Mutational analysis of DinG-family helicase YoaA: its expression and interaction with replication clamp-loader protein HolC in *E. coli*

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ABSTRACT: The XP-D/DinG family of DNA helicases participate in a variety of ways to preserve genomic stability in all three domains of life. We investigate here the genetic role of one of these proteins, YoaA, of *Escherichia coli.* In *E. coli*, YoaA has been identified as having a role in tolerance to the nucleoside azidothymidine (AZT), a DNA replication inhibitor. It is of particular interest because of its physical interaction with a component of the DNA polymerase III holoenzyme, HolC (or χ). We have proposed that this interaction competes with HolC's interaction with HolD (or ψ) and the rest of the replisome. In this work, we map the residues of YoaA that are required for HolC interaction to the C-terminus of the protein by yeast two-hybrid analysis. We also confirm by gene fusions that YoaA is induced as part of the SOS response to DNA damage and define an upstream "LexA box" sequence in its regulation. Induction of YoaA by AZT is biphasic throughout growth of the culture with an immediate response after treatment and a slower response that peaks in the late log phase of growth. This growth-phase dependent induction by AZT is not blocked by the *lexA3* (Ind$^-$) allele, which normally negates its self-cleavage, implying another means to induce the DNA damage response that responds to the nutritional state of the cell.

INTRODUCTION

The YoaA protein of *Escherichia coli* is a member of the XP-D/DinG family of DNA helicases, with members found in all three domains of life. These are superfamily 2 helicases with shared property of 5' to 3' translocation on ssDNA and an intrinsic Fe-S cluster. In humans, these proteins play various roles in DNA repair and the maintenance of genomic stability, the loss of which results in a variety of genetic diseases (Lehmann 2003; Wu et al. 2009; Bharti et al. 2016; Abe et al. 2018).

The bacterium *Escherichia coli* encodes two paralog proteins of this family, both of which appear to be induced by DNA damage as part of the SOS response, DinG and YoaA (Lewis et al. 1992; Lewis and Mount 1992; Courcelle et al. 2001). DinG encodes a structure-specific DNA helicase with the ability to unwind D-loops, R-loops and G-quadruplex sequences (Voloshin et al. 2003; Voloshin and Camerini-Otero 2007; Thakur et al. 2014). Despite its induction by UV irradiation, *dinG* mutants show only a slight sensitivity to UV (Voloshin et al. 2003). Along with two other SF1 helicase proteins, UvrD and Rep, DinG appears to enhance survival of head-on replication/ transcriptional collisions in vivo (Boubakri et al. 2010), when highly transcribed regions of the chromosome are inverted.

YoaA was identified in a genetic screen for factors that promote tolerance to the chain-terminating nucleoside azidothymidine (AZT) in *Escherichia coli* (Brown et al. 2015). AZT is incorporated during DNA replication and, since it blocks DNA chain elongation, produces single-strand DNA (ssDNA) gaps at the replication fork; cells can tolerate certain levels of AZT through its removal from DNA by exonuclease III (Cooper and Lovett 2011). Mutants in *yoaA* are viable but are strongly sensitive to AZT (Brown et al. 2015) as well as to MMS (Watanabe et al. 2016). Mutants in *dinG* are only very slightly AZT sensitive but do further enhance the sensitivity of *yoaA* mutants when combined.

YoaA is of particular interest because it physically interacts with the replisome protein, HolC (χ) of DNA polymerase III (Butland et al. 2005; Brown et al. 2015; Sutera et al. 2020). Increased expression of HolC, like YoaA, promotes tolerance to AZT in vivo (Brown et al. 2015). HolC is purified as an intrinsic component of the DNA polymerase III, where its serves as an accessory protein to the clamp loader complex (Reyes-Lamothe et al. 2010; McHenry 2011). It is the one component of the replisome that interacts with single-strand DNA binding protein, SSB (Kelman

et al. 1998). In addition to its interaction with SSB, HolC forms an heterodimeric complex with HolD (ψ); it is HolD that links this accessory dimer to the clamp loader and to the rest of the replisome (Xiao et al. 1993; Gao and McHenry 2001).

The HolC/YoaA complex and the HolC/HolD complex appear to be mutually exclusive structures. The same residues buried at the HolC/HolD interface, F64 and W57 (Gulbis et al. 2004), are also required to form a complex with YoaA and essential for AZT tolerance in vivo (Sutera et al 2020). Expression of YoaA/HolC/HolD yields two complexes, HolC/HolD and HolC/YoaA, with no evidence of a ternary complex (Sutera et al 2020). This finding led to the hypothesis that HolC forms two complexes, HolC/HolD dedicated to replication and HolC/YoaA to repair, both recruited to ssDNA through HolC's SSB interaction.

In this study we investigate further the genetic role of YoaA. Using yeast two-hybrid analysis, we map residues required for HolC interaction to the C-terminal 18 amino acids of YoaA and show that they are required for YoaA function in vivo. We assay *yoaA* for effects on mutagenesis, genomic rearrangements and homologous recombination. By gene fusions, we confirm LexA regulation of YoaA expression, induced by AZT; mutation of the putative LexA box at 24 nucleotides upstream of the open reading frame yields constitutively high expression even in the absence of damage. A non-inducible allele of LexA, *lexA3*, blocks the bulk of AZT-induction of *yoaA* expression, although there remains some residual induction of P*yoaA* by AZT, especially during the transition of the culture from exponential growth to stationary phase, suggesting an alternative mechanism for overcoming LexA repression at the locus, induced by starvation.

Results

Determination of HolC binding residues within YoaA: The alignment of *Escherichia coli* DinG and YoaA proteins (Figure 1) shows 29% identity over the length of the two proteins, including all helicase motifs (Q motif, I, Motifs Ia, II, III, IV, V, VI, and P motif), the two helicase HD1 and HD2 domains, and the 4 cysteine residues that coordinate Fe-S binding (Cheng and Wigley 2018). In our prior study, we showed that K51 (Walker A, motif I), C168 (Fe-S cluster) and D225 (Walker B, motif II) (see Figure 1) were required for *yoaA* to promote AZT tolerance in vivo (Brown et al. 2015). The most diverged regions of the YoaA and DinG proteins are the Arch domain (between Motifs II and III) and the C-terminus. A clue to the YoaA region that binds HolC came from prior pulldown experiments, where we noted that a C-terminally truncated proteolytic fragment of YoaA present in the extracts failed to pulldown with HolC as did full-length YoaA (Brown et al. 2015).

We deleted the C-terminal 18 amino acids of YoaA on a plasmid-expressed His_6-tagged allele and assayed the ability of it to complement the AZT-sensitivity phenotype of *yoaA* mutants (Figure 2). Whereas the wild-type allele enhanced survival at 37.5 ng/ml dose of AZT almost 1000-fold relative to the plasmid vector control, the *yoaA*Δ619-636 allele failed to complement, with a plating efficiency on the AZT medium similar to the vector control. We mutated a number of individual residues within this region and found that both R619A and T620A destroyed YoaA function as measured by AZT tolerance, whereas D622A, R625A and V627A has no effect; F629A and T620I showed a partial loss of complementation.

