# Effect of the *uvrC* gene in mutation induction by 5-formyldeoxyuridine in *Escherichia coli*

By

Nabin Ghimire

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Faculty of science and Technology

Department of Mathematics and Natural Science

Centre of organelle Research (CORE)

University of Stavanger

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# MASTER'S THESIS

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# Abstract

The oxidation of the methyl group of thymine by reactive oxygen species forms the mutagenic base residue 5-formyluracil (fU). fU can be removed from DNA *in vitro* by AlkA (3-methyl DNA glycosylase II) and the Fpg, Nth and Nei proteins of *Escherichia coli*, which initiate the base excision repair pathway. In addition, it has been reported that the mammalian nucleotide excision repair (NER) system exhibits activity for fU in DNA *in vitro*. We recently found that the *E. coli* NER protein UvrA is involved in 5-formyldeoxyuridine (fdU)-mediated mutagenesis in *E. coli*, which indicates a role in fU repair *in vivo*.

In this study, spontaneous mutagenesis and mutagenesis caused by obtained of 0.1 mM fdU to the culture medium of *E. coli* deficient in the NER gene *uvrC* were investigated by scoring resistance to rifampicin. It was found that fdU does not affect the relative growth rate of *uvrC* cells significantly confirming the moderate toxicity of fdU. The mutation rate increased slightly by addition of fdU to the *uvrC* cells, as previously shown for *uvrA* cells. This contrasted with wild-type, *alkA* and *uvrB* cells which we found doubled their mutation rates by fdU supplementation. Thus the results show that mutagenesis caused by 0.1 mM fdU is highly dependent on the UvrC (and UvrA) protein, in contrast to AlkA or UvrB which only affect mutagenesis slightly. The almost lack of induction of AT $\rightarrow$ GC transitions in *uvrC*, which may be regarded as the "signature" mutation of fdU, underscore this characteristics. Compared to the results obtained on AlkA, this is surprising, because evidence of a similar important role for UvrC protein in fU repair has hitherto not been apparent.

## Acknowledgement

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## Abbreviations

AP: apurininc/apyrimidinic

AlkA: 3-methyladenine-DNA glycosylase II

BER: base excision repair

Fpg: formamidopyrimidine-DNA glycosylase

fdU: 5-formyl-2'-deoxyuridine

fU: 5-formyluracil

hUNG: human uracil-DNA glycosylase

hSMUG: single-strand selective monofunctional uracil-DNA glycosylase

MMR: mismatch repair

Nei: endonuclease VIII

NER: nucleotide excision repair

Nth: endonuclease III

OD: optical density

8-oxoG: 7, 8-dihydro-8-oxoguanine

$Rif^R$: rifampicin resistance

ROS: reactive oxygen species

SAM: S-adenosyl methionine

ssDNA: single-stranded DNA

dsDNA: double-stranded DNA

# Contents

# 1. Introduction

## 1.1 DNA damage formation

DNA is subjected to damage and decomposition by various endogenous and exogenous agents. The processes of hydrolysis, oxidation and non-enzymatic methylation of DNA which occurs often *in vivo* are the cause of DNA damage [6].

Hydrolysis breaks the N-glycosyl bond in DNA and deamination of DNA base residues. The main targets for deamination are cytosine and its homologue 5-methylcytosine which converts to uracil and thymine, respectively. The deamination of purine residues is minor reactions which occur at low rates. Adenine is converted to hypoxanthine which is mutagenic as it forms stable base pair with cytosine, while conversion of guanine to xanthine is rarer and occurs at slower rate than adenine deamination. DNA is damaged by non-enzymatic methylation by small endogenous agents such as S-adenosylmethionine (SAM). The latter is the cellular methyl group donor used as cofactor in most transmethylation reactions. These non-enzymatic methylations occur at a slow rate and attack nitrogen ring atoms of purine base residues yielding $N^7$-methylguanine and $N^3$-methyladenine as major DNA lesions [6].

