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Investigating a Link Between DNA Repair and DNA Replication in Escherichia coli Using a Bacterial Two-Hybrid System

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Investigating a Link Between DNA Repair and DNA Replication in *Escherichia coli* Using a Bacterial Two-Hybrid System

By

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Abstract

Cells rely on nucleotide excision repair (NER) to remove bulky DNA lesions, such as those caused by UV light. In *Escherichia coli*, NER involves the UvrABC complex, and includes two main pathways, global genomic repair and transcription-coupled repair (TCR). While TCR, which preferentially removes lesions that block RNA polymerase on the transcribed strand of active genes, is well studied, less is known about whether a similar coupling between repair proteins and replisomes blocked by damaged DNA exists. Such a process has been suggested by a high throughput interactome study, using the replisome subunit HolC as bait and the damage recognition repair protein UvrA as prey, but this interaction has not been verified. Here, I use the Bacterial Adenylate Cyclase-Based Two-Hybrid system to look for a direct protein-protein interaction between UvrA and HolC. Genes encoding HolC, UvrA, and UvrB were cloned into plasmid vectors to create fusion proteins that were tagged with either the T18 or T25 subunits of adenylate cyclase at their carboxyl terminus. Interactions between T18 and T25-fused proteins were then screened by observing the color of colonies produced on indicator agar plates. Blue colonies produced by co-transformations indicate a positive protein-protein interaction while white colonies indicate no protein-protein interaction. I observed blue colonies in all hybrid protein pairings involving UvrA-T18 as bait, even when the partner protein used was the T25 subunit alone, suggesting a high level of background expression from the reporter gene that was independent of a true protein-protein interaction. I discuss some experimental modifications that can be made to address the non-specificity of this assay.

Introduction

DNA damage is an inevitable consequence of cellular life. It can be caused by internal cellular errors or external factors such as ultraviolet (UV) radiation. UV-induced DNA damage causes cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts, which impede replication forks and can lead to cell death (Mitchell and Nairn 1989; Setlow et al. 1963; Kcuik et al. 2020; Crowley et al. 2006). Cells have evolved to have many complex mechanisms that detect and repair DNA damage, such as nucleotide excision repair (NER). In *Escherichia coli*, NER is encoded by the UvrABC endonuclease complex, which is made up of repair proteins UvrA, UvrB, UvrC, and UvrD. UvrA and UvrB function in damage recognition, while UvrC performs dual incisions on the 3' and 5' ends of the damaged DNA fragment. UvrD then removes the excised fragment, DNA Polymerase I resynthesizes new bases to fill the gap, and DNA ligase seals the nick to restore DNA (Sancar and Tang 1993; Seck et al. 2023; Kraithong et al. 2021). A model of this pathway is adapted from Selby et al. (2023) and shown in Figure 1.

Transcription-coupled repair (TCR) is an NER sub-pathway that preferentially removes lesions from transcribed template strands of DNA in actively transcribed genes (Mellon and Hanawalt 1989; Selby and Sancar 1993; Selby and Sancar 1994). In *E. coli*, Mfd recognizes the stalled RNA polymerase at a damaged site and translocates the RNA polymerase away from the lesion, while recruiting the NER complex through a direct interaction with UvrA, which then initiates repair by the global genome repair pathway (Figure 1; Mellon and Hanawalt 1989; Selby and Sancar 1993; Selby and Sancar 1994; and reviewed in Selby et al. 2023).

DNA damage encountered during replication has a particularly high possibility of resulting in mutagenesis or cell death (Courcelle et al. 2004; Cortez 2019). It has been hypothesized that the NER pathway may be coupled to replication as a mechanism to prevent

errors and lethality during this particularly sensitive time, similar to what has been described for transcription-coupled repair (Courcelle et al. 1997; Courcelle et al. 1999; Courcelle et al. 2003). One possible way of coupling repair to replication may involve recruiting the repair machinery to the DNA Polymerase III holoenzyme when it is blocked or stalled by DNA lesions (Figure 1). The holoenzyme is made up of DNA Polymerase III, proofreading subunits, the processivity clamp, and the clamp loader complex (Cooper et al. 2021; Argiriadi et al. 2006).