After inducing plasmid expression with IPTG, Western blotting of biotin-binding domain tagged YoaA, YoaAΔ619-636, YoaA R619A and YoaA T620A showed levels of soluble YoaA protein

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                          Q motif                   Motif I
Eco YoaA 11  LAKAIPGFKPREPQRQMA--VAVTQAIEKGQPLVVEAGTGTGKTYAYLAPAL----RAKK  64
             L + IP F PR PQRQM   VA T A E+G+ L +EA TG GKT +YL P +      +K
Eco DinG 18  LQEQIPDFIPRAPQRQMIADVAKTLAGEEGRHLAIEAPTGVGKTLSYLIPGIAIAREEQK  77

                                                        Motif Ia
Eco YoaA 65  KVIISTGSKALQDQLYSRDLPTVSKA---LKYTGNVALLKGRSNYLCLERLEQQALAGGD  121
              +++ST + ALQDQ+YS+DLP + K    LK+T       GR  Y+C   L   ALA  +
Eco DinG 78  TLVVSTANVALQDQIYSKDLPLLKKIIPDLKFTAAF----GRGRYVCPRNL--TALASTE  131
                                                          *

Eco YoaA 122 LPVQ-----ILSDVILLRSWSNQT----VDGDISTC--------VSVAEDSQAWPLVTST  164
              P Q      L D +   +   Q     + GD+ T           +A D   W  +++
Eco DinG 132 -PTQQDLLAFLDDELTPNNQEEQKRCAKLKGDLDTYKWDGLRDHTDIAIDDDLWRRLSTD  190


Eco YoaA 165 NDNCLGSDCPMYKDCFVVKARKKAMDADVVVVNHHLFLADMVVK-ESGFGELIPEADVMIF  224
               +CL  +C  Y++C    AR++  +A+VVV NH L +A M    ES      P+  +++
Eco DinG 191 KASCLNRNCYYYRECPFFVARREIQEAEVVVANHALVMAAM----ESEAVLPDPKNLLLVL  247
                *    *     *

             Motif II
Eco YoaA 225 DEAHQLPDIASQYFGQSLSSRQLLDLAKDITIA-YRTELKDTQQL-QKCADRLAQSAQDFR  283
             DE H LPD+A          R  L+++ +IT   YR +L    +L       +A   + FR
Eco DinG 248 DEGHHLPDVA----------RDALEMSAEITAPWYRLQLDLFTKL-------VATCMEQFR  291

Eco YoaA 284 LQLGEP--------GYRGNLRELLAN---------PQIQRAFL------LLDDTLELCYD---- 320
              +   P         +   L EL+A+         P  Q A        L D+ LE+C
Eco DinG 292 PKTIPPLAIPERLNAHCEELYELIASLNNILNLYMPAGQEAEHRFAMGELPDEVLEICQR-----351

Eco YoaA 321 VAKLSLGRSALLD----------AAFERATLYR---------------TRLKRLKEINQP  355
             +AKL+     L +           + +   L+R               ++L RL  + Q
Eco DinG 352 LAKLTEMLRGLAELFLNDLSEKTGSHDIVRLHRLILQMNRALGMFEAQSKLWRLASLAQS  411

                                                       Motif III
Eco YoaA 356 G---YSYWYECTSR----HFTLALTPLSVADKFKELMAQKPGSWIFTSATLSVNDDLHHF  408
                  + W     R    H       + V+D+ + L+ +     I TSATL   +
Eco DinG 412 SGAPVTKWATREEREGQLHLWFHCVGIRVSDQLERLLWRSIPHIIVTSATLRSLNSFSRL  471

                                                       Motif IV
Eco YoaA 409 TSRLGIEQAES---LLLPSPFDYSRQALLCV--LRNLPQT-NQPGSARQLAAMLRPIIEA  462
                 G+++      + L SPF++  Q  + +  +R  P   N+     ++AA  R  +E+
Eco DinG 472 QEMSGLKEKAGDRFVALDSPFNHCEQGKIVIPRMRVEPSIDNEEQHIAEMAAFFRKQVES  531

Eco YoaA 463 NN--GRCFMLCTSHAMMRDLAEQFRATMT---LPVLLQGET--SKGQLLQQFVSAGNALLV  516
                 G   +  +  AM R L   +   + L +L+QG+  +  +L++    +  +   ++LV
Eco DinG 532 KKHLGMLVLFASGRAMQRFL--DYVTDLRLMLLVQGDQPRYR-LVELHRKRVANGERSVLV  589

                     Motif V          P motif
Eco YoaA 517 ATSSFWEGVDVRGDTLSLVIIDKLPFTSPDDPLLKARMEDCRLRGGDPFDEVQLPDAVIT  576
                SF EG+D++GD LS V I K+ F   D P++    E  +     PF+   LP A
Eco DinG 590 GLQSFAEGLDLKGDLLSQVHIHKIAFPPIDSPVVITEGEWLKSLNRYPFEVQSLPSASFN  649


               Motif VI                                      C-terminal
Eco YoaA 577 LKQGVGRLIRDADDRGVLVICDNRLVMRPYGATFLASLPPAP 618 RTRDIARAVRFLAIPSSR
             L Q VGRLIR     G +VI D RL+ + YG   L +LP  P
Eco DinG 650 LIQQVGRLIRSHGCWGEVVIYDKRLLTKNYGKRLLDALPVFP 691 IEQPEVPEGIVKKKEKTKSPRRRRR
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Figure 1.BLAST Alignment of YoaA and DinG proteins. Conserved helicase motifs are indicated above and cysteine residues of the FeS cluster are marked below with an asterisk. YoaA R619 and T620, implicated in HolC binding, are shown in bold at the C-terminus.

