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Investigating the role of Holoenzyme C in restoring replication following UV-induced DNA damage in Escherichia coli

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by

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ABSTRACT

The recovery of replication following UV-induced DNA damage in *Escherichia coli* correlates with the time at which the lesions are repaired, suggesting that the two processes may be coupled. *holC* encodes the Chi-subunit of replicative DNA polymerase III, and is nonessential for viability, but renders cells hypersensitive to UV-induced damage when mutated, suggesting it may play a specific role in restoring replication when it encounters DNA damage. Here, I characterized the role of HolC in restoring DNA replication following disruption by UV-induced damage. I found that survival following UV in strains deleted for *holC* was similar to that of strains deleted for *recF*, a protein that is specifically required to restore replication when it is disrupted by DNA damage. Further, similar to *recF* mutants, *holC* mutants fail to resume DNA synthesis following disruption by UV irradiation. I interpret these observations to be consistent with the idea that the HolC subunit of the replisome is required to restore replication following disruption by DNA damage. I discuss these results in the context of how repair may be recruited to these sites to allow replication to recover.

INTRODUCTION

Ultraviolet light is a form of nonionizing radiation that is capable of causing lesions within DNA strands (13). These lesions can block RNA and DNA polymerases, preventing the essential processes of transcription and replication (8). If not repaired, such lesions in DNA can result in mutagenesis, genomic instabilities, or cell death. In cases where mutagenesis or rearrangements occur, the heritable changes to the genetic code may lead to reprogramming that promotes prolific cell growth and skin cancer in humans (13). Many mechanisms exist within organisms to prevent these deleterious results caused by damaged DNA from occurring. In *Escherichia coli*, a common type of bacteria, a primary error-free mechanism for processing DNA lesions is through repair by the Nucleotide Excision Repair (NER) enzymes. NER is the process that allows for the detection of DNA damage, excision of the lesions, and then resynthesis of the region using the complementary strand as a template. Damage is initially detected by UvrA along with UvrB. UvrB separates the strands near the damaged site, releasing UvrA. UvrC is then recruited to cleave the phosphodiester bonds upstream and downstream of the DNA-damaged site (11).

An alternative subpathway begins with RNA polymerase, which is essential in the process of transcription, and detects DNA damage when it is blocked. An accessory protein of the blocked polymerase, called Mfd, recruits UvrA to initiate the repair process (2). This pathway is called transcription-coupled repair (TCR) as transcription occurs simultaneously with DNA repair.

Whether a similar process occurs when replication is blocked remains an open question, although several lines of evidence suggest it may. In the absence of repair, replication is severely impaired following DNA damage (9). Additionally, several gene products associated with the *recF* pathway serve the purpose of restoring replication following DNA damage. *recF* is required for the resumption of replication at replication forks (6). *E. coli* mutants with *recF recO* or *recR* knockouts show hypersensitivity to UV light and fail to restore replication after DNA damage (6). It was shown that RecF, RecO, and RecR are all necessary to prevent degradation at the replication forks by reassembling a holoenzyme and allowing for replication to continue (6, 9). UvrA is also necessary for RecF's role in the resumption of replication after DNA damage has occurred. For the recovery of replication to begin, the lesions need to first be excised (7). Cells exhibiting RecF expression without nucleotide excision repair, are able to protect the DNA at replication forks but are not able to resume replication (7).

Holoenzyme C is the chi subunit of DNA polymerase III and is also integral to the replisome. HolC interacts with single-stranded binding proteins (SSB) and has been reported to exhibit impaired replication under some conditions (12, 16). HolC has been shown to interact with YoaA, a helicase protein from the DinG family with potential roles in DNA repair (15). For example, YoaA functions to limit the effects of nucleoside azidothymidine (AZT) which is a DNA replication inhibitor (15). Mutants of *E. coli* without the *holC* gene are also hypersensitive to UV (15). Intriguingly, a genome-wide interactome study reported an interaction between UvrA and HolC. This raises the possibility that HolC may serve to recruit repair at blocked DNA polymerases in a manner that is similar to how Mfd recruits repair to blocked RNA polymerases. Additional literature has also shown that HolC forms complexes with HolD to recruit the clamp loader, then allowing for the DNA polymerase III enzyme to bind and continue replication (16). The complexes that HolC forms with other proteins are of great interest and importance as it allows for proper replication to occur within the genome.