DNA is regularly exposed to alkylating agents that are produced endogenously (as mentioned above) or exogenously. There are two types of alkylating agents, SN1 and SN2, depending on the mechanism of attack on the DNA bases. The SN1 agents N-methyl-N-nitrosurea (MNU) and N-methyl-N'-nitrosoguanidine (MNNG) are generally highly mutagenic and cytotoxic, the latter because of its ability to block replication and/or transcription. On the other hand, the SN2 agent methylmethane sulphonate is cytotoxic but less mutagenic. The most abundant damage produced by these agents is $N^7$-methylguanine, which however is nontoxic and easily removed by spontaneous depurination. The toxic

abasic sites formed through such spontaneous depurination are repaired enzymatically [7] [8] [9]. This contrasts with $N^3$-methyladenine, the second most abundant lesion, which can block DNA replication. Both $N^3$-methyladenine and $N^7$-methylguanine are removed by 3-methyladenine-DNA glycosylases I (Tag) and II (AlkA) in *Escherichia coli* [10] [11].

### 1.1.1 Oxidative DNA damage

All aerobic organisms produce oxidation damage to macromolecules including DNA by reactive oxygen species (ROS) produced during (eukaryotic mitochondrial) respiration and photosynthesis. Such damage is also induced by photosensitization reactions which involves ultraviolet (UV) and visible light as well as by ionizing radiation and certain chemical agents. ROS include several entities including the highly reactive hydroxyl radical (•OH) which is especially devastating. As an unfavourable side-reaction, molecular oxygen receives one electron to yield the relatively non-reactive superoxide anion radical ($O_2^-$•), which is converted to hydrogen peroxide ($H_2O_2$). Then •OH is formed from $H_2O_2$ and $Fe^{2+}$ in the Fenton reaction. ROS are also produced when $O_2^-$• reacts with nitric oxide (NO•) resulting in the peroxynitrite anion ($ONOO^-$). Protonated peroxynitrite anion are a significantly reactive oxidant for biological molecules including DNA [12] [13]. Other DNA oxidants are produced by the myeloperoxidase-$H_2O_2$-chloride system and by eosinophil peroxidase [5].

The oxidation of DNA bases causes formation of various products which may be cytotoxic or mutagenic. Some of the most studied are 7, 8-dihydro-8-oxoguanine (8-oxoG), thymine glycol, 5-hydroxyuracil, cytosine glycol and 8-hydroxyadenine (Figure 1). 8- oxoG is the major lesion produced by oxidation of guanine. It forms base pair with adenine rather than cytosine, which causes GC $\rightarrow$ TA transversion mutations if not removed before replication. Cytosine glycol and thymine glycol are important ring-saturated pyrimidine derivatives. They are formed when the oxygen radical attacks the 5,6-double bond of the pyrimidine ring structure [6]. The instability of cytosine glycol results in deamination to uracil glycol, while dehydration of uracil glycol results in the formation of 5-

hydroxyuracil. Hydroxylation of adenine at the C8 position results in the major DNA damage product 8-hydroxyadenine. Pyrimidines like thymine and 5-methylcytosine can be oxidised in its 5-methyl group. 5-(hydroxymethyl)uracil and 5-formyluracil (fU) is produced from thymine this way [5], where the latter is discussed in the next chapter.