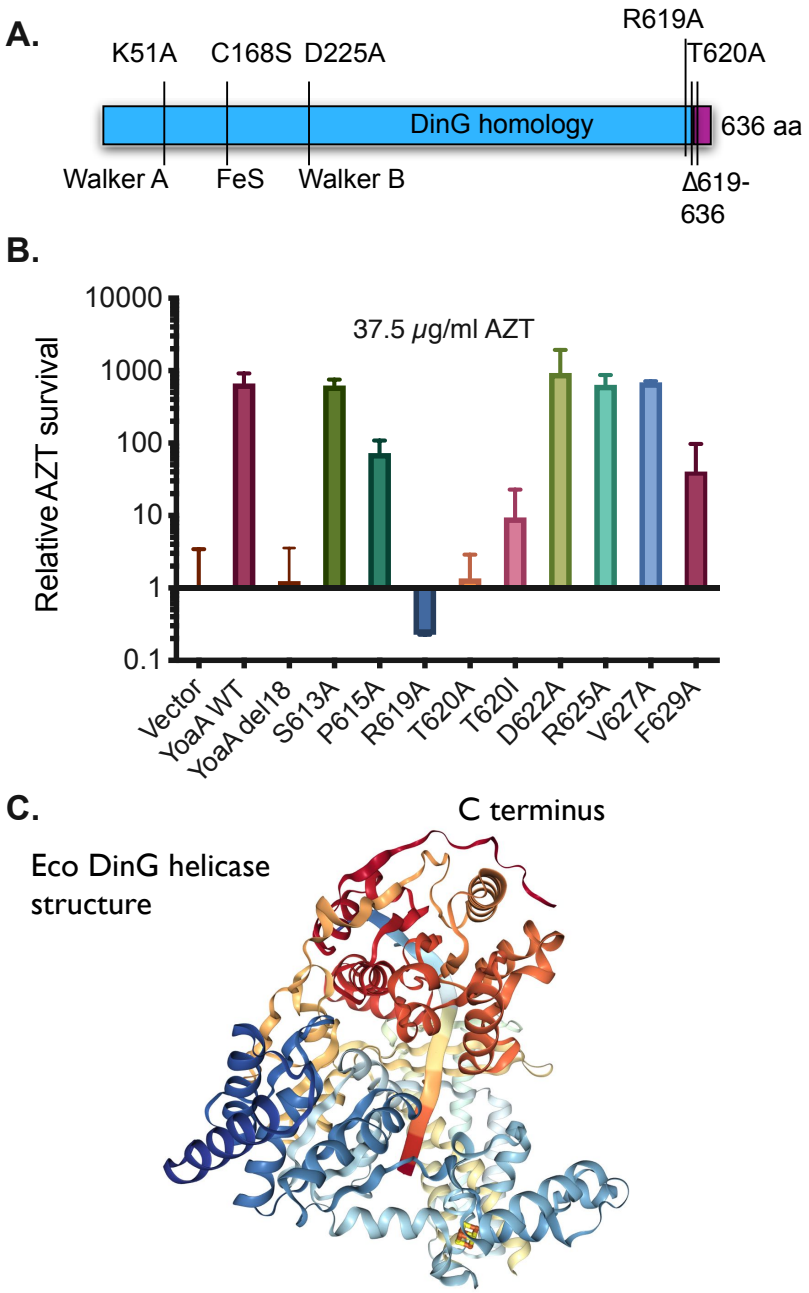

Figure 2. YoaA complementation assays. A. Schematic of the *yoaA* gene showing previously identified non-complementing mutations (Brown et al. 2015) and those identified in this study. The C-terminal region dissimilar to Eco DinG is denoted in purple. B. Complementation assay. Plating efficiency at 37.5 ng/ml AZT of strains carrying the designated *yoaA* plasmid alleles in a *yoaA*Δ strain relative to the vector control. Fractional survival at this dose was 0.66 for *yoaA*$^+$ and 0.0009 for the vector control. Error bars represent standard deviations. C. Eco DinG structure. Image from the RCSB PDB (rcsb.org) of PDB ID 6FWR (Cheng and Wigley 2018), with the C-terminus indicated. DNA within the structure is shown as a flat ribbon.

comparable to wild-type, indicating that failure to complement was not a result of protein degradation (Figure 3).

Based on its similarity with DinG for which there is structural information, the location of the C terminus of the YoaA is likely to be on the outside of the protein at a site distinct from those involved in ATP and DNA binding. The corresponding C-terminus of DinG is partially visible in crystal structures (PDB ID 6FWS, 6FWR) on the exterior surface of the HD2 domain where it overlies helix 18, containing helicase motif IV. (Figure 2C. Image from the RCSB PDB (rcsb.org) of PDB ID 6FWR (Cheng and Wigley 2018).)

We transferred these *yoaA* alleles to yeast two-hybrid fusions to ascertain whether these YoaA alleles retain the ability to interact with HolC, as we have demonstrated previously (Brown et al. 2015; Sutera et al. 2020). Whereas wt YoaA showed an interaction with HolC, indicated by growth on the -His plates, YoaA R619A, T620A and the YoaAΔ619-637 C terminal truncation did not. All strains grew equally well on -Trp Leu plates, which select for the presence of the two plasmids. Control plating of individual plasmids combined with a vector control partner were performed in parallel and yielded negative results (data not shown) so the interaction requires both HolC and YoaA fusion partners. We obtained similar results whether the activation domain was fused to either HolC or YoaA, with the corresponding partner fused to the DNA binding domain, in three independent plating experiments (Figure 4).

Expression of YoaA: To study the regulation of the *yoaA* gene, we fused its upstream region (intergenic with divergently transcribed *yoaB,* with a length of 132 bp) to the *Photorhabdus luminescens* luciferase *luxCDABE* operon on a plasmid and measured luminescence and OD through the growth of the culture, with and without AZT addition (to 1.25 ng/mL) at time zero.

This is a sublethal concentration of AZT for wt strains, where cells continue to proliferate. Expression was induced rapidly after addition of AZT to culture, with a peak approximately 60 minutes after treatment, as we have observed for other similar fusions to SOS-regulated promoter regions of *recA* and *dinB* (Cooper and Lovett 2011). In contrast to those fusions, for *yoaA::lux* we consistently saw a biphasic induction curve, with a slower second induction in late phases of growth, reaching a maximum approximately 150-180 minutes after treatment. A LexA mutation, *lexA3*, affecting

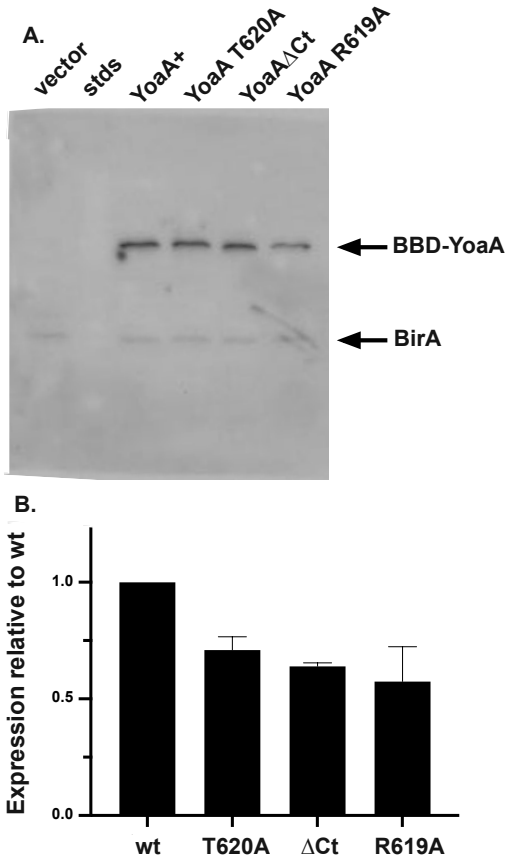

Figure 3. Western blot of BBD-fused YoaA carrying the indicated alleles ("ΔCt"= Δ619-636 of YoaA) using Neutravidin detection, compared to cells expressing vector. BirA is biotin binding protein of E. coli. A. Representative gel. B. Quantitation of Western blots, showing average of 2 independent experiments.

the proteolytic cleavage site that renders the SOS response non-inducible, negates most induction of expression by AZT, especially that soon after treatment, although the second slow phase of induction remained partially intact (Figure 5A). At 180 min after AZT addition,