If this process does occur, several observations suggest that *holC* encoding the χ subunit of the clamp loader complex, could play a central role in recruiting repair. HolC is the only protein on the holoenzyme that directly binds to single-strand DNA binding protein (SSB) (Duigou et al. 2014). Additionally, interaction of HolC with SSB, along with the proofreading 3' exonuclease DnaQ, has been shown to be required for resistance to the DNA damaging agent, 3'-azidothymidine (AZT) (Brown et al. 2015). Although HolC is not necessary for cellular viability, research showed that mutants that lacked HolC were less stable and more sensitive to DNA damage, thus emphasizing that HolC may play a key role in coupling repair to replication (Cooper et al. 2021).

Finally, a genomic interactome survey suggested HolC may physically interact with UvrA (Butland et al. 2005), although this interaction has not been verified. If this interaction was verified, it would provide strong evidence to support the idea that HolC acts as a recruiting factor to form complexes with repair proteins such as UvrA to clear obstructions and lesions caused by DNA damage (Figure 1).

Based on these observations, I hypothesized that when replication is impeded by DNA damage, HolC recruits repair proteins to these sites to aid in repair. Here, I cloned the genes encoding UvrA and HolC into expression vectors that were used as bait and target plasmids,

respectively, and used a bacterial two-hybrid system approach to characterize whether such an interaction between HolC and UvrA occurs in cells.