Here I characterized the role of HolC after UV-induced damage. I generated *E. coli* mutants featuring gene knockouts in *holC* and subjected them to increasing doses of UV light to characterize their phenotypes with respect to survival and ability to resume replication. I also compared these phenotypes to that of *recF* mutants, which has been shown previously to be critical for cells to resume replication post-DNA damage

MATERIALS & METHODS

Strains and growth media. The strains used in this study are derived from BW25113 and are described in Table 1. BW25113 and JW4216 were obtained from the National BioResource Project in Japan and have been previously described (1). Luria-Bertani (LB) was used as a rich medium and phosphate-buffered Davis with 0.4% glucose and 0.2% casamino acids (DGC) was used as the defined medium.

UV Survival Assays. Strains were diluted 1:100 from fresh overnight cultures and grown in 5-ml DGC medium at 37ºC until mid-logarithmic phase. At this time, 0.1-ml aliquots of each culture were removed and serial 10-fold dilutions were made into DGC medium. Triplicate 10-µl aliquots of each dilution were then spotted on LB plates and exposed to increasing doses of UV as indicated. The UV incidence dose was 1 J/m^2/sec and was delivered using a 15-W germicidal lamp (254 nm). Viable colonies were counted following overnight incubation at 37ºC.

Replication Recovery. Fresh overnight cultures were diluted 1:100 into 30-ml DGC medium and grown at 37ºC for 2.5 hours. Following this outgrowth period, cultures were divided equally and one-half of the cells were mock irradiated, while the other half of the cells was UV-irradiated with 30 J/m^2. At the times indicated, duplicate 0.5-ml aliquots of culture were removed and pulse-labeled with ^{3}H-thymidine at a concentration of 1µCi/ml for 2 min at 37ºC before 5% trichloroacetic acid (TCA) was added to lyse the cells and precipitate the DNA. Samples were filtered onto glass fiber filters to collect ^{3}H-labeled DNA. The amount of ^{3}H on each filter was then determined by scintillation counting.

Growth Curve. 0.1-ml aliquots of fresh overnight cultures were serially 10-fold diluted in LB medium. Duplicate 100-µl aliquots of each dilution were pipetted into a 96-well microtiter plate and incubated at 37ºC with agitation. Absorbance readings at 600 nm were taken over time using a BIO-Whittaker ELx808 plate reader. The number of viable cells in each overnight culture was determined at the start of every experiment, and the growth of equal numbers of viable cells for each strain was compared.

RESULTS

Table 1: List of strains used in this study.

Strain	Genotype	Source of Reference
WT (BW25113)	F^- Δ(*araD-araB*)567 Δ*lacZ4787*::(rrnB-3) LAM^- *rph-1* Δ(*rhaD-rhaB*)568 *hsdR514*	Baba et al. 2006
CL579	SR108 *recF*::tet	Courcelle et al. (2003)
CL4271	BW25113 *recF*::tet	P1 (*recF*::tet from CL579) x BW25113 Hilton, Courcelle (unpublished)
JW4216	BW25113 *holC*::kan (Δcodons2-141, Keio)	Baba et al. 2006
CL5103	BW25113 *holC*::kan (Δcodons2-141, Keio) *recF*::tet	P1 (*recF*::tet from CL579) x JW4216 This work
CL4877	BW25113 *holC* (Δcodons2-74)	This work
CL4950	BW25113 *holC* (Δcodons2-74) *recF*::tet	P1 (*recF*::tet from CL579) x CL4877 This work

holC recF mutants are more sensitive to UV than either *holC* (Keio) or *recF* single mutants.

HolC is a known accessory protein of the replication machinery and the absence of this gene has been previously shown to sensitize cells to the DNA replication arresting agent, 3'-azidothymidine (AZT) (15). RecF, which is known to be required for replication recovery after DNA damage, shows hypersensitivity to DNA damaging agents, particularly in actively replicating cells (6, 7). To characterize HolC's phenotype with regards to replication recovery, I constructed a *holCrecF* double mutant and compared the UV survival of this strain to isogenic wild-type (WT), *holC*, and *recF* mutants. If HolC is crucial for the resumption of replication post UV-induced DNA damage, I expect that knockout mutants of this strain would exhibit UV sensitivity, potentially similar to UV sensitivity levels of *recF*.