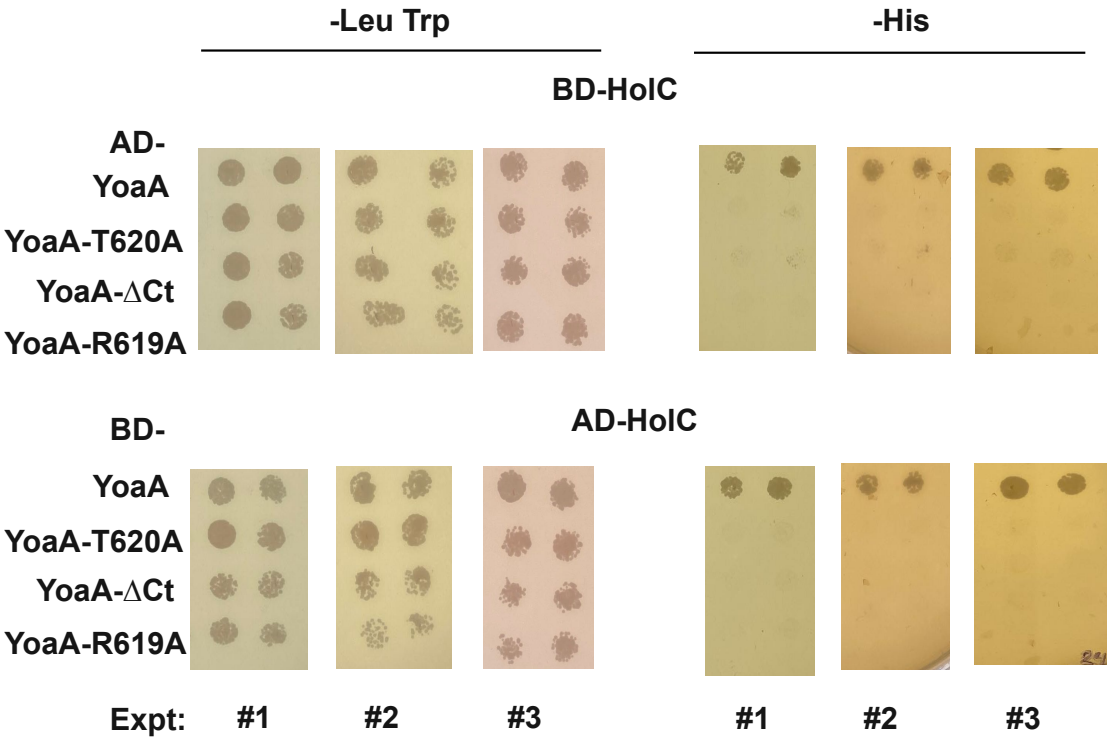

Figure 4. Yeast two-hybrid analysis of YoaA and HolC interaction. Shown at the left are segments of -Leu Trp plates that select for the two hybrid plasmids; at right are those from -His plates that selection for a functional interaction. The top row shows results from hybrids in which the HolC is fused to the Gal4 DNA binding domain (BD) and YoaA (wt and three mutants alleles) is fused to the Gal4 activation domain (AD). YoaAΔCt is the YoaA truncation at amino acid 618, YoaAΔ619-636. In the bottom series HolC is fused to the activation domain and YoaA and its alleles are fused to the DNA binding domain. Three independent experiments are shown.

expression in the *lexA3* genetic background is approximately 30% that of wt and 6-fold higher in treated vs. untreated cells. Note that the data are normalized to culture OD, so the slow increase in expression is not due to expansion of the culture. In a second experiment we compared the induction of *yoaA* expression by AZT from an intact upstream region with one in which we mutated the putative LexA box (replacing the two triplets of invariant LexA box sequence CTG$(N)_{10}$CAG with CCC$(N)_{10}$GGG; $(N)_{10}$ = ttcaaatcaa for *yoaA*). As expected for a LexA-repressed gene, we saw high constitutive expression in the absence of any damage, with no further increase by the addition of AZT (Figure 5B).

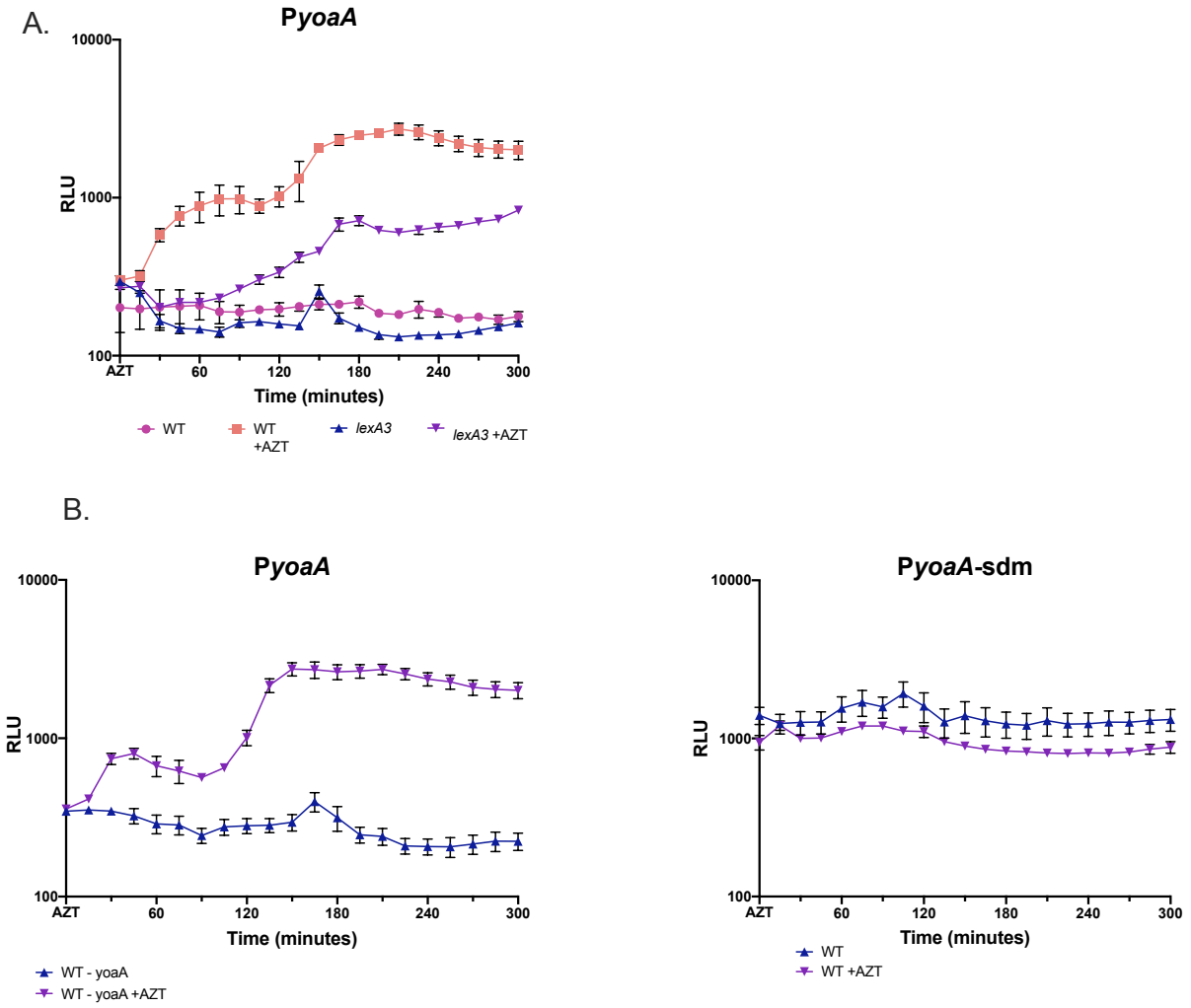

Figure 5. YoaA promoter (P*yoaA*) expression as measured by *lux* operon fusions during growth of the culture in LB, with and without addition of AZT at time 0. Values are expressed as relative luminescence units, with luminescence cpm divided by OD_{600} of the culture at that time. The average of 4 replicates is plotted, with errors indicating standard error of the mean. A. Expression from the *yoaA* upstream intergenic region in wt strains, with and without AZT compared to *lexA3* (noninducible), with and without AZT. B. Left panel: expression of the P*yoaA* promoter region from wt *yoaA* in wt strains with and without AZT; right panel and performed in parallel with the left: expression of the promoter region with a site-directed mutation, P*yoaA*-sdm to remove the putative LexA box at -24, with and without AZT.

YoaA does not affect recombination or base substitution mutagenesis:

Because a number of eukaryotic members of the XPD/DinG helicase family affect homologous recombination or mutation rates, we examined whether loss of *yoaA* had effects on several assays for recombination or genetic instability (Figure 6). We measured crossover