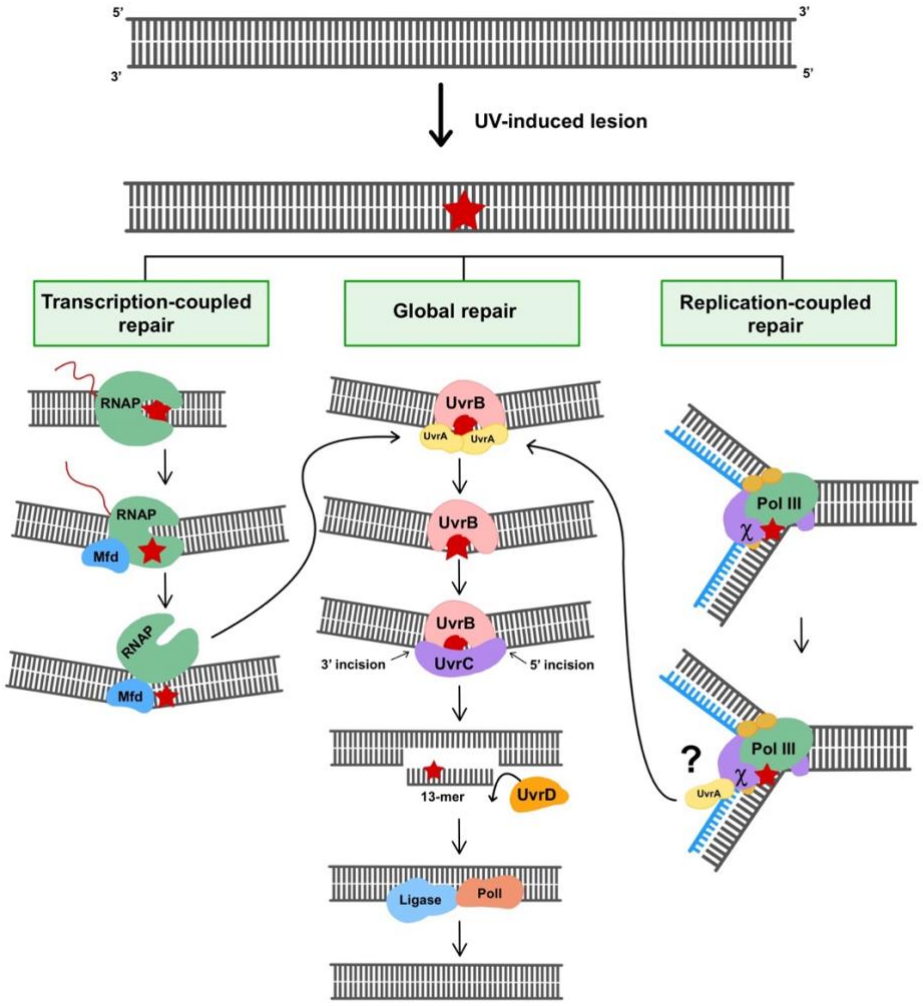

Figure 1: Model of nucleotide excision repair in *E. coli* cells, including a proposed replication-coupled repair (RCR) pathway. Adapted from Selby et al. (2023). In global genomic repair (center), UvrA and UvrB recognize a damaged nucleotide in the genome. UvrA dissociates while UvrB remains. UvrC is recruited and performs dual 3' and 5' incisions around the lesion. UvrD helicase then removes the damaged fragment. DNA polymerase I resynthesizes the gap and DNA ligase seals the nick. In transcription-coupled repair (TCR, left), the RNA polymerase stalls at a lesion and is recognized by Mfd recruiting factor. The RNA polymerase gets lifted, exposing a high-affinity UvrA binding site on Mfd. When UvrA binds, ATP-dependent removal of Mfd and RNAP is initiated and UvrB binds. Following DNA damage verification by UvrB, the global repair pathway removes the lesion from the transcribed strand. In the proposed replication-coupled repair pathway (RCR, right), a lesion encountered at the replication fork may trigger HolC, the χ subunit of the holoenzyme, to recruit UvrA, facilitating global repair at the stalled fork to resolve the damage.

Materials and Methods

Bacterial strains and plasmids

JM109 cells were used to clone plasmids expressing candidate *E. coli* genes fused to adenylate cyclase T25 or T18 subunits. BTH101 (F-, *cya-99, araD139, galE15, galK16, rpsL1* (Strr), *hsdR2, mcrA1, mcrB1*) was used as the reporter strain in bacterial two-hybrid screens as previously described (Matthews and Simmons, 2018). The plasmid vectors used were pUT18 (ampicillin resistant) and pKNT25 (kanamycin-resistant) for the construction of plasmids expressing candidate proteins fused to the T18 and T25 subunits of adenylate cyclase, respectively.

Circular Polymerase Extension Cloning

The primer sequences used to amplify *uvrA, uvrB, holC*, and vectors by polymerase chain reaction (PCR) were designed to share overlapping double-stranded sequences at both ends of inserts and vectors. A wild-type *E. coli* strain was used as a template for *uvrA, uvrB, holC;* cells stably transformed with pUT18 or pKNT25 served as templates for vector amplification. Vectors and inserts were amplified using primers shown in Table 1.

Table 1: List of primers used in plasmid constructions.

pUT18-pKNT25 Forward	5’ catgcctgcaggtcgactct
pUT18-pKNT25 Reverse	5’ cgtaatcatggtcatagctg
pUT18-UvrA Forward	5’ taacaatttcacacaggaaacagctatgaccatgattacgatgGATAAGATCGAAGTTCG
pUT18-UvrA Reverse	5’ tcggtacccggggatcctctagagtcgacctgcaggcatgCAGCATCGGCTTAAGGAAGC
pKNT25-UvrB Forward	5’ taacaatttcacacaggaaacagctatgaccatgattacgatgAGTAAACCGTTCAAACT
pKNT25-UvrB Reverse	5’ tcggtacccggggatcctctagagtcgacctgcaggcatgCGATGCCGCGATAAACAGCT
pKNT25-HolC Forward	5’ taacaatttcacacaggaaacagctatgaccatgattacgatgAAAAACGCGACGTTCTA
pKNT25-HolC Reverse	5’ tcggtacccggggatcctctagagtcgacctgcaggcatgTTTCCAGGTTGCCGTATTCA

Plasmids containing genetic fusions of candidate proteins to the N-termini of T18 or T25 fragments were cloned using circular polymerase extension cloning as previously described (Quan and Tian 2014). Briefly, inserts and vectors were PCR amplified, gel purified, and then quantified. Then, 200 ng of purified vector was mixed in an equal molar ratio with insert DNA and subjected to PCR in a total volume of 20 µL. Conditions for assembly PCR were as follows: initial denaturation at 94°C for 3 minutes; 15 cycles 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 3 minutes; final extension at 72°C for 10 minutes. 2 µL of each mixture was then used to transform 50 µL of electrocompetent JM109 cells and a fraction of these cells was then plated on Lysogeny Broth (LB) agar plates supplemented with either 100 µg/mL of ampicillin or 50 µg/mL of kanamycin.