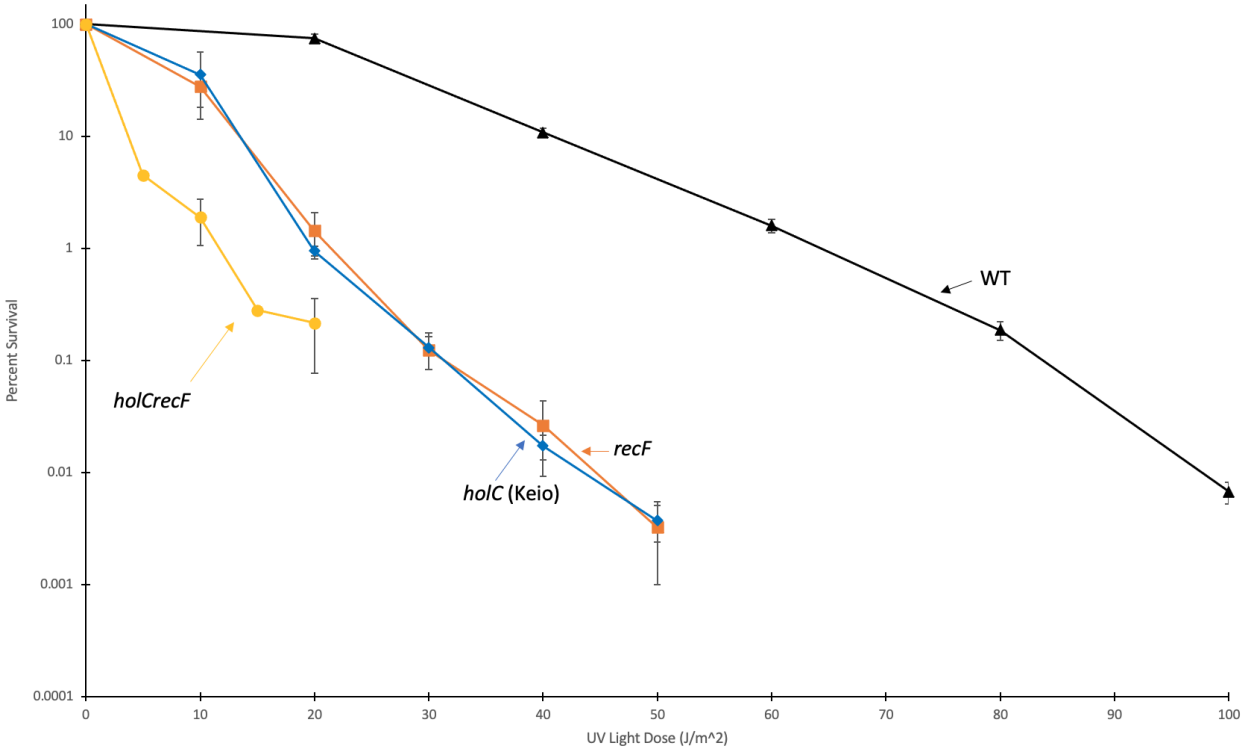

Figure 1: *holC recF* mutants are as sensitive to UV as *holC* (Keio) and *recF* single mutants. Survival of wild-type (WT) (black triangle), *holC* (Keio) (blue diamond), *recF* (orange square), and *holC* (Δ2-141) *recF* (yellow circle) is plotted at the UV doses indicated. Graph represents the average of at least three individual experiments. Error bars represent standard error.

As previously reported, the *recF* single mutant showed moderate hypersensitivity to UV irradiation (5 and Fig. 1). The *holC* single mutant from the keio collection showed a similar trend in sensitivity to UV irradiation to *recF* cells across all UV doses tested (Fig. 1). At a UV dose of 50 J/m^2, I observed a 1000-fold difference in percent survival between wild-type cells and either *recF* or *holC* single mutants (Fig. 1). The percentage of surviving *holC* or *recF* cells at 50 J/m^2 was comparable to wild-type cells irradiated with 100 J/m^2. In contrast, the absence of both *holC* and *recF* increased the UV sensitivity of cells by approximately 10-fold relative to each of the single mutants alone. In fact, the extreme UV hypersensitivity exhibited by the double mutant strain required lowering the UV doses applied to study this strain. Further, although I assessed the viability of the *holC recF* mutant out to 25 J/m^2 UV, any viable colonies were below this assay's detection limit. The observation that *holC recF* double mutants are more sensitive to UV irradiation compared to *holC* and *recF* single mutants suggests that these proteins are acting on separate genetic pathways that facilitate survival following UV-induced DNA damage.

holC (Keio) and *recF* mutants do not recover replication following UV irradiation.

My survival data shows that RecF and HolC contribute to cell survival in the presence of UV-induced DNA damage, however, the survival assay only allowed me to assess whether a gene was required for viability and not if it played a direct role in DNA replication resumption following DNA damage. To test the roles of RecF and HolC directly, I measured the rates of replication recovery post-UV-induced DNA damage in *recF* and *holC* (Keio) mutant strains. To

this end, duplicate aliquots of cultures were pulse-labeled with ^{3}H-thymidine for 2 minutes at various times following irradiation with 30 J/m^2 and the rate of DNA synthesis at each time (^{3}H incorporation/2 min) was then determined. A mock-irradiated control was included in all experiments to ensure that any observed differences in DNA synthesis were related to UV treatment.