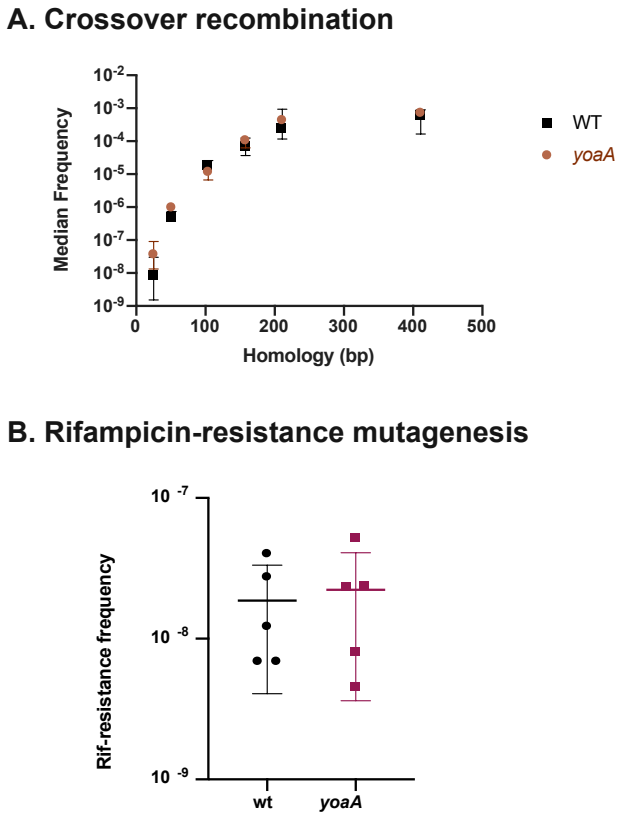

Figure 6. A. Recombination frequencies in wt and *yoaA* strains at differing amounts of homology B. Mutagenesis to rifamipicin resistance is not affected by *yoaA*. Bars represent means and standard deviations.

recombination using an assay previously developed that varies the amount of homology between the recombining loci (Lovett et al. 2002). Recombination at limiting homology often reveals genetic effects not apparent with larger homologies (Lovett et al. 2002; Dutra et al. 2007). This assay primarily measures the RecFOR pathway of homologous recombination, which is believed to be the primary pathway in *E. coli* for recombination at single-strand gaps in DNA (reviewed in Persky and Lovett 2008). We saw no influence of *yoaA* at any homology length using this crossover assay (Figure 6A). Likewise, when we examined mutation frequencies to rifampicin-resistance, we saw no effect of *yoaA*. Rifampicin-resistance selects a variety of base substitution within the *rpoB* gene, encoding the β submit of RNA polymerase (Jin and Gross 1988). Thus, it would appear that *yoaA* mutants do not exhibit a mutator phenotype, although we cannot rule out an effect on specific classes of mutational events.

DISCUSSION

In this study we examine further the genetic effects of the *yoaA* gene of *E. co*li. We mapped the residues of YoaA required for HolC binding to the C-terminal 18 amino acids of the protein, a region in which it is distinct from its paralog protein, DinG. Mutation of two specific residues R619 and T620 to alanine also abolished interaction with HolC, as determined with yeast two-hybrid analysis. Mutants in *yoaA* are sensitive to the replication inhibitor AZT; the ability of YoaA to interact with replisome protein HolC must be required for AZT tolerance, since YoaA R619A and T620A and C terminal deletions fail to complement this defect. These mutations had only minor effects on YoaA protein accumulation in vivo and, based on similarity of the protein to the DinG helicase for which there is structural information (Cheng and Wigley 2018), likely affect a peripheral region of the helicase. Whether this interaction is merely a means for recruitment of the helicase to persistent ssDNA gaps, through a HolC/SSB interaction, or in someway alters the properties of the enzyme remain to be determined.

We confirm that *yoaA* is a DNA-damage inducible gene by promoter fusions to a luciferase (*luxCDABE*) operon, as suggested by previous microarray experiments, with and without UV exposure (Courcelle et al. 2001). We also demonstrate for the first time LexA-regulation of the gene, with a non-inducible *lexA3* allele reducing AZT induction and constitutive high expression when the predicted LexA box is mutated. One advantage of *luxCDABE* luciferase fusions is that the expression signal can be detected in live cells (because the substrate is an endogenous metabolite), allowing us to follow expression during growth of the culture with a sublethal concentration of AZT. Following AZT treatment of an early log phase culture, *yoaA* shows an unusual bimodal induction of expression, with the first increase within 60 minutes (as is typical of SOS genes (see Cooper and Lovett 2011)). AZT induces replication gaps and is a strong inducer of the SOS response via the RecAFOR pathway (Cooper and Lovett 2011) that

promoted the cleavage of the LexA repressor. In addition to the immediate response, we observed a slower secondary increase as the culture begins to approach stationary phase. Note that because AZT is incorporated during replication, it would not be expected to affect stationary phase, non-replicating cells. The *lexA3* mutation, which prevents its cleavage (Little et al. 1980), abolishes the immediate mode of induction, but does not negate the slow and more gradual induction at late phases of growth. We hypothesize that there is nutritional modulation of LexA repression of the *yoaA* gene, such that induction of the gene is triggered more easily as the culture ages. We suspect that this is advantageous because the last rounds of replication may become more difficult to complete as cells begin to starve. Because this induction persists in the non-cleavable *lexA3* strain, there may be an alternative mechanism to relieve LexA gene repression in addition to RecA-stimulated self-cleavage. To our knowledge, this phenomenon has not been reported previously, although most studies have not been conducted with late log phase cultures. The generality and the mechanism of this second phase of induction therefore remains be be determined. The regulation of YoaA is likely important because of its competition with HolD and the replisome for interaction with HolC/SSB and YoaA expression may therefore interfere with replication.

Although YoaA promotes tolerance of ssDNA gaps caused by AZT incorporation in DNA, we do not find that it alters the efficiency of the RecAFOR pathway of homologous recombination during normal growth. In addition, YoaA does not affect the fidelity of replication, as assayed by base substitution mutagenesis in the *rpoB* gene to rifampicin resistance.

MATERIALS AND METHODS

Strains, plasmids, growth conditions: For this study we used *Escherichia coli* K-12 strains MG1655 as wild-type (wt, *rph*-1) and isogenic strains STL9813 (*yoaA*Δ::FRT) and STL22918 (*lexA3 malF3089*::Tn10). LB (Miller 1992) Lennox formulation was used for standard growth media. Plate media included the addition of Bacto-agar at 2%. For plasmid selection, the following antibiotics were employed at the given concentrations: ampicillin (Ap) at 100 μg/ml, kanamycin (Km) at 60 μg/ml, tetratcycline (Tc) at 15μg/ml, phleomycin (Phleo) at 5 μg/ml, and chloramphenicol (Cm) at 15 μg/ml. Strains were grown at 37°C.

For budding yeast, YEPD media (complete) and Drop Out Media (synthetically deficient) followed traditional recipes (Sherman et al. 1987). Strains were incubated at 30°.
Site-directed mutagenesis of plasmid pCA24N-YoaA+ was used to create YoaA mutants at specific residues. Plasmids and primers are listed in Table 1. The forward primer was phosphorylated with T4 polynucleotide kinase (New England Biolabs) and used in a high fidelity PCR reaction (see below) with its complement primer. Following DpnI digestion (New England Biolabs, the PCR product was purified (BioBasic Inc.), ligated with T4 DNA ligase (New England Biolabs) and and transformed into the host strain XL1-Blue by electroporation (Dower et al. 1988). PCR reactions employed Pfu DNA polymerase from Agilent Technologies®, using the guidelines provided from the manufacturer. To create each *yoaA* site-directed mutant, primers that were used are listed in Table 1. Plasmids (listed in Table 1) from bacterial transformants were isolated using BioBasic® Inc. plasmid purification kits and the procedure of the manufacturer. DNA sequence analysis (GeneWiz) confirmed the presence of each particular *yoaA* site-directed mutant and no other change to the sequence.

Complementation assays: Plasmids including pCA2N vector, pCA2N-YoaA+ and the indicated YoaA site-directed mutants were introduced strain STL9813 (*yoaA*Δ::FRT). Cultures

were grown in LB +Cm to log phase, OD600 =0.4 upon which time they were serially diluted into 56/2 buffer and plated on LB medium containing 37.5 ng/ml AZT.

