant) might also explain several DNA replication defects observed in the present work [25].

The *ybbN* mutant displays a filamentation phenotype accompanied by nucleoid irregularities and the occurrence of anucleated cells. These phenotypes are similar to those of a *dnaQ* mutant (deficient in the ε proofreading subunit of DNA polymerase 3) [21]. It is possible that YbbN, through its interaction with the θ subunit, indirectly affects the biogenesis of the ε subunit, as well as perhaps the formation of the core polymerase (α, ε, θ) [14], which might explain the mutator and the filamentation phenotypes of the *ybbN* mutant.

Thioredoxins are involved in several aspects of DNA metabolism. They function as hydrogen donors for ribonucleotide reductase [5] the activity of which is linked to DNA replication [26]. Since Trx1, Trx2 and glutaredoxin 1 are involved as hydrogen donors for ribonucleotide reductase, it is unlikely that YbbN plays an important role in this function, especially since its oxidoreductase activity is weak [4]. Thioredoxin 1 forms a 1:1 complex with T7 DNA polymerase to which it confers processivity (which increases from 15 nucleotides in the absence of thioredoxin to 2000 in its presence). Since the beta clamp is the processivity factor of DNA polymerase 3 [27], YbbN may not function as a processivity factor for this polymerase, although its involvement in the biogenesis of the beta clamp might indirectly affect DNA polymerase 3 processivity.

In summary, we propose that the DNA replication and cell division defects observed in the *ybbN* mutant, might best be explained by chaperone functions of YbbN in the biogenesis of DNA polymerase 3 holoenzyme. In particular, these defects may occur on the β, θ and δ′ subunits that are reported to specifically interact with YbbN [13].

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