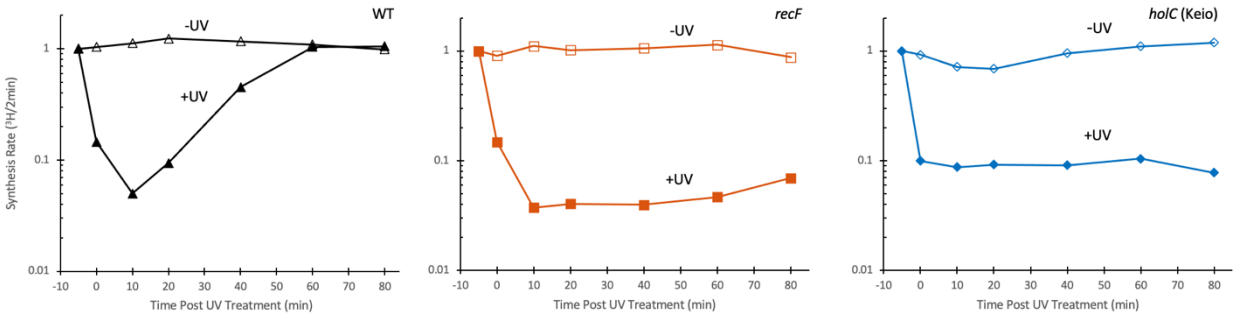

Figure 2: *holC* (Keio) and *recF* mutants do not recover replication following UV irradiation. ^{3}H-thymidine at 1µCi/ml was used as a radiolabel and added to cultures for 2 minutes at time points -5, 0, 10, 20, 40, 60, and 80 minutes following UV irradiation at time 0. The rate of DNA synthesis (^{3}H-thymidine incorporated/2min) relative to pre-treatment levels is plotted for wild-type and mutant strains over time. Open symbols, mock irradiation; closed symbols, 30 J/m^2 UV.

As expected, the rate of DNA synthesis was unaffected by mock irradiation and remained constant over time for all strains (Fig. 2). Immediately post-UV exposure, the DNA synthesis rate decreased by greater than 90% in wild-type cells before recovering 20 minutes post-UV treatment and exhibiting full replication recovery by 60 minutes (Fig. 2). In the absence of RecF, I observed a similar decrease in the rate of replication immediately following UV irradiation but unlike wild-type cells, *recF* mutants did not restore replication over the remainder of the 80-min time course (Fig. 2). When I measured the rate of synthesis in UV-irradiated *holC* mutants from the keio collection, I observed a lack of recovery in replication throughout the course of the experiment (Fig. 2). These observations suggest that HolC and RecF are necessary for the resumption of replication after UV-induced DNA damage.

holC (new construct) mutant grows better than *holC* (Keio).

holC deletion mutants are viable, however, they exhibit poor growth and have been observed to accumulate suppressor mutations and local genetic rearrangements (4). To determine whether any suppressor mutations had accumulated in the *holC* mutant I was characterizing, I submitted genomic DNA prepared from this mutant for genome sequencing. Several additional mutations in the Keio collection *holC* mutant were found including a spontaneous *recF* mutation that is predicted to delete amino acids 272-313 of RecF (see Table 2 for a complete list of mutations). In addition, I found that the design for the Keio *holC* mutation would be predicted to remove the two promoters of *valS*, which is essential for *E. coli* growth and is the gene immediately downstream of *holC* (Fig. 3). Based on the sequence data showing secondary mutations in Keio *holC* and the potential for altered *valS* expression in this mutant, I decided to construct a new *holC* mutant. To do this, I used the recombineering technique described by Baba et al. (1) to target a kanamycin resistance cassette into *holC*, resulting in a loss of codons 2 to 74

of this gene while preserving the two *valS* promoters (Fig. 3). Genomic DNA from this newly constructed *holC* mutant, designated *holC* (new construct) to distinguish it from the Keio collection *holC* (*holC* (Keio)) for the remainder of my thesis, was submitted for sequencing and found to only contain the designed *holC* mutation (Table 2).

Table 2: List of mutations found in *holC* from the Keio collection and newly constructed *holC*.

