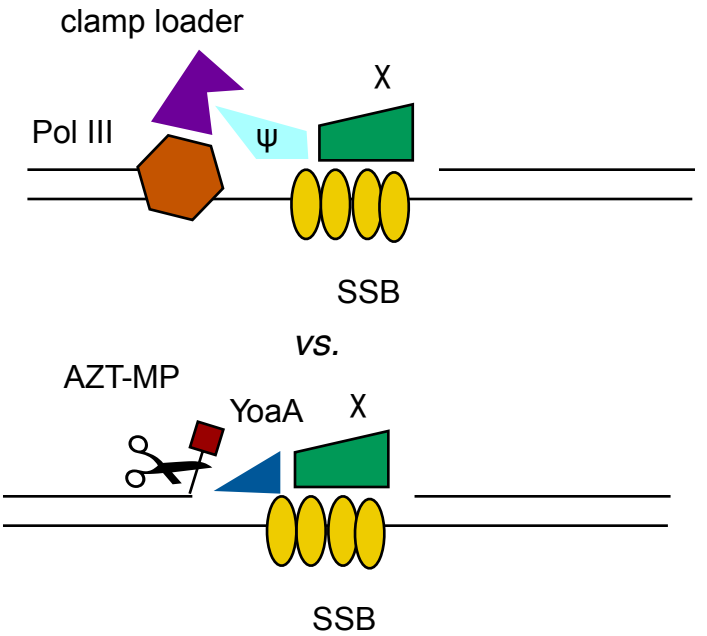
HIGHLIGHTS

- HolC binds to two exclusive partners, the clamp-loader protein, HolD or the repair helicase, YoaA.
- HolC residues W57 and F64 are required for interaction with YoaA as well as HolD.
- These HolC residues do not affect its binding to single-strand DNA binding protein, SSB.
- HolC can recruit alternative complexes to balance replication vs. repair reactions at the replication fork.

Graphical Abstract



Alternative complexes formed by the *Escherichia coli* clamp loader accessory protein HolC (χ) with replication protein HolD (ψ) and repair protein YoaA

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ABSTRACT

Efficient and faithful replication of DNA is essential for all organisms. However, the replication fork frequently encounters barriers that need to be overcome to ensure cell survival and genetic stability. Cells must carefully balance and regulate replication vs. repair reactions. In *Escherichia coli*, the replisome consists of the DNA polymerase III holoenzyme, including DNA polymerase, proofreading exonuclease, processivity clamp and clamp loader, as well as a fork helicase, DnaB and primase, DnaG. We provide evidence here that one component of the clamp loader complex, HolC (or χ) plays a dual role via its ability to form 2 mutually exclusive complexes: one with HolD (or ψ) that recruits the clamp-loader and hence the DNA polymerase holoenzyme and another with helicase-like YoaA protein, a DNA-damage inducible repair protein. By yeast 2 hybrid analysis, we show that two residues of HolC, F64 and W57, at the interface in the structure with HolD, are required for interaction with HolD and for interaction with YoaA. Mutation of these residues does not interfere with HolC's interaction with single-strand DNA binding protein, SSB. In vivo, these mutations fail to complement the poor growth and sensitivity to azidothymidine, a chain-terminated replication inhibitor. In support of the notion that these are exclusive complexes, co-expression of HolC, HolD and YoaA, followed by pulldown of YoaA, yields a complex with HolC but not HolD. YoaA fails to pulldown HolC-F64A. We hypothesize that HolC, by binding with SSB, can recruit the DNA polymerase III holoenzyme through HolD, or an alternative repair complex with YoaA helicase.

1. INTRODUCTION[1]

The sliding clamp, known as "β" in bacteria and "proliferating cell nuclear antigen" (PCNA) in eukaryotes and archaea, is one of the most conserved structures in DNA replication [1, 2] By encircling DNA and tethering DNA polymerases, the sliding clamp confers processivity to DNA synthesis. In addition, the clamp binds a number of proteins involved in DNA repair or cell cycle regulation. In *E. coli*, β binds all five of its DNA polymerases, as well as DNA ligase, DnaA and Hda, which regulate DNA replication initiation, and MutS/MutL of the mismatch repair pathway [3]. The sliding clamp does not self-assemble on DNA; assembly requires an ATP-hydrolyzing pentameric clamp-loader complex, known as the γ/τ complex in bacteria and RFC (Replication Factor C) in eukaryotes and archaea. In *E. coli*, the core clamp loader consists 3 subunits of DnaX protein (in its two forms, τ and γ) and one subunit each of HolA (δ) and HolB (δ)'. The *E. coli* core clamp loader also binds a dimer of two additional accessory proteins, HolC (χ) and HolD (ψ)[4, 5].

The biological role of the accessory clamp loader proteins, HolC and HolD, is not altogether clear. Genes for HolC- and HolD-related proteins are only found in the gamma-proteobacteria, although it is possible that more unrelated bacterial proteins play the same function in other species. HolC is of particular interest because it is the only protein of the *E. coli* replicative DNA

[1] Abbreviations: SSB, single-strand DNA binding protein; AZT, azidothymidine; Y2H, yeast two-hybrid

polymerase III holoenzyme known to bind to single-strand DNA binding protein, SSB [6], which is expected to coat the lagging strand template during replication and both templates after blocks to DNA synthesis.

In contrast to the subunits of the core clamp loader complex that are essential for viability, those of the accessory complex are not essential under certain conditions and genetic backgrounds [7-11]. In general, slow growth and low temperatures enhance the viability of both *holC* and *holD* mutants [8]. Even when viable, both mutants are constitutively induced for the SOS DNA damage response and rapidly accumulate genetic suppressors; for example, a duplication of the *ssb* gene suppresses both *holC* and *holD* null mutants. [8]. Rendering the SOS response to DNA damage non-inducible suppresses *holD*; the SOS genes that reduce viability in the *holD* mutant strain include two of the bypass DNA polymerases, Pol II (*polB*) and Pol V (*dinB*). Additional, less understood, genetic suppressors of *holD* null mutants [10, 11] include *trkA* and *rfaP (*affecting potassium import), *sspA* (a transcriptional regulatory protein) and *rarA (*an ATPase found associated with DNA replication forks) [12]. Even under conditions that minimize their growth defects, *holC* mutants show 5-16 fold elevated rates of genetic rearrangements [7], an indication of perturbed replication.

Biochemical studies of the clamp loader suggest a number of roles for the HolCD accessory complex. The accessory complex may assist in clamp loader assembly: HolC and HolD increase the affinity of the DnaX subunits of the core complex (γ, τ) with HolA (δ)and HolB (δ′)[13]. Although the core clamp loader complex is sufficient for clamp loading and unloading, the core

plus accessory complex is more efficient [14]. In vitro, HolC and its interaction with SSB facilitate the hand-off of an RNA primer from primase to DNA polymerase III [15] and overall stabilizes the binding of the Pol III replisome to SSB-coated templates [6, 16, 17]. HolD, which interacts directly with the DnaX subunits of the core clamp loader, promotes conformational states with higher affinity for the clamp and for DNA [18, 19].

In a genome-wide screen, we isolated HolC and YoaA as genes that confer tolerance to the chain-terminating replication-inhibitor azidothymidine [20]. Subsequently, a similar suppressive effect was observed for survival to methyl methane sulfonate (MMS) [21]. YoaA is a paralog of the structure-specific DinG DNA helicase [22-25], a member of a larger group of Fe-S cluster-containing helicases in all three domains of life, implicated in DNA repair and genomic integrity [26]. An interaction between HolC and YoaA was identified by mass spectrometry of epitope-tagged proteins [27]; we confirmed that the two proteins interact, by yeast two-hybrid analysis and by pulldown experiments [20]. In this study, we define some residues within HolC that are required for interaction with YoaA. HolC F64 and W57 are both required for interaction with YoaA, as assayed in the yeast 2-hybrid system. (Y2H). In the crystal structure of the HolC/HolD complex, these residues are at the interface of the two subunits, with HolC F64 buried deeply into a cleft of HolD. In this and our previous work, these residues are required for interaction with HolD, but not the interaction with SSB, as assayed by Y2H. These findings suggest that YoaA and HolD both bind to the same surface of HolC. Because of this, the complexes formed with HolC, HolC/YoaA and HolC/HolD, are most likely exclusive. Overexpression of HolC HolD YoaA proteins and subsequent pulldown of YoaA shows that HolC but not HolD binds to YoaA.