### 1.1.2 5-Formyluracil

Kasai et al. first detected fU as a new type of 5-methyl-oxidised thymine

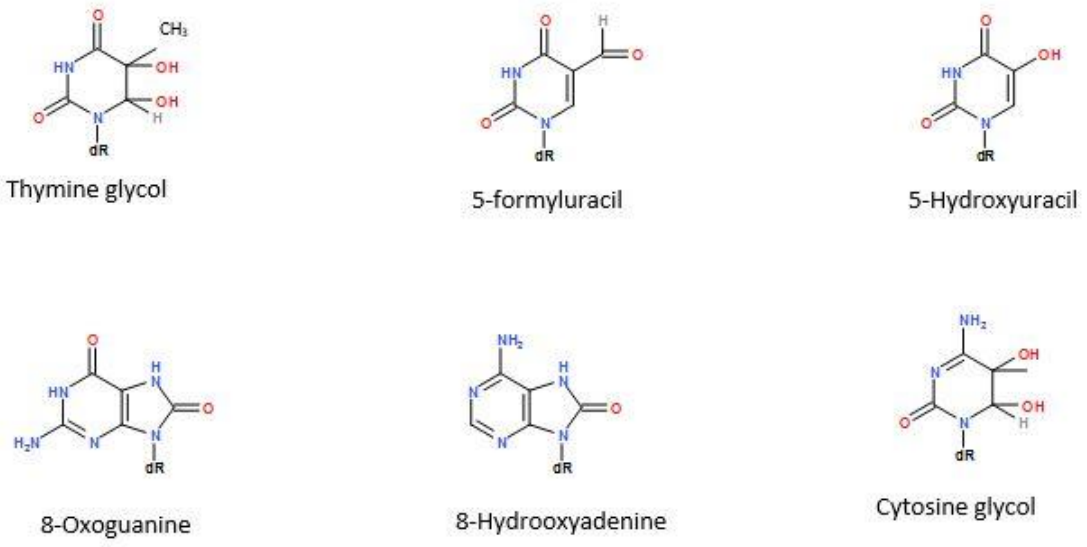


**Figure 1: Some example of important oxidative DNA base damage. Adapted from [5]**

residue in DNA formed by ionizing radiation [14], and later it was shown to be formed by quinone-mediated UVA photosensitization [15]. The oxidation of thymine on the 5-methyl group yields products with intact aromatic ring structure. One of these, 5-(hydroperoxymethyl)uracil, decomposes to the more stable products 5-(hydroxymethyl)uracil and fU [5]. Like thymine in DNA, thymine in DNA precursors can also be similarly oxidised. Thus, 5-formyl-2'-deoxyuridine (fdU) is formed by γ-irradiation in the presence of $O_2$ and also by quinone-mediated UV-A photosensitization of thymidine [16] [4]. fU can be found in an

enol, keto and ionized form. The conversion of fU from keto to ionized form increases with pH, and a considerable amount of the latter exists at physiological pH.

The different forms of fU mentioned above exhibit different base-pairing abilities (Figure 3). The common keto form base-pairs with cognate adenine like thymine. This contrasts with the ionized form which can form a specific kind of mispair with guanine. Tentative mispair with non-cognate cytosine and thymine has been suggested (Figure 4) [2] [17] [4]. The possible mutations resulting from this promiscuous base pairing ability of fU have been investigated by culturing certain mutagenesis tester strains of *E. coli* in the presence of fdU, scoring for reversions at position 461 in the *lacZ* gene. The results showed that fdU induces several base substitutions at different frequencies in the following order: AT $\rightarrow$ GC > GC $\rightarrow$ AT > GC$\rightarrow$ AT > GC $\rightarrow$ TA >>> AT $\rightarrow$ T.A > AT $\rightarrow$ CG [2]. Another study on the effect of fU was done by construction of vector plasmid pSVK3 containing fU. The plasmid vector thus constructed was incorporated into E. coli AB1157 or MS23 by the calcium chloride method. The study shows the formation of the base substitutions AT $\rightarrow$ GC and AT $\rightarrow$ TA, and that fU induces deletion mutations [18].