Strain	Mutation List
BW25113 *holC* (Δcodons2-141, Keio)	Isogenic BW25113 *ypjF/ypjA*(Δ1bp), *waaQ*(W113), *holC* (Δcodons2-141), *recF* (Δcodons272-313)
BW25113 *holC* (Δcodons2-74, new construction)	Isogenic BW25113 *holC* (Δcodons2-74)

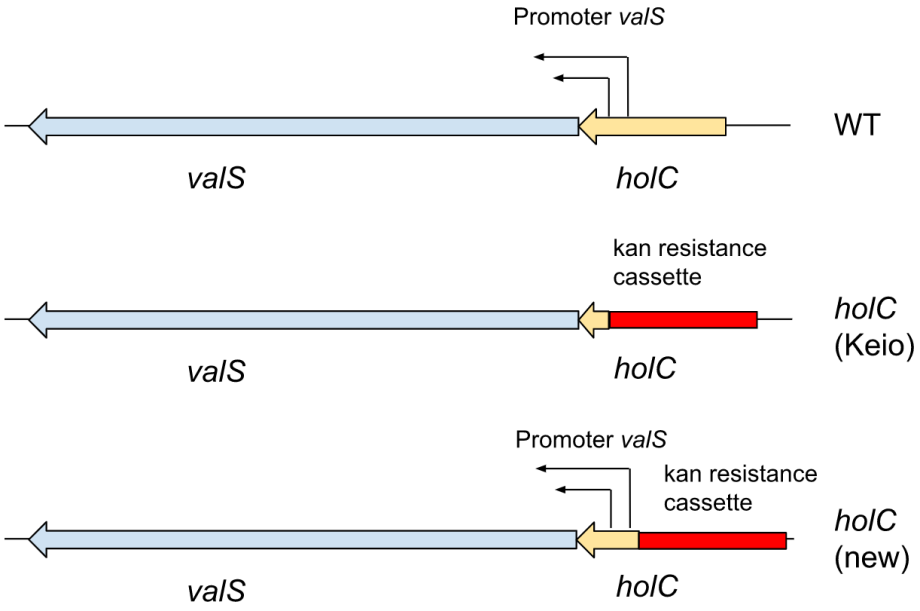

Figure 3: Model of the *holC-valS* region in wild-type (WT), *holC* (Keio), and *holC* (Δ2-74 (new construct)). Two putative promoters for *valS* are found within *holC* (WT). *holC* (Keio) contains a deletion of *holC* codons 2-141 that also deletes the *valS* promoters. *holC* (new construct) was designed to include the *valS* promoter.

Our sequencing data showed that *holC* (new construct) did not contain any secondary gene alterations, therefore I wanted to determine how this strain fared in terms of growth in the absence of any additional mutations. If the cause of slow growth in *holC* (Keio) was due to dysregulation of *valS* expression and mutation in *recF*, I hypothesized that *holC* (new construct) would have an increased growth rate and decreased lag in growth relative to *holC* (Keio). Further, to distinguish the contribution of *recF* mutation to the observed slow growth in *holC* (Keio), I constructed an isogenic *holC* (new construct) *recF* double mutant. To begin characterizing the effect of *holC* mutation on cell growth and viability, I compared the growth of *holC* (new construct), *holC* (new construct) *recF*, *holC* (Keio), and *holC* (Keio) *recF*.