Protein expression and Western blots: BBD-YoaA wildtype (pET104.1DEST-yoaA), BBD-YoaA mutants (pET104.1 yoaA T620A, pET104.1 yoaA T3, pET104.1DEST-yoaA R619A) and the empty pET104.1 vector (listed in Table 1) were expressed from the *E. coli* BL21(DE3) strain. The strains were grown in LB media at 37°C with a final concentration of 100 µg/mL ampicillin, and the cells were induced with 1 mM IPTG for 2 hours. The cells of all overexpressing strains were concentrated by centrifugation at 4700 rpm for 30 minutes at room temperature, concentrated 1:100 in Tris-Sucrose (50 mM Tris-HCl, 10% w/v sucrose, pH 7.5), and stored at -80°C.

Crude cell extracts were prepared with lysozyme lysis using 1µM DTT and 0.1 mg/mL lysozyme (United States Biochemical) in Tris- Sucrose. After a 5-minute incubation on ice ,NaCl was added to a final concentration of 0.2 M, and the extract was incubated an additional 25-minute incubation, after which the cells were heat shocked at 37°C for 15 seconds, then transferred to on ice for 30 seconds. Following two heat shocks, the lysed cells were centrifuged, and the crude lysate supernatant was collected. BBD-YoaA protein samples, combined with an equal volume of 2X FSB (0.12 M Tris-HCl pH 6.8, 3.8% SDS, 19% glycerol, 1.43 M beta-mercaptoethanol, 1mg/ml Bromophenol Blue). Samples were subject to polyacrylamide gel electrophoresis in 12% polyacrylamide gels and transferred to PVDF membrane utilizing Bio-rad transblot apparatus at 100 volts and 400 milliamps for 75 minutes. The western blot analysis was performed according to the QIAexpress detection kit and the protocol (Qiagen) with the following modifications: the BBD-YoaA was detected with a dilution of 1:1000 Neutratavidin Antibody (ThermoFisher) in 10% nonfat milk in for 1 hour. Gel was then washed for 4x 10

minutes with TBS-T wash buffer (20mM 1M Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20, 0.2% Triton X-100). Imaging was performed on a Bio-Rad ChemiDoc system.

Construction of GAL4 Activation Domain and Binding Domain fusions to HolC and YoaA For Yeast Two-Hybrid System:

Bacterial colony PCR with high fidelity DNA polymerase Phusion (New England Biolabs) was used to obtain the wild type alleles of *holC* and *yoaA* for construction of GAL4 fusions for the yeast two hybrid analysis as previously described (Sutera et al. 2020). Gateway cloning technology from Invitrogen® was used to transfer the mutations created in pCA24N-YoaA to the GAL4 activation and binding domain plasmids, pGADT7GW and pGBKT7GW, respectively. Primers were used in a high fidelity PCR reaction using Pfu DNA polymerase obtained from Agilent®. Subsequent PCR reactions were subjected to DpnI to destroy the template plasmid. Following purification of the PCR products (BioBasic kits), *yoaA* mutant fragments (*attB1-yoaA* mutant– *attB2*) were cloned into pDONRZEO using the enzyme BP Clonase II and subsequently into either the pGADT7GW or pGBKT7GW vectors, using LR Clonase II .

Yeast Two-Hybrid Analysis: We followed procedures and used controls from the Matchmaker Gold Yeast Two-Hybrid System from Takara Bio, with the following modifications. Negative controls consisted of the ones suggested in the Matchmaker Gold Yeast Two-Hybrid System with the addition of *holC* and *yoaA* activation domain hybrid plasmids paired with the binding domain empty plasmid. Similarly *holC* and *yoaA* binding domain plasmids were paired with the activation domain empty plasmid vector. A single colony of all controls and combinations of the activation and binding domain plasmids were grown in 5 mls either leucine or leucine and tryptophan dropout media for 20 hours. Following such incubation cultures were diluted 1:5 and 1:50 in sterile water. 1/20 of this dilution was plated on the following

media: YEPD, -leucine, -tryptophan, -leucine and tryptophan, -histidine and -adenine. YEPD is the universal medium for which all cultures will grown. The plates that lack either leucine, tryptophan or both leucine and tryptophan are controls to test plasmid retention. The histidine deficient plates test for lower stringency protein-protein interactions while adenine deficient plates test for a higher stringency. Plates were incubated for two or three days at 30°C.

Luciferase gene expression assays: Luciferase fusion construct plasmids were based on plasmid pDEW201 (Van Dyk et al. 2001) with an inserted GATEWAY-cloning *attR* site-specific recombination cassette. Plasmid construction was completed using GATEWAY cloning (Life Technologies) from PCR amplified products with the primer pair yoaApromoterGWF 5'-ggggacaagtttgt acaaaaaagc aggcttcCATTTTGTCCTCA TTATACTTCC AT-3' and yoaApromoterGWR 5'- ggggaccact ttgtacaaga aagctgggtc ACTACCCCCT GTTGATTTGA ACAGG-3'. Products were recombined with the BP reaction into the pDONR201 GATEWAY plasmid vector. Verified pDONR201GW-yoaAp were recombined using the LR reaction into the GATEWAY pDEW201 Ap LuxCDABE vector. To create the yoaApSDM with the mutated LexA box, site directed mutagenesis (Quikchange Agilent Technologies) on the pDEW201GW-yoaAP was conducted according to the manufacturer's instructions, using overlapping PCR primers with the target mutation, yoaALexAp1 5'-GCGCCCTCAT CCTGACATAA TGTCCCTTCA AATCAAGGGA CGGTAGTGTG ACGGAC-3' and yoaALexAp2 5'- GTCCGTCACA CTACCGTCCC TTGATTTGAA GGGACATTAT GTCAGGATGA GGGCGC-3' and amplification with the Phusion High-Fidelity DNA Polymerase PCR kit (New England BioLabs). Constructs were sequence verified.

Luminescence and OD600 were measured using BioTek Cytation 1 Plate Reader and Costar 96 Well Assay Plate (treated polystyrene, black plate, clear bottom). Colonies were inoculated in

LB media in tubes, shaking until OD_{600} = .5 after which they were diluted 1:100 in LB and grown again to ensure log phase growth. In the 96 well plates, cells were diluted 1:100 and grown for 2 hours, before being treated with 1.25 ng/mL AZT. Bioluminescence was measured and normalized to the OD_{600} yielding relative luminescence units (RLU) every 15 minutes and data are averages of 4 independent replicate cultures.

Recombination and mutagenesis assays. Recombination assays were performed as previously described (Lovett et al. 2002), with crossovers between plasmids carrying various homologies, selected by Tc-resistance. Wt (MG1655) and *yoaA* (STL9813) mutant strains were assayed in parallel on multiple days. Rifampicin resistance mutational assays were performed with MG1655 and STL9813, as previously described (Viswanathan and Lovett 1998) using selection on LB contained 50 μg/ml rifampicin (Sigma-Aldrich).