Pulldowns also confirm that YoaA does not bind to HolC-F64A. This finding suggests that HolC, by binding with SSB, can recruit the DNA polymerase III holoenzyme through HolD, or an alternative repair complex with YoaA helicase.

2. METHODS

2.1 Bacterial and yeast growth media:

Strains used in this study are given in Table 1. Luria broth [28] and minimal glucose media were used for the bacterial strains used in this study. Minimal media contain 60 mM Na_2HPO_4, 40 mM KH_2PO_4, 0.02% $MgSO_4 \bullet 7H_2O$, 0.2% $(NH_4)SO_4$, 0.001% $Ca(NO_3)_2$, 0.00005% $FeSO_4 \bullet 7H_20$, 0.2% glucose, and 0.001% of vitamin B1 (thiamine). Plate media included the addition of Bacto-agar at 2%. For plasmid selection, the following antibiotics were employed at the given concentrations: ampicillin (Amp, Ap) at 100µg/ml, kanamycin (Kan, Km) at 60 µg/ml, tetratcycline (Tet, Tc) at 15µg/ml, phleomycin (Phleo) at 5µg/ml, and chloramphenicol (Cat, 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 [29]. Yeast strains were incubated at 30°.

Table 1: *Escherichia coli* and *Saccharomyces cerevisiae* strains used in genetic analysis.

Strain	Relevant Genotype	Source
A. *E. coli* strains		
AG11[a]	*recA1 endA1 gyrA96 thi-1 hsdR17(rK"mKþ) supE44 relA1*	[30]
BL21[b]	*ompT hsdS(r_B-m_B-) dcm- gal* λ *(DE3) F-*	[31]
DB3.1[a]	*gyr*A462 *end*A1 D(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(rB-,MB-)	Invitrogen™

	*sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Smr) *xyl*-5 lambda	
	- leu mtl-1 *F-*	
DH5α [a]	*phi80d lacZ*Δ*M15* Δ*(lacZYA-argF)U169 endA1 recA1 hsd17 F-*	CGSC Stock Center
MG1655[a]	*F- rph-1*	[32]
STL18822 [a]	*holC::*Δ*FRT Kan F- rph-1*	[20]
XL1-Blue[a]	F' [*proAB lacIq lacZ*Δ M15 ::Tn10] *recA1 endA1 gyrA96 thi*	CGSC Stock Center
	hsdR17 supE44 relA1 lac	
B. *S. cerevisiae* strains		
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ,	[33], A. Holtz, unpublished
	*gal80*Δ, *LYS2* : : a-*GAL1*TATA-*HIS3*,	
	*GAL2*UAS-G*AL2*TATA-ADE2, *URA3* : : *MEL1*UAS-*MEL1*	
	TATA-laca	

[a] Bacterial strains derived from *Escherichia coli* K12

[b] Bacterial strains derived from *Escherichia coli* B

2.2 **Construction of pCA24N-holC site-directed mutants via PCR:**

Plasmid pSTL426 (pCA24N-holC+) was subjected to site-directed mutagenesis to create *holC* mutants at specific residues. Plasmids are listed in Table 2; primers used in construction are listed in Table 3. 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. The original template was removed by DpnI digestion (New England Biolabs; the PCR product was purified using PCR purification kits (BioBasic Inc.), ligated with T4 DNA ligase (New England Biolabs) and and introduced into the host strain XL1-Blue by electroporation [34].

Pfu DNA polymerase from Agilent Technologies® was used in these PCR reactions. Using the guidelines provided from the manufacturer, reactions were set up using 10X Pfu buffer diluted to 1X, 0.2mM dNTPS, 10 ng of pSTL426, 2.5 units of Pfu DNA polymerase, 100 ng of forward primer and 100 ng of the reverse primers. To create each *holC* site-directed primers (Table 3) were used in the following pairs, 9+11, 10+11, 12+14, 13+14, 15+16, 17+18, 19+18, 20+21, 22+23, and 24+25). The PCR reaction contained 25 cycles.

Plasmids (listed in Table 2) from bacterial transformants were isolated using BioBasic® Inc. plasmid purification kits and the procedure of the manufacturer. DNA sequence analysis (GeneWiz) using primers 26 and 27 confirmed the presence of each particular *holC* site-directed mutant and no other change to the sequence.

Table 2: Plasmids used in genetic analysis

pSTL#	Name	Derivation
-	pCA24N-yoaA[a]	ASKA 6XHIS collection clone, National Institute of Genetics[30]
-	pCL1[b]	Plasmid control, Clontech, Takara Bio USA
-	pDONRZEO[c]	Plasmid Invitrogen, ThermoFisher
-	pGADT7GW[d]	Gateway Y2H plasmid, Addgene

-	pGADT7-T[e]	Plasmid control Clontech, Takara Bio USA
-	pGBKT7-53[f]	Plasmid control, Clontech, Takara Bio USA
-	pGBKT7GW[g]	Gateway Y2H plasmid, Addgene
-	pGBKT7-Lam[h]	Plasmid control, Clontech by Takara Bio USA
393	pCA24N[i]	Contained parC which was removed creating empty plasmid
426	pCA24N-holC+[a]	Plasmid obtained through ASKA 6XHIS collection clone
394	pCA24N-holC F6A[j]	Created with PCR primers 9+11
395	pCA24N-holC Y7A[c]	Created with PCR Primers 10+11
396	pCA24N-holC S19A[j]	Created with PCR Primers 12+14
397	pCA24N-holC A20D[j]	Created with PCR Primers 13&14
398	pCA24N-holC C26A[j]	Created with PCR Primers 15&16
399	pCA24N-holC C43A[j]	Created with PCR Primers 17&18
400	pCA24N-holC D45A[j]	Created with PCR Primers 19&18
401	pCA24N-holC E54A[j]	Created with PCR Primers 20&21
402	pCA24N-holC W57A[j]	Created with PCR Primers 22&23
403	pCA24N-holC F64A[j]	Created with PCR Primers 24&25
404	pDONRZEO-holC[+k]	BP[l] rxn pDONRZEO & PCR of pSTL426 using primer set 3&4
405	pDONRZEO-holC F6A[k]	BP[l] rxn pDONRZEO & PCR of pSTL394 using primer set 3&4
406	pDONRZEO-holC E54A[k]	BP[l] rxn pDONRZEO & PCR of pSTL401 using primer set 3&4
407	pDONRZEO-holC W57A[k]	BP[l] rxn pDONRZEO & PCR of pSTL402 using primer set 3&4

408 pDONRZEO-holC F64A[k] BP[l] rxn pDONRZEO & PCR of pSTL403 using primer set 3&4

409 pGADT7GW-holC+[m] LR[n] rxn pSTL404 & pGADT7GW

410 pGADT7GW-holC E54A[m] LR[n]rxn pSTL406 & pGADT7GW

411 pGADT7GW-holC W57A[m] LR[n] rxn pSTL407 & pGADT7GW

412 pGADT7GW-holC F64A[m] LR[n] rxn pSTL408 & pGADT7GW

413 pGBKT7GW-holC+[o] LR[p] rxn pSTL404 & pGBKT7GW

414 pGBKT7GW-holC E54A[o] LR[p] rxn pSTL406 & pGBKT7GW

415 pGBKT7GW-holC W57A[o] LR[p] rxn pSTL407 & pGBKT7GW

416 pGBKT7GW-holC F64A[o] LR[p] rxn pSTL408 & pGBKT7GW

417 pDONRZEO-holD+[k] BP[l] rxn pDONRZEO & *E. coli* colony[q] PCR using primer set 5&6