Plasmid DNA from potential clones was purified using alkaline lysis and analyzed by agarose gel electrophoresis. Candidate clones were sent out for sequencing to verify construction.

Analysis of protein-protein interactions using a bacterial two-hybrid system

Ten ng of each recombinant plasmid encoding UvrA fused to T18, and UvrB or HolC fused to T25 was transformed into 50 µL of electrocompetent BTH101 cells. Following a one-hour recovery at 37°C, serial dilutions were made up to 10^{-4}, then 10 µL of each dilution was spotted on LB agar plates supplemented with 0.1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG), 40 µg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-d-glactopyranoside), 100 µg/mL ampicillin, and 50 µg/mL kanamycin and incubated at 30°C for 24 to 48 hours before scoring for the presence of blue colonies.

Results

To determine whether *E. coli* cells encode a replication-coupled repair pathway, I used the Bacterial Adenylate Cyclase-Based Two-Hybrid (BACTH) system to look for protein-protein interactions between HolC and UvrA. The BACTH system involves cloning the genes encoding proteins of interest into vectors expressing either the T25 or T18 subunits of *Bordetella pertussis* adenylate cyclase, creating two separate plasmids that could then express fusion proteins. Separation of the T25 and T18 subunits results in a catalytically inactive adenylate cyclase that is unable to convert ATP to cyclic AMP (cAMP) (Figure 2A). On the other hand, when candidate proteins interact, the T25 and T18 segments of the adenylate cyclase are brought into proximity of one another and activate a cAMP signaling cascade. The cAMP produced interacts with Catabolite Gene Activator Protein (CAP) to form a complex that binds to and activates expression from the promoters of a number of catabolic operons, including *lacZ* (Figure 2B), which produces β-galactosidase (Karimova et al. 1998). β-galactosidase allows *E. coli* cells to hydrolyze lactose, including its chromogenic analog X-gal, to produce blue bacteria colonies. Thus, blue colonies indicate a positive protein-protein interaction, while white colonies indicate no interaction (Figure 2D).