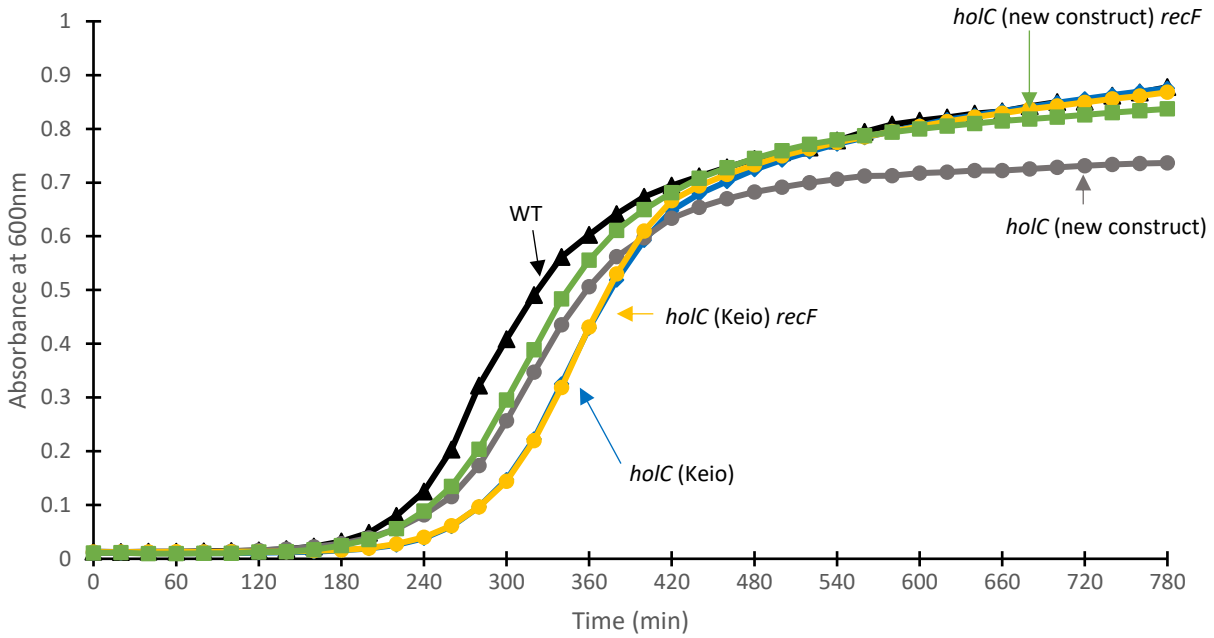

Figure 4: *holC* (new construct) mutant grows better than *holC* (Keio). Absorbance at 600 nm for wild-type (black triangles), *holC* (new construct, gray circles), *holC* (new construct) *recF* (green squares), *holC* (Keio, blue diamond), *holC* (Keio) *recF* (yellow circles) cultures grown at 37°C is plotted over time.

Wild-type cells begin exponential growth at 200 minutes and exhibited the greatest change in absorbance at 280 minutes with a rate of 0.005 Δabsorbance/min (Fig. 4). The *holC* (new construct) strain showed a lag in growth relative to wild-type cells and showed a maximum change in absorbance of 0.0045 Δabsorbance/min at 320 minutes (Fig. 4). The *holC* (new construct) *recF* mutant strain showed a similar growth pattern to *holC* (new construct) with a maximum growth rate of 0.0047 Δabsorbance/min at 320 minutes (Fig. 4). In contrast, *holC* (Keio) and *holC* (Keio) *recF* exhibited a delay in onset of exponential growth relative to both wild-type and *holC* (new construct) (Fig. 4). While *holC* (Keio) and *holC* (Keio) *recF* were slower to begin exponential growth, both strains were still able to replicate with wild-type kinetics once they started growing, showing a maximum rate of 0.0053 Δabsorbance/min at 340 minutes. Taken together, my results suggest that adding a *recF* deletion has no effect on the growth rates of either *holC* (Keio) or *holC* (new construct) strains. Additionally, the absence of the *valS* promoters in *holC* (Keio) exacerbates the general slow growth of *holC* mutants.

holC (new construct) *recF* shows increased sensitivity relative to its single mutants.

To further investigate whether HolC and RecF interact following UV-induced DNA damage, I compared survival of *recF*, *holC*, and *holCrecF* double mutants exposed to various doses of UV irradiation.