418 pGADT7GW-holD[m] LR[n] rxn pSTL417 & pGADT7GW

419 pGBKT7GW-holD[o] LR[n] rxn pSTL417 & pGBKT7GW

420 pDONRZEO-ssb+[k] BP[l] rxn pDONRZEO & *E. coli* colony[q] PCR using primer set 7&8

421 pGADT7GW-ssb+[m] LR[n] rxn pSTL420 & pGADT7GW

422 pGBKT7GW-ssb+[o] LR[n] rxn pSTL420 & pGBKT7GW

423 pDONRZEO-yoaA+[k] BP[l] rxn pDONRZEO & *E. coli* colony[n] PCR using primer set 1&2

424 pGADT7GW-yoaA+[m] LR[n] rxn pSTL423 & pGADT7GW

425 pGBKT7GW-yoaA+[o] LR[n] rxn pSTL423 & pGBKT7GW

a. Plasmid obtained from ASKA library of *E. coli* ORF 6XHIS tag clonesu nder T5 promoter expression; confers Cm^R.

b. Yeast two hybrid plasmid control that contains both the GAL4 activation and binding domains along with the leucine gene

c. Gateway donor plasmid obtained through Invitrogen by ThermoFisher; confers $Zeocin^R$ and $Cm.^R$

d. Gateway yeast two hybrid plasmid containing GAL4 activation domain, leucine gene, Cm and Ap resistance genes, obtained from Addgene.

e. Yeast two hybrid plasmid positive control containing the GAL4 activation domain along with the leucine gene. Can be used in conjunction with pGBKT7-53 as a positive control or pGBKT7-Lam as a negative one.

f. Yeast two hybrid plasmid positive control containing the GAL4 binding domain along with the tryptophan gene.

g. Gateway yeast two hybrid plasmid containing GAL4 binding domain, tryptophan gene, Cm and Km resistance genes, obtained from Addgene.

h. Yeast two hybrid plasmid negative control containing the tryptophan gene but not containing the GAL4 binding domain along.

i. Original plasmid pCA24N-parC from ASKA library of *E. coli* ORF 6XHIS tag clones. *parC* was excised from pCA24N creating empty vector negative control; confers Cm^R.

j. pCA24N-holC site-directed mutants under T5 promoter expression; confers Cm^R.

k. All Gateway donor vectors constructed confer Zeocin^R

l. Gateway reaction using BP Clonase II reaction mix, specified donor vector, and indicated primer sets

m. All gateway destination vectors constructed using pGADT7GW contain the leucine gene and confer Ap^R

n. Gateway reaction using LR Clonase II reaction mix, specified donor vector, and pGADT7GW

o All gateway destination vectors constructed using pGBKT7GW contain the tryptophan gene and confer Ap^R.

p. Gateway reaction using LR Clonase II reaction mix, specified donor vector, and pGBKT7GW.

q. *E. coli* colony PCR technique is described in further details in Methods

Table 3: DNA Primers used in genetic analysis

Primer Name	Primer #	Sequence
yoaA fusion attB1	1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCacggacgattttgcaccagac
yoaA fusion attB2	2	GGGGACCACTTTGTACAAGAAAGCTGGGTCttacctggaggatggtatcgc
holC fusion attB1	3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCaaaaacgcgacgttctacctt
holC fusion attB2	4	GGGGACCACTTTGTACAAGAAAGCTGGGTCttatttccaggttgccgtatt

holD fusion attB1 5

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCacatcccgacgagactggca

g

holD fusion attB2 6

GGGGACCACTTTGTACAAGAAAGCTGGGTCtcagtcgtttcgagggaagaa

ssb fusion attB1 7

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCgccagcagaggcgtaaaca

ag

ssb fusion attB2 8

GGGGACCACTTTGTACAAGAAAGCTGGGTCtcagaacggaatgtcatcatc

holC_F6A_F	9	AACGCGACGgccTACCTTCTGGACAAT
holC_Y7A_F	10	AACGCGACGTTCGCCCTTCTGGACAAT
holC_F6A_Y7A_L8A_R	11	TTTGGCCCTCAGGGCCGGATCCGTATG
holC_S19A_F	12	GTCGATGGCTTAGCCGCCGTTGAGCAA
holC_A20D_F	13	GTCGATGGCTTAAGCGACGTTGAGCAA
holC_S19A_A20D_R	14	GGTGGTGTCATTGTCCAGAAGGTAGAA
holC_C26A_F	15	GTTGAGCAACTGGTGGCTGAAATTGCC
holC_C26A_R	16	GGCGCTTAAGCCATCGACGGTGGTGTC
holC_C43A_F	17	ATCGCCGCTGAAGATGAAAAGCAGGCT
holC_C43A_D45A_R	18	GAGCACGCGCTTACCGCTGCGCCAACG
holC_D45A_F	19	ATCGCCTGTGAAGCTGAAAAGCAGGCT
holC_E54A_F	20	CTGGATGCAGCCCTGTGGGCGCGTCCG

holC_E54A_L56A_R	21	CCGGTAAGCCTGCTTTTCATCTTCACA
holC_W57A_F	22	GAAGCCCTGGCGGCGCGTCCGGCAGAA
holC_W57A_R	23	ATCCAGCCGGTAAGCCTGCTTTTCATC
holC_F64A_F	24	CCGGCAGAAAGCGCTGTTCCGCATAAT
holC_F64A_R	25	ACGCGCCCACAGGGCTTCATCCAGCCG
pCA24N FOR seq	26	CATTAAAGAGGAGAAATTAACTATGAGAGG
pCA24NrrnBT1 REV seq	27	ATGTGTCAGAGGTTTTCACCGTCATCAC
pGADT7_Seq_FOR	28	CGACTCACTATAGGGCGAGCG
pGADT7_Seq_REV	29	GTGCACGATGCACAGTTGAAGTGAAC
pGBKT7_Seq_FOR	30	GCCGCCATCATGGAGGAGCAG
pGBKT7_Seq_REV	31	CCCGGAATTAGCTTGGCTGCAAGC
holD KO confirm F	32	AGGTCATCCTGTAAGTCTCCGGCAAACAGA
holD KO confirm R	33	GATGTTCCAGCAGCGCCCTTCCCAATCCCT 2.3

Complementation and Toxicity Assays:

Plasmids pSTL 393, 426, 394, 395, 396,397, 398, 399, 400, 401, 402, and 403 were introduced by using the bacterial electroporation transformation into strain backgrounds MG1655 (*holC*+) and STL18822 (*holC*Δ). In parallel, plasmids pSTL393 and pSTL426 were introduced, representing the vector and *holC*$^{+}$ controls, respectively.

To assay toxicity of the plasmid-encoded *holC* gene, a single colony was inoculated in 2 mls of minimal glucose Cm media and incubated w at 37°C for 18 hours. Following this incubation cultures were re-inoculated and diluted 1:100 into 2 mls of fresh minimal minimal Cm media and grown for 2 hours. Each

culture was split, with one culture receiving 1mM IPTG to promote induction of *holC* and the other untreated. Each culture was subjected to an additional 2 hours of growth before serial dilution and plating in duplicate on a minimal Cm plate. Results were compared to the positive and negative controls of this assay.