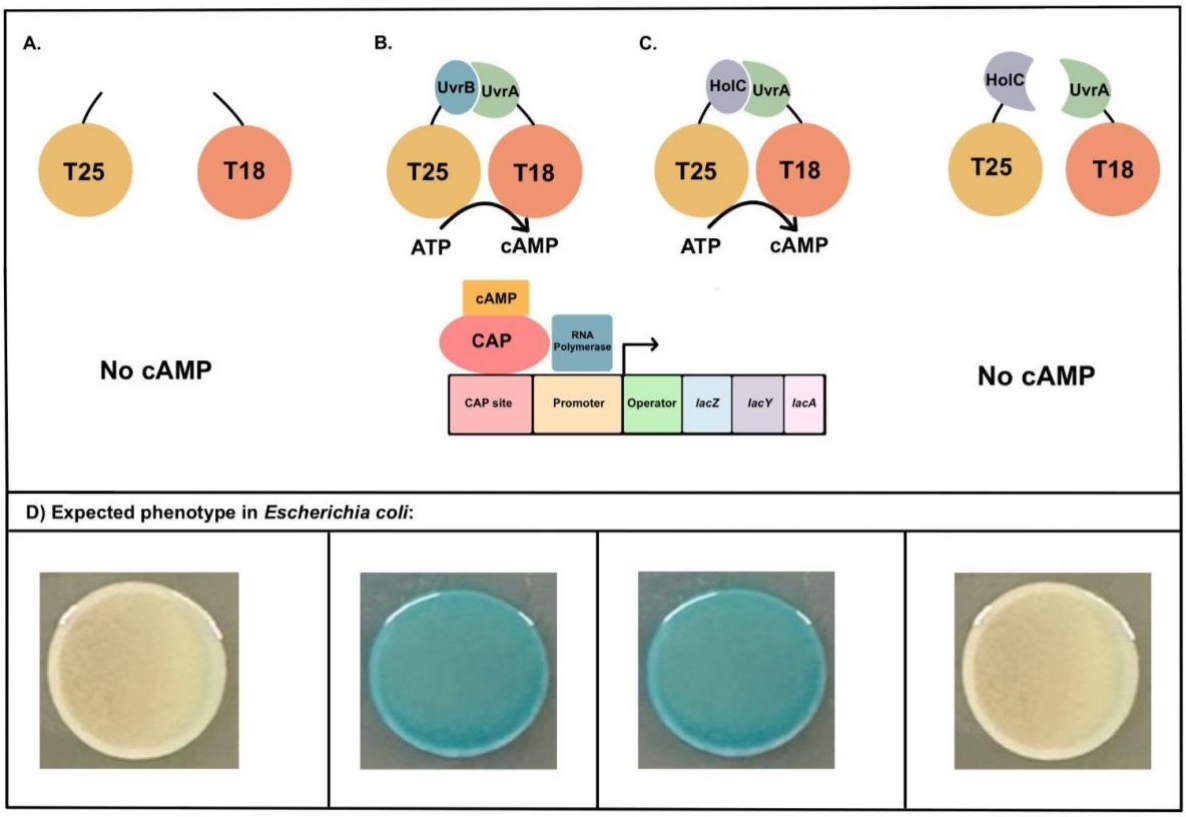

Figure 2: Model describing the Bacterial Adenylate Cyclase Two-Hybrid system. A) Empty vectors pKNT25 and pUT18. Without protein-protein interaction, cAMP is not produced, therefore *lacZ* will not be expressed. Without *lacZ* expression, β-galactosidase will not be made and cells are unable to process the chromogenic substrate X-gal, so the bacterial colonies will be white in color. B) Positive interaction between pKNT25-UvrB and pUT18-UvrA. This leads to cAMP production. cAMP binds with CAP, which binds to the CAP site on DNA upstream of the *lac* promoter, leading to expression of *lacZ*. β-galactosidase is produced by *lacZ*, which allows cells to hydrolyze X-gal, resulting in bacterial colonies that are blue in color. C) Two possible scenarios for a UvrA-HolC two-hybrid screen. If they do interact, cAMP production will occur resulting in *lacZ* expression leading to blue bacteria. If they do not interact, cAMP will not be produced and *lacZ* will not be expressed, resulting in white bacteria. D) Examples of colonies showing predicted results from co-transformation of empty T25 and T18 vectors; a positive interaction between known protein partners UvrA and UvrB; hypothesized interaction between UvrA and HolC; expected result if UvrA and HolC do not interact.

I cloned the genes encoding UvrA, UvrB, and HolC into plasmid vectors. UvrA was cloned into the pUT18 vector, while UvrB and HolC were cloned into pKNT25 vectors using circular polymerase extension cloning. Figure 3 shows plasmid DNA prepared from my candidate clones and separated by agarose gel electrophoresis. As predicted, linearized pUT18 empty vector alone produced a band at 3.0 kb, while clones of pUT18-UvrA gave a band at 5.9 kb. Linearized pKNT25 empty vector produced a band at 3.46 kb, while clones of pKNT25-UvrB were 5.5 kb and pKNT25-HolC were 4.0 kb in size, respectively. Candidate clones from each attempted construction were verified by Sanger sequencing.