Table 1 Plasmids

pSTL#	Name	Derivation
-	pCA24N-holC+	ASKA 6XHIS collection clone, National Institute of Genetics(Kitagawa et al. 2005)
-	pCA24N-yoaA+	ASKA 6XHIS collection clone, National Institute of Genetics(Kitagawa et al. 2005)
-	pCL1	Plasmid control, Clontech, Takara Bio USA
-	pDEW201	(Tina Van Dyk et al. 2001)
-	pDONR201	Plasmid Invitrogen, ThermoFisher
-	pDONRZEO	Plasmid Invitrogen, ThermoFisher
-	pGADT7GW	Gateway Y2H plasmid, Addgene
-	pGADT7-T	Plasmid control Clontech, Takara Bio USA
-	pGBKT7-53	Plasmid control, Clontech, Takara Bio USA
-	pGBKT7GW	Gateway Y2H plasmid, Addgene
-	pGBKT7-Lam	Plasmid control, Clontech by Takara Bio USA
330	pBR322d2	(Lovett et al. 2002), recombination plasmid 5'Δ*tetA*
331	pACYC184up2	(Lovett et al. 2002), recombination plasmid *tetA*Δ3' (25 bp homology to pSTL330)
332	pACYC184up3	(Lovett et al. 2002), recombination plasmid *tetA*Δ3' (51 bp homology to pSTL330)
333	pACYC184up4	(Lovett et al. 2002), recombination plasmid *tetA*Δ3' (104 bp homology to pSTL330)
334	pACYC184up4.5	(Lovett et al. 2002), recombination plasmid *tetA*Δ3' (158 bp homology to pSTL330)
335	pACYC184up5	(Lovett et al. 2002), recombination plasmid *tetA*Δ3' (211 bp homology to pSTL330)
336	pACYC184up6	(Lovett et al. 2002), recombination plasmid *tetA*Δ3' (411 bp homology to pSTL330)
393	pCA24N	Empty plasmid
404	pDONRZEO-holC+	(Sutera et al. 2020)
409	pGADT7GW-holC+	(Sutera et al. 2020)
413	pGBKT7GW-holC+	(Sutera et al. 2020)
423	pDONRZEO-yoaA+	(Sutera et al. 2020)
424	pGADT7GW-yoaA+	(Sutera et al. 2020)

pSTL#	Name	Derivation
425	pGBKT7GW-yoaA+	(Sutera et al. 2020)
428	pCA24N- yoaA Δ619-636	Created by whole plasmid PCR Primer sets 19&20
429	pCA24N- yoaA S613A	Created by whole plasmid PCR Primer sets 29&30
430	pCA24N- yoaA P615A	Created by whole plasmid PCR Primer sets 23&24
431	pCA24N- yoaA R619A	Created by whole plasmid PCR Primer sets 25&26
432	pCA24N- yoaA T620A	Created by whole plasmid PCR Primer sets 31&32
433	pCA24N- yoaA T620I	Created by whole plasmid PCR Primer sets 31&32
434	pCA24N- yoaA D622A	Created by whole plasmid PCR Primer sets 21&22
435	pCA24N- yoaA R625A	Created by whole plasmid PCR Primer sets 27&28
436	pCA24N- yoaA V627A	Created by whole plasmid PCR Primer sets 33&34
437	pCA24N- yoaA F629A	Created by whole plasmid PCR Primer sets 15&16
438	pGBKT7GW-yoaA Δ619-636	LR Gateway rxn pSTL454 & pGBKT7GW
439	pGBKT7GW-yoaA R619A	LR Gateway rxn pSTL452 & pGBKT7GW
440	pGBKT7GW-yoaA T620A	LR Gateway rxn pSTL453 & pGBKT7GW
441	pGADT7GW-yoaA Δ619-636	LR Gateway rxn pSTL454 & pGADT7GW
441	pET104.1 DEST yoaA Δ619-636	LR Gateway rxn pSTL454 & pET104.1DEST
442	pGADT7GW-yoaA R619A	LR Gateway rxn pSTL452 & pGADT7GW
442	pET104.1DEST-yoaA R619A	LR Gateway rxn pSTL452 & pET104.1DEST
443	pGADT7GW-yoaA T620A	LR Gateway rxn pSTL453 & pGADT7GW
443	pET104.1DEST yoaA T620A	LR Gateway rxn pSTL453 & pET104.1DEST
444	pET104.1DEST-yoaA+	LR Gateway rxn pSTL423 & pET104.1DEST
447	pDEW201-GW	pDEW201 with gateway cassette B from Invitogen Thermofisher inserted at EcoK53i site
448	pDEW201-GW YoaAp	LR Gateway rxn pSTL450 & pSTL447
449	pDEW201-GW YoaApSDM	Created by whole plasmid PCR Primer sets 37&38
450	pDONR201-GW YoaAp	BP Gateway rxn pDONR201-GW & native yoaA PCR product from primer sets 35&36
451	pET104.1 (-ccdb)	Created by whole plasmid PCR Primer sets 39&40

pSTL#	Name	Derivation
452	pDONRZEO-yoaA R19A	BP Gateway rxn pDONRZEO & yoaA R619A PCR product
453	pDONRZEO-yoaA T20A	BP Gateway rxn pDONRZEO & yoaA T620A PCR product
454	pDONRZEO-yoaA Δ619-636	BP Gateway rxn pDONRZEO & yoaA Δ619-636 PCR product

Table 2 Primers

Primer Name	Primer #	Sequence of Primer
DB 09 YoaA b817R	1	cagtcgctgccaagacagttgtcg
DB 10 YoaA b693F	2	tacaaatcttaagcgatgtgatcc
DB 11 YoaA b1715F	3	ggtcgttgttttatgctttgtacc
DB 12 YoaA b1825R	4	ctgacaaattgctgcaacagttgc
holC fusion attB1	5	ggggacaagtttgtacaaaaaagcaggcttcaaaaacgcgacgttctacctt
holC fusion attB2	6	ggggaccactttgtacaagaaagctgggtcttatttccaggttgccgtatt
holD KO confirm F	7	aggtcatcctgtaagtctccggcaaacaga
holD KO confirm R	8	gatgttccagcagcgcccttcccaatccct
pCA24N FOR seq	9	cattaaagaggagaaattaactatgagagg
pCA24NrrnBT1 REV s	10	atgtgtcagaggttttcaccgtcatcac
pGADT7_Seq_FOR	11	cgactcactatagggcgagcg
pGADT7_Seq_REV	12	gtgcacgatgcacagttgaagtgaac
pGBKT7_Seq_FOR	13	gccgccatcatggaggagcag
pGBKT7_Seq_REV	14	cccggaattagcttggctgcaagc
YoaA F629A F	15	cgtgcggttcgtgcccttgcgatacca
YoaA F629A R	16	tggtatcgcaagggcacgaaccgcacg
yoaA fusion attB1	17	ggggacaagtttgtacaaaaaagcaggcttcacggacgattttgcaccagac
yoaA fusion attB2	18	ggggaccactttgtacaagaaagctgggtcttacctggaggatggtatcgc
YoaA Truncation 3 R	19	tggcgcgggcggcagactggcgag

YoaA Truncation F	20	taacctatgcggccgctaagggtc
YoaAD622AF	21	ccacgcacccgtgccattgcccgtgcg
YoaAD622AR2	22	cgcgggcggcagactggcgagaaacgt
YoaAP615AF	23	ctcgccagtctggcgcccgcgccacgc
YoaAP615AR2	24	aaacgtcgcgccgtaaggacgcatca
YoaAR619AFOR	25	ccgcccgcgccagccacccgtgacatt
YoaAR619AREV	26	cagactggcgagaaacgtcgcgccgtaaggacgcatcacc
YoaAR625AF	27	cgtgacattgccgctgcggttcgtttc
YoaAR625AR2	28	ggtgcgtggcgcgggcggcagact
YoaAS613AF	29	acgtttctcgccgctctgccgcccgcg
YoaAS613AR2	30	cgcgccgtaaggacgcatcacca
YoaAT620AF	31	cccgcgccacgcgcccgtgacattgcc
YoaAT620AR2	32	cggcagactggcgagaaacgtcgc
YoaAV627AF	33	attgcccgtgcggctcgtttccttgcg
YoaAV627AR2	34	gtcacgggtgcgtggcgcgggcggca
yoaApromoterGWF	35	ggggacaagtttgtacaaaaaagcaggcttccattttgtcctcattatacttcc at
yoaApromoterGWR	36	ggggaccactttgtacaagaaagctgggtcactacccccctgttgatttgaaca gg
yoaALexAp1	37	gcgccctcatcctgacataatgtcccttcaaatcaagggacggtagtgtgacg gac
yoaALexAp2	38	gtccgtcacactaccgtcccttgatttgaagggacattatgtcaggatgagggc gc
pET104.1 (-ccdb) Forward	39	atgtcaggctccgttatacacagccagtct
pET104.1 (-ccdb) Reverse	40	ttcaccagtccctgttctcgtcagcaaaag

Acknowledgments: This work was support by NIGMS R01 grant GM51753 to STL and T32 GM007122 to THS. We thank Deani Cooper and Laura Brown for initial work on the expression of YoaA and Kyle McSweeney and McKay Shaw for construction of some of the site-directed mutants.