To assay for *holC* complementation and for genetic dominance of the plasmid allele, a single colony was inoculated in 2 mls of LB Cm media standing at 37°C for 18 hours. Following this incubation cultures were re-inoculated and diluted 1:100 into 2 mls of fresh LB Cm media using the standing cultures. Cultures were then grown for 2 hours. At the end of this time period culture were then split with one culture receiving 1mM IPTG to promote induction of *holC*+ or the site directed mutants while the other set was not. Each culture was subjected to an additional 2 hours of growth before serial dilution and plating in duplicate on a LB Cm plate with no or various concentrations of azidothymidine (AZT), 7.5,12.5, 25 and 37.5 ng/µl. Results were compared to the positive (pSTL426) and negative (pSTL393) controls and complementation was determined with fraction survival of those cells exposed to AZT versus those that were not for every dose. AZT-sensitivity in STL18822 indicates lack of complementation of *holC*Δ.

2.4 **Construction of GAL4 Activation Domain and Binding Domain fusions to HolC, HolD, SSB 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, holD, ssb* and *yoaA* for construction of GAL4 fusions for the yeast two hybrid analysis. A small portion of a live single colony of MG1655 was briefly submerged in 10µl of sterile water. This mixture was then subjected to 1000 Watts of microwave for 30 seconds to aid in the breaking up of the cell wall and its contents. This 10 µl denatured mixture was then used as a template for the high fidelity PCR reaction.

Gateway cloning technologies from Invitrogen® were used to transfer the mutations created in pCA24N-holC to the GAL4 activation and binding domain plasmids, pGADT7GW and pGBKT7GW, respectively. Primers 3+4 were used in a high fidelity PCR reaction using Pfu DNA polymerase obtained from Agilent®. Subsequent PCR reactions were subjected to *Dpn*I to destroy the template plasmid. Following purification of the PCR products (BioBasic kits), *holC* mutant fragments (attB1-*holC* 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 .

2.5 **Yeast Two-Hybrid Analysis:**

Procedures, instructions, and controls followed the Matchmaker Gold Yeast Two-Hybrid System obtained from Takara Bio with the following modifications. Negative controls consisted of the ones suggested in the Matchmaker Gold Yeast Two-Hybrid System along with *holC, holD, yoaA*, and *ssb* that were cloned in the activation domain plasmid paired with the binding domain empty plasmid. Likewise *holC, holD, yoaA*, and *ssb* cloned into the binding domain plasmid was paired with the activation domain empty plasmid. 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 low stringency protein-protein interactions while adenine deficient plates test for a higher stringency. Plates were incubated for two or three days at 30°C.

2.6 **Overexpression and Purification of HisYoaA with HolC and HolD or with HolC F46A:** The *holC* gene in pET15b-holC was mutated to convert Phe-64 to Ala using the

NEB Q5 Site-Directed Mutagenesis Kit as per the manufacturer's directions. *E. coli* BL21(DE3) cells were transformed with two vectors, pCOLADuet-HisYoaA along with pETDuet-holC/holD or pET15b-holCF46A to co-express His-tagged YoaA (HisYoaA) along with HolC and HolD or HolC-F64A, respectively. Cells were grown at 37°C in Terrific Broth to an OD600 of about 0.6 at 37°C and protein expression induced by addition of IPTG (1 mM) and cells were grown for an additional 4 hrs at 25°C. At the time of induction, iron (II) sulfate and ammonium ferric citrate were added to final concentrations of 0.1 mg/ml each. Cells were harvested by centrifugation and frozen at -80°C. Thawed cells were resuspended in cell lysis buffer (20 mM NaPi pH 7.5, 40 mM imidazole pH 8.0, 500 mM NaCl, 10% glycerol, Sigma FAST Protease Inhibitor Cocktail EDTA-free, and 2 mM DTT at pH 7.8) and lysed by French Press. The cell lysate was clarified by centrifugation and loaded on to a HisTrap FF 5 mL column and washed with 40 mL of cell lysis buffer which has a low imidazole concentration. The samples were eluted with a high imidazole buffer (20 mM NaPi pH 7.5, 250 mM imidazole, 500 mM NaCl, 10% glycerol, and 2mM DTT at pH 7.8). Fractions from the high 250 mM imidazole elution were pooled and dialyzed against 25 mM Tris-Cl pH 7.8, 250 mM NaCl, 10% glycerol, and 2 mM DTT. The protein in Fig. 5A lane E was used as a reference in the SDS-PAGE analysis in Fig. 5B (HisYoaA-χ).

2.7 **SDS-PAGE and Western Blot -** Protein samples from the soluble fraction of the cell lysate, the flow-through from the HisTrap FF column, and the high-concentration

imidazole buffer elution (described in the purification above) were separated by SDS-PAGE and transferred to a PVDF membrane. Two separate gels were run, one to measure HisYoaA and a second to measure HolC and HolD (when present), using the same samples. An antibody to His-tags (Invitrogen MAI-21315 X-Histag from mouse) was used to detect HisYoaA. The secondary probe for HisYoaA was Biorad IgG-HRP conjugate anti-mouse antibody from goat. A HolC rabbit antibody, a gift of Charles McHenry's laboratory, was used to detect HolC and HolC-F64A. The secondary probe for HolC was Biorad IgG-HRP conjugate anti-rabbit antibody from goat. A HolD antibody, a gift of Charles McHenry's laboratory, from mouse and secondary anti-mouse antibody (Biorad IgG-HRP conjugate anti-mouse antibody from goat) was used to detect HolD. All antibodies were diluted in 5% milk with Tris-buffered saline with Tween 20 (TBST). 2.6 Overexpression and Purification of YoaA, Chi, Psi and YoaA HolC F46A. The *holC* gene was mutated to convert Phe-64 to Ala using the NEB Q5 Site-Directed Mutagenesis Kit as per the manufacturer's directions. *E. coli* BL21(DE3) cells were transformed with two vectors, either pCOLADuet-HisYoaA and pETDuet-holC/holD or pCOLADuet-HisYoaA and pET15b-holCF46A. The first pair expresses N-terminally 6X His-tagged YoaA (HisYoaA) along with HolC and HolD, and the second co-expresses HisYoaA and HolC-F64A. Cells were grown at 37°C in Terrific broth to an OD600 of about 0.6 at 37°C and protein expression induced by addition of IPTG (1 mM) and cells grown for an additional 4 hrs at 25°C. At the time of induction, iron (II) sulfate and ammonium ferric citrate were

added to final concentrations of 0.1 mg/ml each. Cells were harvested by centrifugation and frozen at -80°C. Thawed cells were resuspended in cell lysis buffer (20 mM NaPi pH 7.5, 40 mM imidazole pH 8.0, 500 mM NaCl, 10% glycerol, Sigma FAST Protease Inhibitor Cocktail EDTA-free, and 2 mM DTT at pH 7.8) and loaded on to a HisTrap FF 5 mL column and washed with cell lysis buffer. The samples were eluted with a high imidazole buffer (20 mM NaPi pH 7.5, 250 mM imidazole, 500 mM NaCl, 10% glycerol, and 2mM DTT at pH 7.8). Fractions from the 250 mM imidazole elution were pooled and dialyzed against 25 mM Tris-Cl pH 7.8, 250 mM NaCl, 10% glycerol, and 2 mM DTT.