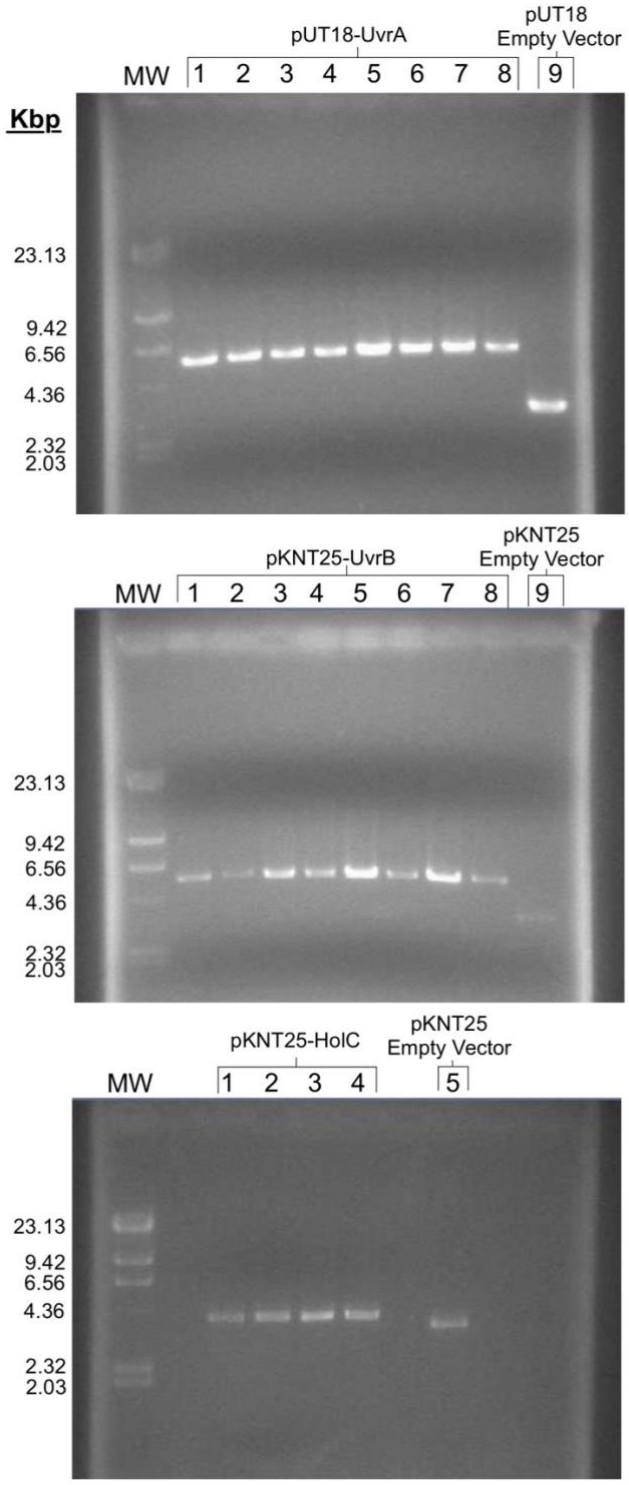

Figure 3: Agarose gel analysis of purified plasmid preparations. A) Lanes 1 to 8: pUT18-UvrA candidate clones; Lane 9: pUT18 empty vector; B) Lanes 1 to 8: pKNT25-UvrB candidate clones; Lane 9: pKNT25 empty vector; C) Lanes 1 to 4: pKNT25-HolC candidate clones; Lane 5: pKNT25 empty vector. MW – Lambda DNA digested with HindIII.

Plasmids were co-transformed into *E. coli* BTH101 cells, which lack adenylate cyclase activity, using UvrA as the "bait" protein, and UvrB and HolC as the targets. Transformed cells were spotted on indicator plates and assessed for the accumulation of blue pigment after 24 to 48 hours incubation at 30°C. As modeled in Figure 2C, an interaction between UvrA and HolC would bring T18 and T25 subunits in proximity of each other and result in blue bacterial colonies; no interaction between these subunits would result in white colonies. Empty vector pairings were used as negative controls and as a measure of background expression of *lacZ*, while co-transformations of pUT18-UvrA and pKNT25-UvrB were used as a positive control.

When pUT18 empty vector was paired with either pKNT25-UvrB or pKNT25-HolC, I observed white colonies, consistent with a lack of interaction between the T18 subunit and T25, UvrB-T25 fusion protein, and HolC-T25 fusion protein, respectively (Figure 4). As expected from the known in vivo interaction between UvrA and UvrB, cells co-transformed with pUT18-UvrA and pKNT25-UvrB produced blue colonies. However, the pairing of pUT18-UvrA with pKNT25 empty vector also unexpectedly produced blue colonies. This suggests that there is some unintended background expression of the *lac* operon in presence of UvrA-T18 fusion protein. Co-transformations of pUT18-UvrA and pKNT25-HolC produced blue colonies, however the interaction cannot be confirmed from this experiment due to the unintended expression in a negative control, as it could have been a result of the same unintended background expression in pUT18-UvrA (Figure 4).