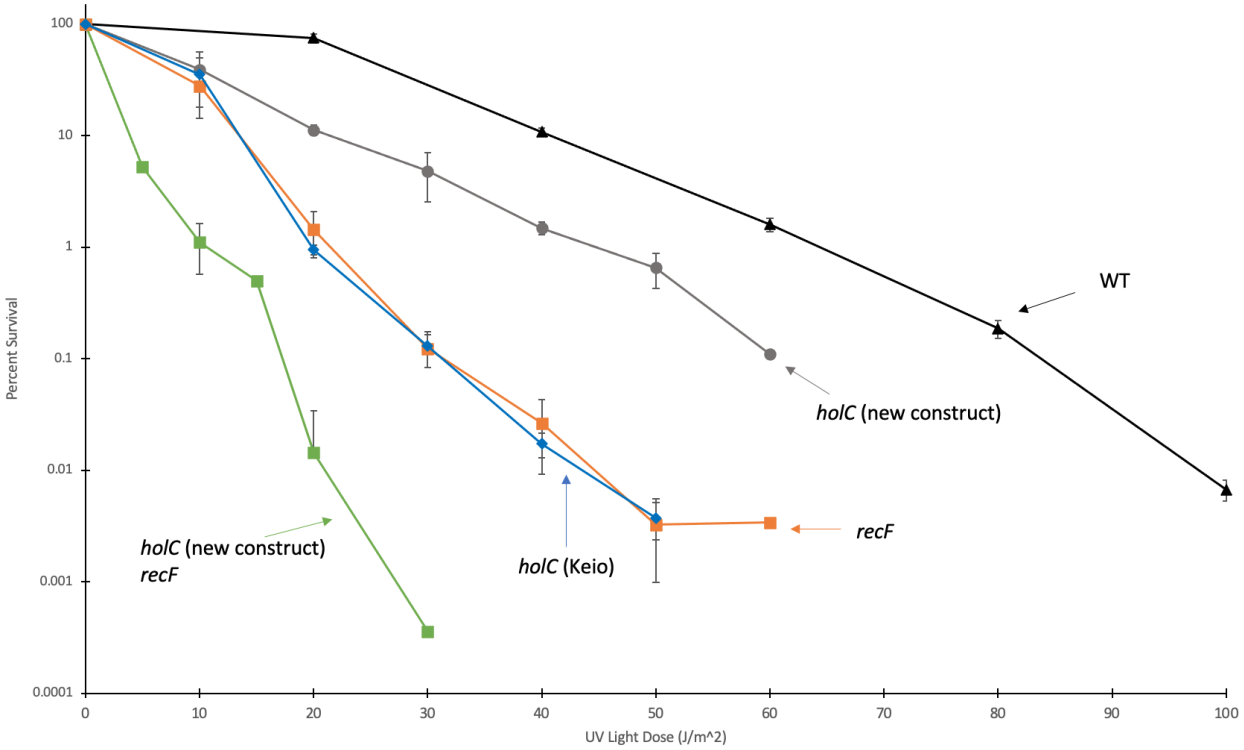

Figure 5: *holC* (new construct) *recF* shows increased sensitivity relative to its single mutants. Survival of wild-type (black triangles), *holC* (new construct) (gray circles), *holC* (new construct) *recF* (green squares), *holC* (Keio), blue diamond), and *recF* (orange squares) is plotted at the UV doses indicated. Survival data for wild-type, *recF* and *holC* (Keio) was reproduced from Figure 1. Graphs represent the average of at least three individual experiments. Error bars represent standard error.

The *holC* (new construct) mutant strain was less sensitive to UV than either *recF* or *holC* (Keio), however, it was approximately 10-fold more UV sensitive than its wild-type parent at every dose examined (Fig. 5). The *holC* (new construct) *recF* mutant strain was hypersensitive to UV irradiation (Fig. 5). In contrast to what is observed for the wild-type strain, the shape of the UV survival curve for *holC* (new construct) *recF* cells does not exhibit a shoulder but instead decreases rapidly beginning at 5 J/m^2, the lowest UV dose assayed (Fig. 5). The UV hypersensitivity of *holC* (new construct) *recF* suggests that HolC and RecF operate on separate cellular pathways to allow for replication to recover post DNA damage by UV irradiation.

holC (new construct) mutants are delayed for replication recovery.

The survival curve for *holC* (new construct) showed a slight sensitivity of this mutant to UV light, however this data did not allow me to assess whether this sensitivity was due to an effect on replication recovery following UV treatment. To this end, I compared the rates of replication recovery post DNA damage induced by UV irradiation between *recF* and *holC* (new construct) mutant strains using the replication assay described above.

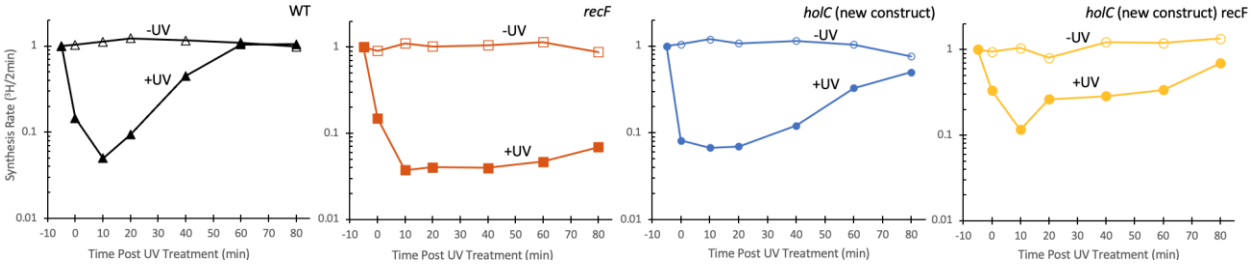

Figure 6: *holC* (new construct) mutants are delayed for replication recovery after UV-induced DNA damage. ^{3}H-thymidine at 1μCi/ml was used as a radiolabel and added to cultures for 2 minutes at time points -5, 0, 10, 20, 40, 60, and 80 minutes following UV irradiation at time point 0 minutes. Time points indicated minutes post UV treatment. The rate of DNA synthesis (^{3}H-thymidine incorporated/2min) is plotted for wild-type cells and mutant strains over time. WT and *recF* plots were reproduced from figure 2 for comparison. Open symbols, mock irradiation; closed symbols, 30 J/m^{2} UV.