3. RESULTS

To identify interaction mutants, we mutated a number of conserved residues in the *holC* gene, expressed from a plasmid (see Figure 1). These mutants were first transformed into a *holC*Δ mutant strain to test for the mutants' ability to complement its AZT-sensitive phenotype, in parallel with the wild-type *holC* and plasmid vector controls. We grew the bacterial cells in minimal medium, which reduces the inviability of *holC*Δ strains. Without IPTG- induction of the *tac* promoter on the plasmid, the wild-type *holC* gene restored survival to AZT to wt levels (about 10% at this dose), compared to the vector control. HolC Y7A, S19A, C26A, C43A mutants had little or no effects on complementation. Complementation defects were observed for HolC mutants F6A, A20D, D45A, E54A, W57A and F64A; we had previously documented F64A, V117F, R128A and Y131L

as a non-complementing mutants [20]. (Figure 2A). Upon IPTG- induction of the strong *tac* promoter on the plasmid, *holC*$^{+}$ expression is toxic, causing a 10,000-fold reduction in plating efficiency after a two-hour exposure (Figure 2B). Many of the mutants that negated complementation of AZT-sensitivity also reduced the toxicity of *holC*, including F6A, A20D, D45A. Mutation Y7A also reduced toxicity, although its effect on complementation was modest. Mutants of HolC affecting SSB-binding, R128A and Y131L [17], caused a dramatic reduction in toxicity when overexpressed, 400- and 6000-fold, respectively, indicating that the toxicity due to results from excess HolC binding to SSB. We presume this toxicity is caused by sequestration of SSB, reducing its ability to bind other partners. Non-complementing mutants E54A, W57A and F64A remained toxic when overexpressed. These are of interest because they appear to retain SSB binding but do not support AZT tolerance. Two of these were chosen for further study.

The mutated *holC* genes were moved into yeast two-hybrid vectors and tested for the ability of their encoded proteins to interact with SSB, HolD and YoaA Gal4-fusions. The yeast two-hybrid analysis of protein interaction is especially useful here because we introduce only one pair of *E. coli* proteins, precluding indirect interactions through other partners. We had previously shown a defect for HolC F64A in interaction with HolD [20]; the crystal structure of the HolC HolD heterodimer shows HolC-F64 buried deep in a hydrophobic pocket of HolD [35], so this is not unexpected. Likewise, HolC-W57A reduced interaction with HolD (Figure 4); W57A is also at the HolC/HolD subunit interface (see Figure 1B) and both of these residues are invariant in HolC from different genera. HolC-F64A and W57A retained the ability to interact with single-strand DNA binding protein, SSB (Figure 3, Figure 4). Surprisingly, HolC-F64A and W-57A also failed to interact with YoaA (Figure 3, Figure 4), indicating that the same surface on HolC may be used for binding to both HolD and to YoaA. These mutants retained the ability to interact with SSB, so their defect in YoaA/HolD interaction is unlikely to be due to misfolding or gross changes to the protein structure. When expressed in *E. coli*, neither mutant affects HolC expression levels (D. Cooper, unpublished data).

YoaA, HolC, and HolD were co-expressed in BL21(DE3) cells to determine whether a ternary YoaA-HolC-HolD complex could form. YoaA was expressed with a N-terminal 6X His-tag to permit isolation of HisYoaA by Ni^{2+} affinity chromatography. Immunoblots were used to monitor the eluent from the Ni^{2+} column and determine which proteins co-purify. All three proteins were present in the soluble fraction of the cell lysate (Figure 5A, CL). HisYoaA bound to the Ni^{2+}

column and was eluted with a high concentration of imidazole and HolC co-eluted with HisYoaA (Figure 5A, E), indicating that HolC was bound to HisYoaA. In contrast, HolD was not present in this fraction. HolD passed through the column along with additional HolC presumably as a HolC-HolD complex (Figure 5A, FT). Because HolC eluted with either HisYoaA or HolD but not both, these results indicate that HolC does not bind YoaA and HolD simultaneously. When the HolC-F64A mutant was overexpressed with HisYoaA, the HolC-F64A mutant was present in the soluble fraction of the cell lysate suggesting that the mutant protein folds. But, the HolC-F64A mutant eluted in the low imidazole loading buffer and was not retained on the Ni^{2+} column with HisYoaA as was wild-type HolC (Figure 5B). It is unlikely that the F64A mutation would affect direct binding of HolC to the Ni^{2+} column such that wild-type HolC would bind the column whereas HolC-F64A would not. Therefore, we conclude that wild-type HolC binds YoaA and is retained on the Ni^{2+} column via binding to HisYoaA, but HolC-F64A does not bind YoaA with high affinity, and therefore flows through the Ni^{2+} column. Taken together, these results support the conclusion that YoaA binds HolC via an interaction that involves HolC Phe-64 such that the binding sites for YoaA and HolD on HolC overlap, making binding of HolC to YoaA and HolD mutually exclusive.

4. DISCUSSION

This work sheds new light on the role of the accessory protein of the *E. coli* clamp loader complex, HolC, and its ability to modulate replication and replication fork repair. Although the HolC protein is nonessential for cell proliferation under certain conditions, it is required to preserve genomic integrity [36]. Loss of the interaction of HolC with SSB causes constitutive SOS induction [17], indicative of the accumulation of fork damage. The work described here

indicates that HolC, via its interaction with SSB, may play a dual role: one in recruiting HolD and, through it, the Pol III holoenzyme, and the other in recruiting the YoaA helicase. Through these alternative binding partners, HolC has the potential to modulate replication and repair of SSB-coated single-strand DNA.

We show here that overexpression of HolC leads to toxicity that is dependent on the proteins's interaction with SSB. This toxicity is not abated in HolC mutants that have lost the ability to interact with YoaA or HolD, so the presence of HolC alone on SSB-coated DNA must be sufficient to cause toxicity. Excess HolC may preclude SSB-binding of a number of proteins required to sustain replication or repair. In addition to HolC, SSB binds a host of DNA replication/repair proteins including DNA primase (DnaG) [15, 37], bypass polymerases (Pol II, PolIV, PolV)[38-40], replication restart proteins (PriA, PriB) [41, 42], recombination proteins (RecO)[43, 44], helicases (RecQ, RecG) [45, 46], nucleases (RecJ, ExoI, ExoIX) [47-49], RNase H [50],[51] ATPases (RarA, RadD)[52, 53], topoisomerases (TopoIII, TopB) [54], uracil N-Glycolyase (Ung) [55] among potentially others [54, 56]. Any one or any combination of these interactions may be required to sustain viability.

In *E. coli,* YoaA and HolC promote tolerance to AZT, a chain-terminating nucleoside [20]. After incorporation into DNA, AZT monophosphate, lacking a 3′ OH, blocks further DNA polymerization, leading to a ssDNA gap in the replication fork [57]. The AZT moiety must be excised from DNA to allow the completion of replication. We propose that HolC and YoaA must in some way assist in this removal process. The AZT tolerance promoted by these two functions

is co-dependent, implicating a common mechanism: AZT-tolerance by overexpressing HolC was dependent on YoaA and that conferred by overexpressing YoaA required HolC [20]. By yeast two-hybrid analysis and pulldowns, we demonstrated a physical interaction between the two proteins [58]. Because of the absence of any other *E. coli* genes in the yeast two-hybrid analysis, this interaction must be direct.

In our previous study, we showed that knock-out mutants in both HolC and YoaA are sensitive to normally sublethal concentrations of AZT. [20] In HolC, the amino acid residues required for SSB interaction in vitro [17], HolC- V117, R128 and Y131, are also required for AZT resistance in vivo. [20] The crystal structure of the HolCD heterodimer [35] strongly implicated HolC-F64 in stabilizing the HolC/HolD interaction, since the residue is bound deeply within a pocket in HolD. HolC-F64A was defective in both the Y2H interaction with HolD and in its ability to promote AZT tolerance [20]. At the time, we interpreted this result with the simplest explanation—that HolD interaction with HolC is important for YoaA to promote AZT survival. However, an alternative explanation is that there are two distinct complexes, HolC with HolD and HolC with YoaA; HolC F64 may be required for interaction with either partner, YoaA or HolD. We provide here evidence to support this latter alternative by showing that two residues of HolC, F64 and W57, are required for HolC's interaction with HolD and with YoaA as measured in the Y2H analysis. After overexpression of all three proteins, we detected HolC but not HolD associated with YoaA, indicating that HolC's binding to one excludes the other, as expected if HolD and YoaA share the same binding surface on HolC.