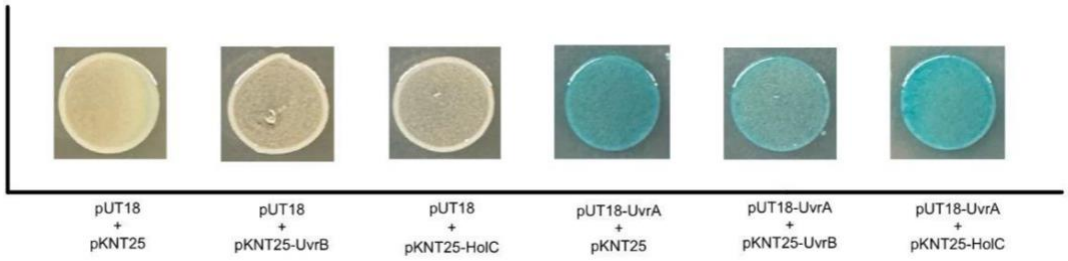

Figure 4: Bacterial adenylate cyclase two-hybrid screens using pUT18-UvrA as bait produced blue colonies even when paired with pKNT25-empty vector. From left to right, colonies produced when combined: pUT18 empty vector and pKNT25 empty vector; pUT18 empty vector and pKNT25-UvrB; pUT18 empty vector and pKNT25-HolC; pUT18-UvrA and pKNT25 empty vector; pUT18-UvrA and pKNT25-UvrB; pUT18-UvrA and pKNT25-HolC were co-transformed into BTH101 reporter cells.

Discussion

A previous interactome study suggested that HolC, the χ subunit of the DNA polymerase holoenzyme, interacted with UvrA, the damage recognition component of the NER pathway (Butland et al. 2005). If an interaction between these proteins could be verified in vivo, it would provide some evidence for a replication-coupled repair pathway in *E. coli*. This study aimed to confirm such an interaction between HolC and UvrA, using a bacterial two-hybrid system. I found that there was no difference in the color of colonies produced following co-transformations with either UvrA-T18 and HolC-T25 fusions, my positive control (UvrA-T18 and UvrB-T25 fusions), or my negative control (UvrA-T18 fusion and unfused T25 subunit). All colonies arising from these co-transformations were uniformly blue and there was no difference in the intensity of the blue pigment.

The bacterial two-hybrid system I used relied on the association of protein partners to reconstitute an active adenylate cyclase domain that could convert ATP to cAMP, followed by an upregulation of *lacZ* as a result of cAMP/CAP binding to the promoter of this reporter gene. Of note in my experimental design is the addition of IPTG, which was used to induce expression of the fusion proteins from their respective plasmids, but would also be expected to increase expression from the *lac* operon (O'Gorman et al. 1980) in the absence of an interaction between my candidate proteins. Although this is an unlikely source of the observed false positive results since the other negative controls used in my experiments produced predicted phenotypes even with IPTG addition, one modification to the experimental design that can be made is to use a different cAMP/CAP responsive gene as a reporter gene. The *mal* operon could be a good candidate since it is not directly induced by IPTG but can be used to screen for interacting hybrid proteins using either colony color on indicator plates or growth on selective medium that

includes maltose as the sole carbon source (Karimova et al. 1998; Ladant 2022).

Another source of false positive results could be that UvrA-T18 interacts with many other cellular proteins in a non-specific manner or self-activates *lacZ* independent of adenylate cyclase. It could be beneficial to test UvrA-T18 with additional T25-fused negative controls and on its own to see if the unintended background *lacZ* expression still occurs. This might involve cloning proteins unrelated to DNA processes into pKNT25 and pairing these with UvrA-T18.

Unlike the Butland et al. paper (2005), which tagged genes in their native location on the *E. coli* genome, *uvrA*, *uvrB*, and *holC* were cloned into plasmids that express these genes in non-physiological quantities. In addition, the pUT18 vector is a high-copy plasmid, while the pKNT25 vector is present at low-copy numbers in cells (Mehla et al. 2017). This differential expression could interfere with the proper stoichiometry required to observe interactions between these proteins and could be addressed by switching the vectors used for cloning, e.g., by fusing UvrA with T25 and HolC with T18.

Despite the inconclusive results, this experiment may serve as a solid foundation for future experiments that aim to determine whether there is a UvrA-HolC interaction. If this interaction could be confirmed, it would provide strong evidence for a replication-coupled repair pathway in *E. coli.*

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