UV-treated *holC* (new construct) mutant was inhibited for replication immediately following irradiation, began to recover replication at 40 minutes post-UV treatment, and restored replication to approximately 50% of pretreatment levels by 80 minutes post-UV (Fig. 6). The rate of replication recovery is delayed in the absence of HolC compared to wild-type cells, which show full recovery by 60 minutes post-UV (Fig. 6). This observation suggests that HolC is required for timely resumption of replication following UV-induced DNA damage. When I examined the effect of *holC* and *recF* double mutants (*holC* (new construct) *recF*) on replication resumption after UV damage, I found an inhibition in replication following treatment followed by a slight increase in the rate of replication beginning at 20 min post-UV (Fig. 6). While this result would suggest that the absence of HolC and RecF rescues the ability of both *holC* and *recF* single mutants to recover replication, the lower level of replication inhibition relative to all other strains assessed and the low ^{3}H counts over background I obtained for a single experiment with this strain require further experimentation to draw any conclusions.

DISCUSSION

Based on the results listed above, HolC appears to contribute to the recovery of replication, although the magnitude of the effect is not as severe as that for RecF. As seen in the survival assays (Fig. 1, 5), the double mutant strains consistently showed a more severe hypersensitive phenotype relative to their respective single mutants. In Figure 6 it can be seen that *holC* (new construct) exhibits a delay in restoring replication, while the delay in *recF* is more severe. This mirrors the hypersensitivity of the two mutants as well (Figure 5). As discussed in the introduction, HolC has the ability to interact with other proteins, such as YoaA, to allow for replication to be repaired following DNA damage by AZT (15). An additional report from a global interactome study suggested HolC may also interact with UvrA (3). The delayed recovery of *holC* mutants is similar to that reported for *recJ* mutants, which encodes a nuclease that partially degrades the nascent DNA and allows the lesion to be repaired (4). In the absence of RecJ, the recovery of replication depends upon translesion synthesis by Pol V, which requires approximately 50 minutes after UV to become activated (14). If Pol V is absent, the delay in *recJ* mutants is more severe and appears similar to that seen in *recF* (4). Speculatively, if HolC is required to recruit UvrA, the delayed recovery that occurs may be similarly dependent on translesion synthesis, similar to *recJ* mutants. This would be interesting to examine in future studies.

The difference in UV sensitivity between *holC* (new construct) and *holC* (Keio) is quite significant (Fig. 5). This is best explained due to the presence and absence of the *valS* promoter region, respectively (Fig. 3). ValS is essential for viability since it is a valine-tRNA ligase, catalyzing the reaction that allows for valine to be incorporated into growing peptides (17). Although some *valS* transcript can be expressed from the promoter upstream of *holC*, loss of the internal promoter likely results in a severe reduction of this enzyme's levels that would greatly limit a cell's ability to produce required proteins. Surprisingly, the *holC* strain from the Keio collection also features a spontaneous mutation in *recF* from codons 272-313 (Table. 2). As a result, RecF's function and expression could be limited, further explaining the increased UV sensitivity of the strain and its ability to not recover replication (Fig. 2). It has previously been shown that Keio *holC* mutants exhibit slow growth (4). This was seen in Figure 4 as the double and single mutant based on the Keio collection showed slower growth than compared to the newly generated strains. This further shows that *valS* expression may be causing the increased lag phase in growth. Lastly, it can be seen that the *holC* (new construct) strain recovers replication, which is far different from the lack of replication recovery seen in the *holC* (Keio) strain (Fig. 2 & 6). However, it is slower to recover than the wild-type strain, indicating that *holC* (new construct) does have a slight phenotype in terms of replication recovery. These observations suggest that the *holC* (new construct) strain is most relevant in gauging HolC's phenotype with regard to UV-induced DNA damage.

This research is significant in terms of furthering scientific knowledge on the role HolC plays in cell survival and replication recovery post-UV-induced DNA damage. It also establishes that the *holC* strain from the Keio collection removes a promoter for *valS*, which is notable as it affects the UV phenotype for survival and replication recovery and causes a greater lag phase in the strain's growth rate. Further experimentation is still needed to determine whether HolC and UvrA physically interact as the characterization of such a complex is important to better understand the replication-coupled repair process.

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