What is the biological reason for these alternative complexes? HolC's interaction with HolD promotes recruitment of the DNA polymerase III holoenzyme through the clamp loader complex, which facilitates efficient clamp loading and DNA replication. In the heterodimer, HolD is the subunit that interacts with DnaX protein of the core clamp loader [18]. On the other hand, interaction of YoaA and HolC would allow the helicase to be recruited, without interference from the DNA polymerase III, to persistent single-strand DNA bound by SSB, as might be expected to accumulate in a fork that needs repair. Consistent with a second repair role, HolC is found in excess relative to HolD protein and the clamp subunits in vivo by about 3-4-fold, a situation that persists well into stationary phase [59]. YoaA is an SOS-gene with a predicted LexA repressor binding site, induced by DNA damage [60], although the magnitude of this up-regulation is unknown. Because of this regulated expression, we suspect that YoaA may bind tighter to HolC than does HolD, although this needs to be measured directly in vitro. HolC, in some way, may modulate the activity of YoaA or it may act as a recruitment factor at the fork to target YoaA to a persistent ssDNA gap. Factors that remodel or process replication forks for repair need to be carefully restricted to those forks experiencing problems, lest they interfere with normal replication.

What is the role of the YoaA helicase in repair? YoaA is a member of a large group of Fe-S cluster-containing DNA helicases (the XPD family) that translocate 5′ to 3′ on ssDNA [61]. At a ssDNA gap, these helicases would unwind the 3′ terminus of the interrupted strand. Radiation and oxidative damage can induce strand breaks with 3′ phosphoglycolate or other modified termini [62, 63]. This type of strand break, like that after incorporation of

azidothymidine monophosphate, cannot be simply ligated. A damaged 3′ terminus of an elongating DNA strand needs to be removed before replication can be resumed and gaps are filled. YoaA/HolC unwinding of 3′ terminated DNA strands may assist this removal. In the Gram-positive, Firmicutes group of bacteria, the YoaA ortholog is fused to a 3′ to 5′ exonuclease domain, similar to that associated with DNA polymerase proofreading. This may indicate that YoaA function is coupled to nuclease action. Unwinding of DNA by YoaA might facilitate "extrinsic proofreading" by non-polymerase associated 3′ to 5′ exonucleases or by flap endonucleases. YoaA, through a ATP-dependent motor activity, may also assist in removing proteins, such as replisome components that remain bound to a nascent strand, DNA secondary structures or bound RNA transcripts. In addition, helicase-mediated unwinding of DNA may promote a template-switch to the sister to overcome blocks in replication [64].

In the gamma-proteobacteria, YoaA has a paralog, DinG, a structure-specific DNA helicase [23] and the two proteins share 29% identity across almost their entire length, including the helicase motifs, HD1 and HD2 domains, and the 4 cysteines that coordinate Fe-S binding [65]. DinG, like YoaA, is induced by DNA damage as part of the SOS response [60, 66, 67]. In *E. coli,* DinG, unlike YoaA, makes little contribution to tolerance of AZT [20] nor to UV [22]. DinG is, however, implicated as one of the helicases that help overcome conflicts between replication and transcription [68], presumably by dissociating DNA/RNA hybrids ("R-loops"), since the phenotype of DinG mutants is suppressed by overexpression of RNaseH [68] Most other bacteria encode only a single member of the DinG/YoaA family. in *Neisseria meningitidis,* the YoaA/DinG ortholog is required for resistance to the DNA cross-linker mitomycin C [25]. In

Mycobacterium tuberculosis, the YoaA/DinG ortholog is able to unwind G-quadruplex structure in vitro, as well as various branched and forked DNA structures [69].

The importance of this group of helicases is seen in other domains of life. Eukaryotes and archaea possess multiple members of Fe-S cluster DNA helicase group [26, 61] with similar functions in repair and maintenance of genomic stability. In humans, these pathways include excision repair and transcription-couple repair (XPD)[70], DNA cross-link repair (FANCJ, DDX11) [71, 72], disassembly of DNA secondary structure and telomere maintenance (RTEL, FANCJ) [73, 74], and homologous recombination (RTEL, DDX11)[72, 74]. Defects in these underlie a number of genetic diseases, including XP-D xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy; FANC-J, Fanconi anemia and breast cancer proneness; RTEL, Hoyeraal-Hreidarsson syndrome; DDX11, Warsaw breakage syndrome.

FIGURE LEGENDS

Figure 1. Schematic of the HolC protein, indicating the conserved residues mutated in this study that were defective in function. Shown below is the crystal structure of the HolC/HolD heterodimer and a blow-up of the subunit interface [38], indicating the position of HolC F64 and W57 interaction residues (in yellow), reproduced with permission of the publisher. HolD/YoaA and SSB interaction residues lie on opposite ends of HolC.

Figure 2. A. Complementation of *holC* AZT sensitivity by the plasmid-borne *holC* alleles indicated. % survival determined by the cfu on glucose minimal medium with 50 ng/ml AZT relative to that without AZT. B. Toxicity of 2 hour expression of plasmid-borne *holC* alleles,

induced by 1 mM IPTG. Toxicity index is the cfu of untreated culture/ cfu of the IPTG-induced culture. Controls for both experiments include cells carrying the plasmid vector (pCA24N) or the plasmid-encoded *holC*+ wild-type gene. Averages of values from 3-6 independent cultures are shown, with standard deviation error bars.

Figure 3. Yeast 2 hybrid analysis of HolC or HolC-F64A fused to the GAL4 activation domain and YoaA top spots) or SSB (bottom spots) fused to the GAL4 DNA binding domain. Growth on -Ade plates indicates a strong interaction; growth on -His plates indicates a weaker interaction. All strains grow on -Leu Trp. Three replicates are shown for each configuration.

Figure 4: Yeast 2 hybrid analysis of HolC or HolC-W57A fused to the GAL4 activation domain and HolD (left spots), YoaA (center spots) or HolD (right spots) fused to the GAL4 DNA binding domain. Growth on -Ade plates indicates a strong interaction; growth on -His plates indicates a weaker interaction. All strains grow on -Leu Trp plates.

Figure 5. HolC but not HolD nor HolC-F64A co-purified with YoaA. Protein-protein interactions were measured by co-expression with 6X His-tagged YoaA (HisYoaA) in the same cells. Cells were lysed and soluble material (CL) was loaded onto a Ni^{2+} affinity column in buffer containing a low concentration of imidazole. Proteins that did not bind Ni^{2+} (or HisYoaA) flowed through the column (FT). HisYoaA was retained on the column and eluted with buffer containing a high concentration of imidazole (E). Samples from each fraction were analyzed by SDS-PAGE and immunoblotting with anti-His, anti-HolC, or anti-HolD antibodies to detect HisYoaA, HolC or HolC-F64A, and HolD, respectively. A) Fast Green-stained blots and immunoblots (IB) of samples from co-expression of HisYoaA, HolC, and HolD are shown. Proteins from the same experiment were loaded on two different gels in different amounts, 3 µg in the left panel and 5 µg in the

right panel. The left panel was immunoblotted with an anti-His antibody to detect HisYoaA, and the right panel with anti-HolC and anti-HolD to detect HolC (χ) and HolD (ψ), respectively. Protein molecular weight markers (MW) and a purified sample of Yχψ proteins were included as standards. B) Fast green-stained blots and immunoblots of samples from co-expression of HisYoaA and HolC-F64A are shown. Different amounts of protein, 2 µg and 5 µg, from the same sample were loaded two different gels in the left and right panels, respectively. The left panel was immunoblotted with anti-His to detect HisYoaA and the right with anti-HolC to detect HolC-F64A. Protein molecular weight markers (MW) and a purified sample of HisYoaA-χ were included as standards..