Bibliography

Abe, T., M. Ooka, R. Kawasumi, K. Miyata, M. Takata *et al.*, 2018 Warsaw breakage syndrome DDX11 helicase acts jointly with RAD17 in the repair of bulky lesions and replication through abasic sites. Proc Natl Acad Sci U S A 115**:** 8412-8417.

Bharti, S. K., S. Awate, T. Banerjee and R. M. Brosh, 2016 Getting Ready for the Dance: FANCJ Irons Out DNA Wrinkles. Genes (Basel) 7.

Boubakri, H., A. L. de Septenville, E. Viguera and B. Michel, 2010 The helicases DinG, Rep and UvrD cooperate to promote replication across transcription units in vivo. EMBO J 29**:** 145-157.

Brown, L. T., V. A. Sutera, Jr., S. Zhou, C. S. Weitzel, Y. Cheng *et al.*, 2015 Connecting Replication and Repair: YoaA, a Helicase-Related Protein, Promotes Azidothymidine Tolerance through Association with Chi, an Accessory Clamp Loader Protein. PLoS Genet 11**:** e1005651.

Butland, G., J. Peregrin-Alvarez, J. Li, W. Yang, X. Yang *et al.*, 2005 Interaction network containing conserved and essential protein complexes in Escherichia coli. Nature 433**:** 531-537.

Cheng, K., and D. B. Wigley, 2018 DNA translocation mechanism of an XPD family helicase. Elife 7.

Cooper, D. L., and S. T. Lovett, 2011 Toxicity and tolerance mechanisms for azidothymidine, a replication gap-promoting agent, in Escherichia coli. DNA Repair 10**:** 260-270.

Courcelle, J., A. Khodursky, B. Peter, P. O. Brown and P. C. Hanawalt, 2001 Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient*Escherichia coli*. Genetics 158**:** 41-64.

Dower, W., J. Miller and C. Ragsdale, 1988 High efficiency transformation of*E.Coli*by high voltage electroporation. Nucleic Acids Research 16**:** 6127-6145.

Dutra, B. E., V. A. Sutera, Jr. and S. T. Lovett, 2007 RecA-independent recombination is efficient but limited by exonucleases. Proc Natl Acad Sci U S A 104**:** 216-221.

Gao, D., and C. S. McHenry, 2001 Tau binds and organizes Escherichia coli replication proteins through distinct domains. Domain III, shared by gamma and tau, binds delta delta ' and chi psi. J Biol Chem 276**:** 4447-4453.

Gulbis, J. M., S. L. Kazmirski, J. Finkelstein, Z. Kelman, M. O'Donnell *et al.*, 2004 Crystal structure of the chi:psi sub-assembly of the Escherichia coli DNA polymerase clamp-loader complex. Euro J Biochem 271**:** 439-449.

Jin, D. J., and C. A. Gross, 1988 Mapping and sequencing of mutations in the Escherichia coli rpoB gene that lead to rifampicin resistance. J Mol Biol 202**:** 45-58.

Kelman, Z., A. Yuzhakov, J. Andjelkovic and M. O'Donnell, 1998 Devoted to the lagging strand-the subunit of DNA polymerase III holoenzyme contacts SSB to promote processive elongation and sliding clamp assembly. EMBO J 17**:** 2436-2449.

Lehmann, A. R., 2003 DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Biochimie 85**:** 1101-1111.

Lewis, L. K., M. E. Jenkins and D. W. Mount, 1992 Isolation of DNA damage-inducible promoters in Escherichia coli: regulation of polB (dinA), dinG, and dinH by LexA repressor. Journal of Bacteriology 174**:** 3377-3385.

Lewis, L. K., and D. W. Mount, 1992 Interaction of LexA repressor with the asymmetric dinG operator and complete nucleotide sequence of the gene. Journal of Bacteriology 174**:** 5110-5116.

Little, J. W., S. H. Edmiston, L. Z. Pacelli and D. W. Mount, 1980 Cleavage of the Escherichia coli lexA protein by the recA protease. Proc Natl Acad Sci U S A 77**:** 3225-3229.

Lovett, S. T., R. L. Hurley, V. A. Sutera, Jr., R. H. Aubuchon and M. A. Lebedeva, 2002 Crossing over between regions of limited homology in Escherichia coli. RecA-dependent and RecA-independent pathways. Genetics 160**:** 851-859.

McHenry, C. S., 2011 DNA replicases from a bacterial perspective. Annu Rev Biochem 80**:** 403-436.
Miller, J. H., 1992 *A Short Course in Bacterial Genetics*. Cold Spring Harbor Press, New York.
Persky, N. S., and S. T. Lovett, 2008 Mechanisms of recombination: lessons from E. coli. Crit Rev Biochem Mol Biol 43**:** 347-370.
Reyes-Lamothe, R., D. J. Sherratt and M. C. Leake, 2010 Stoichiometry and architecture of active DNA replication machinery in*Escherichia coli*. Science 328**:** 498-501.
Sherman, F., G. Fink and J. Hicks, 1987 Methods in Yeast Genetics: a Laboratory Course Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.
Sutera, V. A., S. J. Weeks, E. E. Dudenhausen, H. B. Rappe Baggett, M. C. Shaw *et al.*, 2020 Alternative complexes formed by the Escherichia coli clamp loader accessory protein HolC (x) with replication protein HolD (ψ) and repair protein YoaA. DNA Repair in press.
Thakur, R. S., A. Desingu, S. Basavaraju, S. Subramanya, D. N. Rao *et al.*, 2014 Mycobacterium tuberculosis DinG is a structure-specific helicase that unwinds G4 DNA: implications for targeting G4 DNA as a novel therapeutic approach. J Biol Chem 289**:** 25112-25136.
Van Dyk, T. K., E. J. DeRose and G. E. Gonye, 2001 LuxArray, a high-density, genomewide transcription analysis of*Escherichia coli*using bioluminescent reporter strains. J. Bacteriol. 183**:** 5496-5505.
Viswanathan, M., and S. T. Lovett, 1998 Single-strand DNA-specific exonucleases in Escherichia coli. Roles in repair and mutation avoidance. Genetics 149**:** 7-16.
Voloshin, O. N., and R. D. Camerini-Otero, 2007 The DinG protein from Escherichia coli is a structure-specific helicase. The Journal of biological chemistry 282**:** 18437-18447.
Voloshin, O. N., F. Vanevski, P. P. Khil and R. D. Camerini-Otero, 2003 Characterization of the DNA damage-inducible helicase DinG from Escherichia coli. The Journal of biological chemistry 278**:** 28284-28293.
Watanabe, K., K. Tominaga, M. Kitamura and J. I. Kato, 2016 Systematic identification of synthetic lethal mutations with reduced-genome Escherichia coli: synthetic genetic interactions among yoaA, xthA and holC related to survival from MMS exposure. Genes Genet Syst 91**:** 183-188.
Wu, Y., A. N. Suhasini and R. M. Brosh, 2009 Welcome the family of FANCJ-like helicases to the block of genome stability maintenance proteins. Cellular and molecular life sciences : CMLS 66**:** 1209-1222.
Xiao, H., Z. Dong and M. O'Donnell, 1993 DNA polymerase III accessory proteins. IV. Characterization of chi and psi. J Biol Chem 268**:** 11779-11784.