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The authors declare that there are no conflicts of interest.

6. Author contributions:

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Figure 1

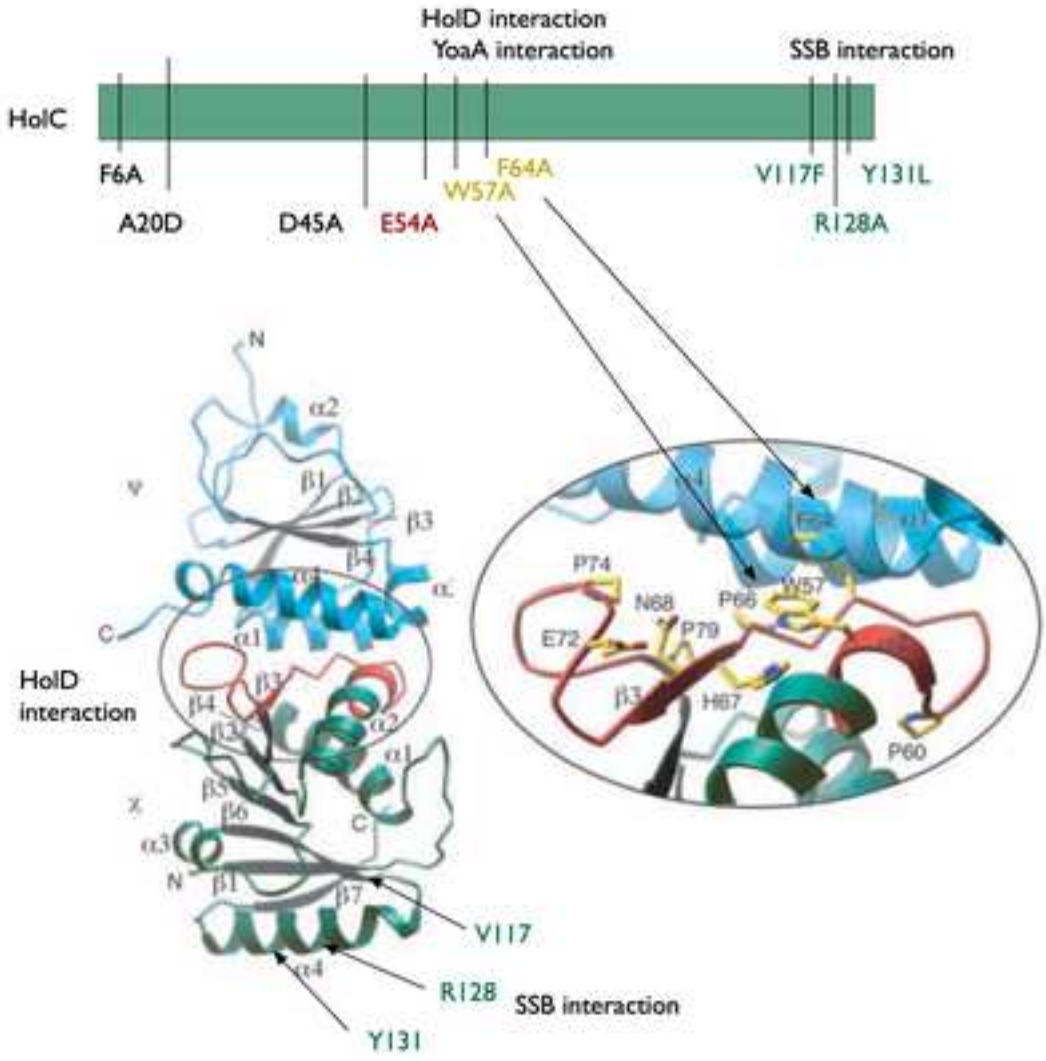
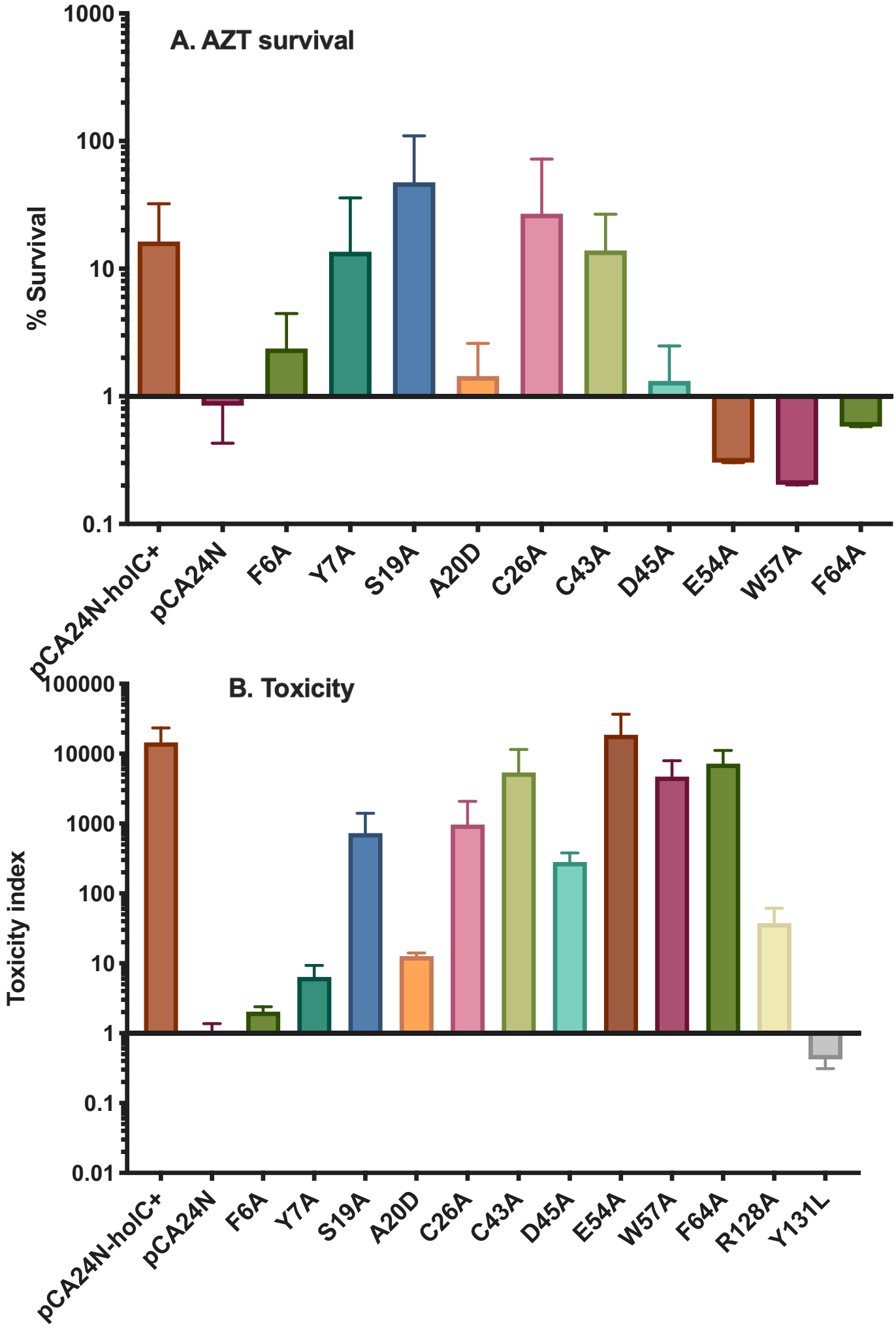


Figure 2


A. AZT survival
% Survival
1000
100
10
1
0.1
pCA24N-holC+
pCA24N
F6A
Y7A
S19A
A20D
C26A
C43A
D45A
E54A
W57A
F64A
B. Toxicity
Toxicity index
100000
10000
1000
100
10
1
0.1
0.01
pCA24N-holC+
pCA24N
F6A
Y7A
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R128A
Y131L

Figure 3

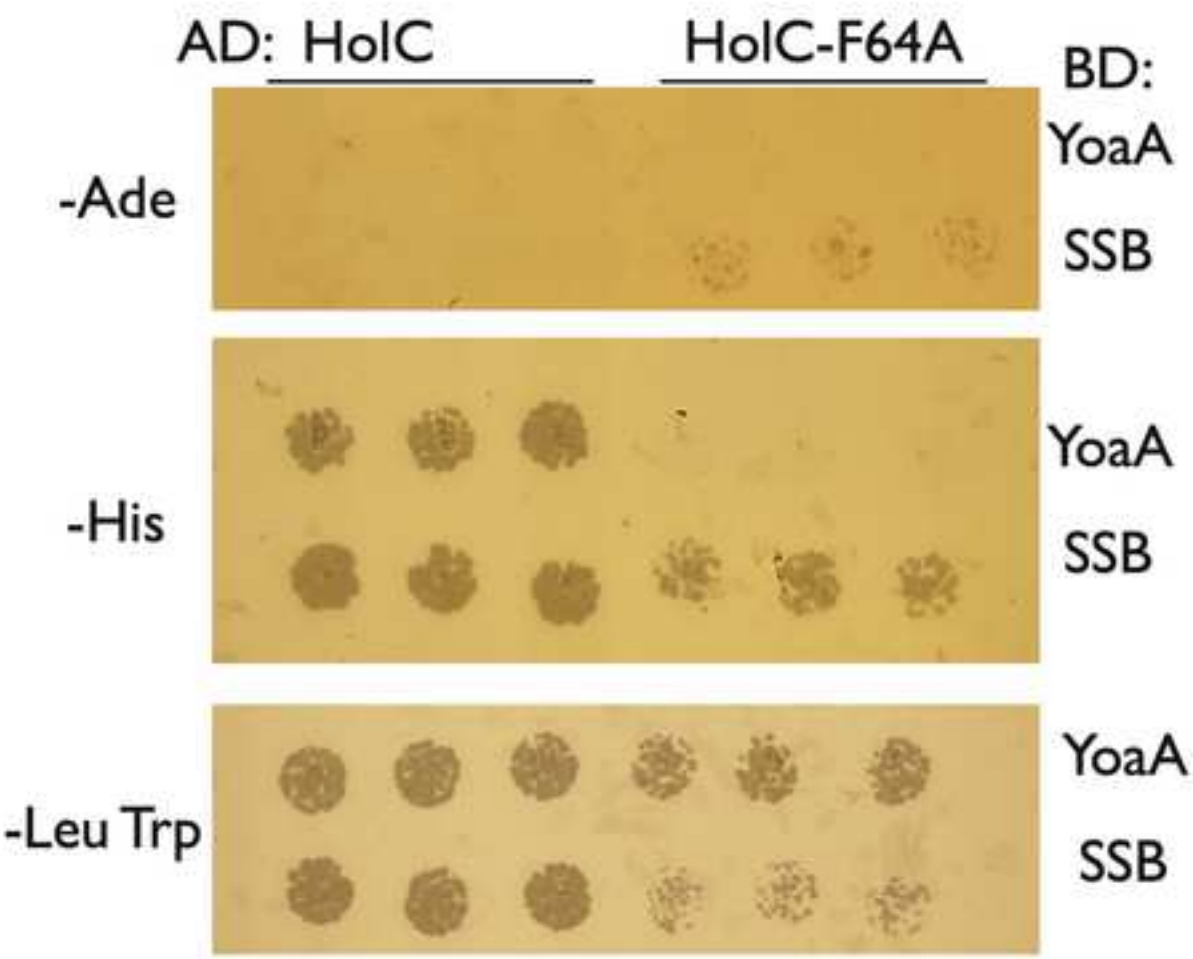


Figure 4

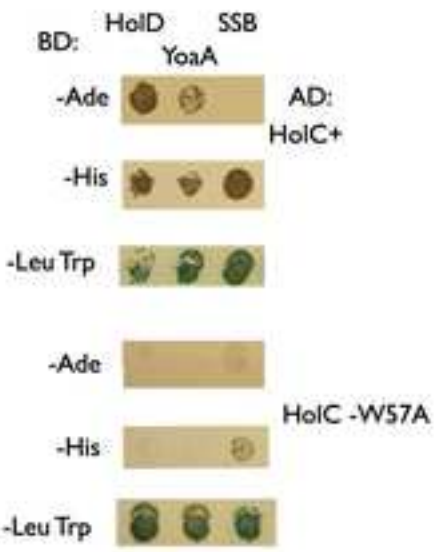
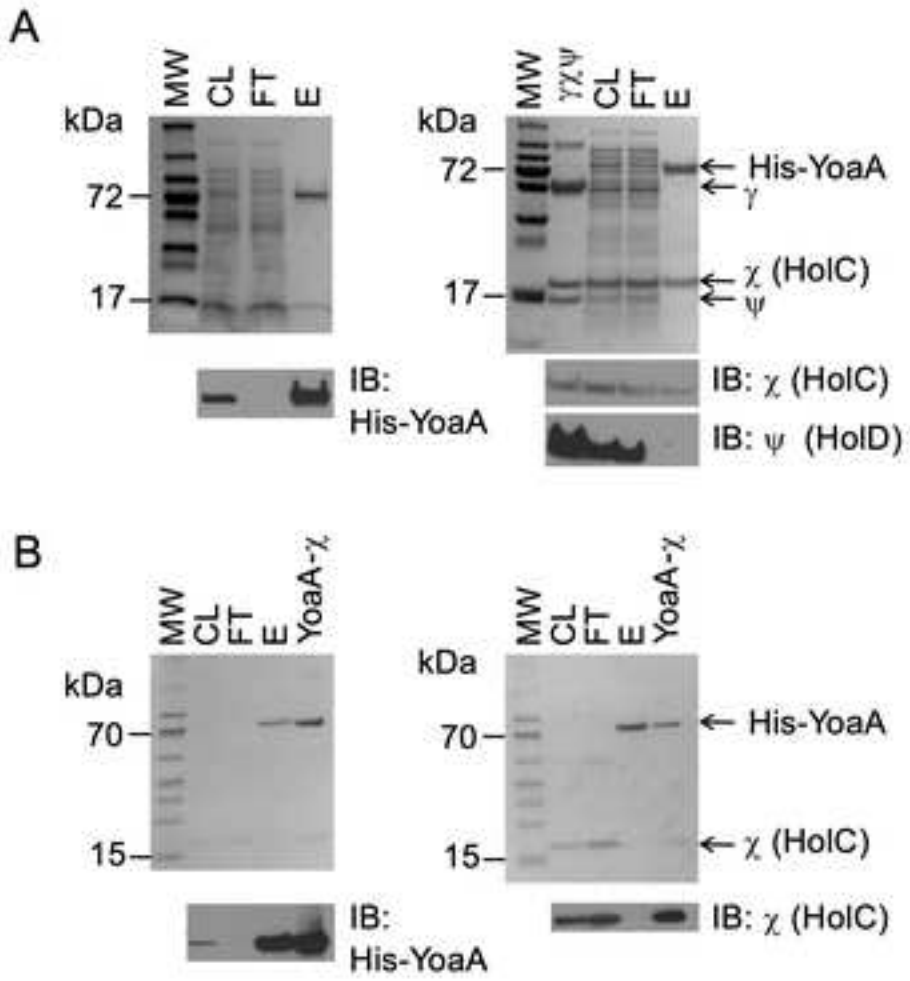


Figure 5



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