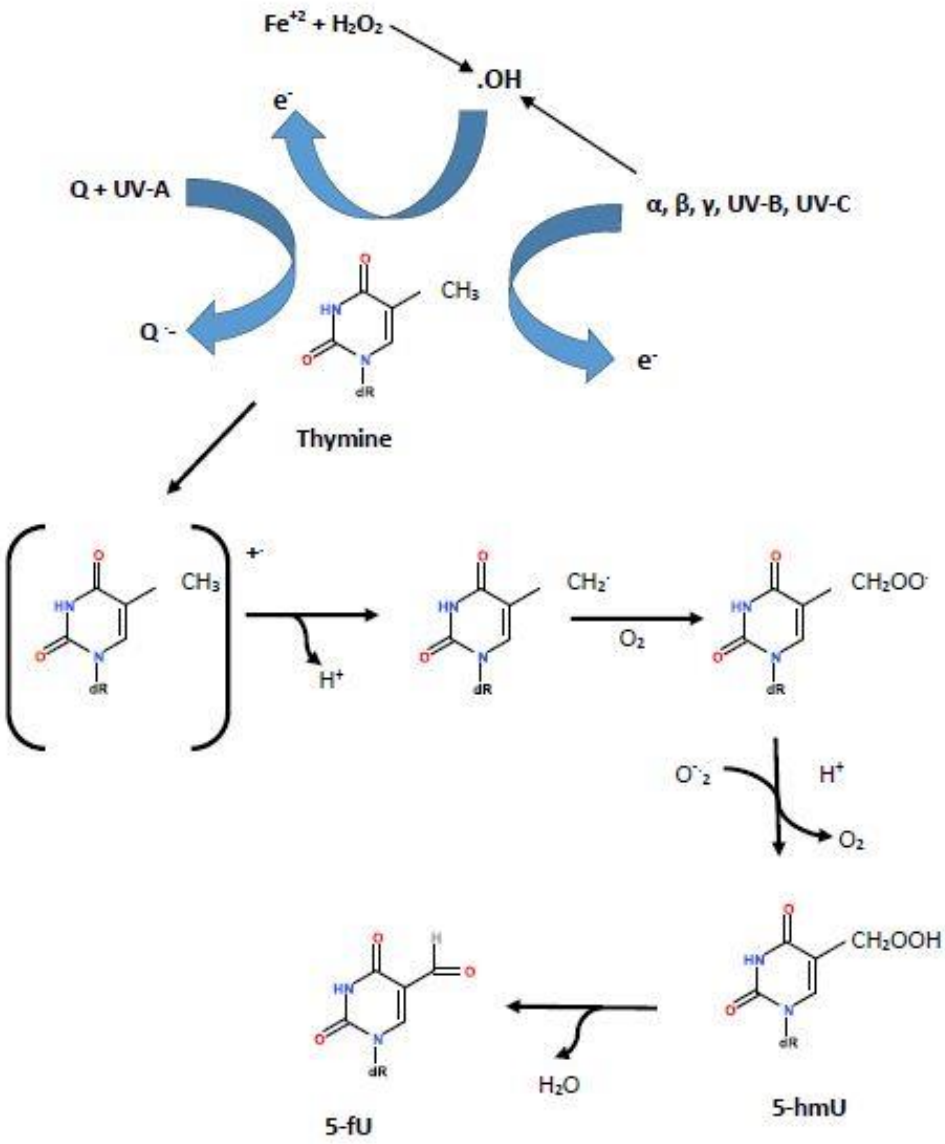


**Figure 2: Mechanism of formation of fU in DNA. Q, Quinone. Adapted from [4]**

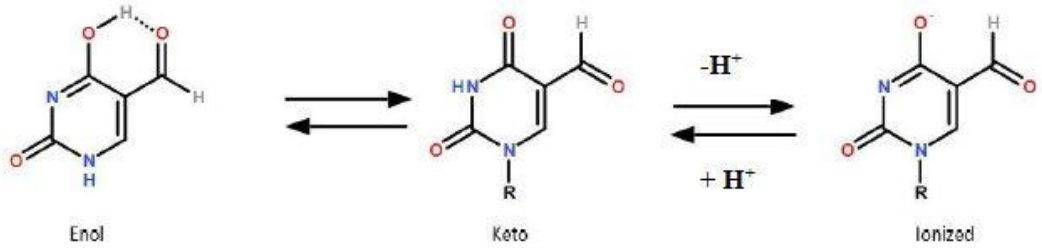


**Figure 3: Conversion of fU from keto to ionized form and keto to enol form. Adapted from [2]**

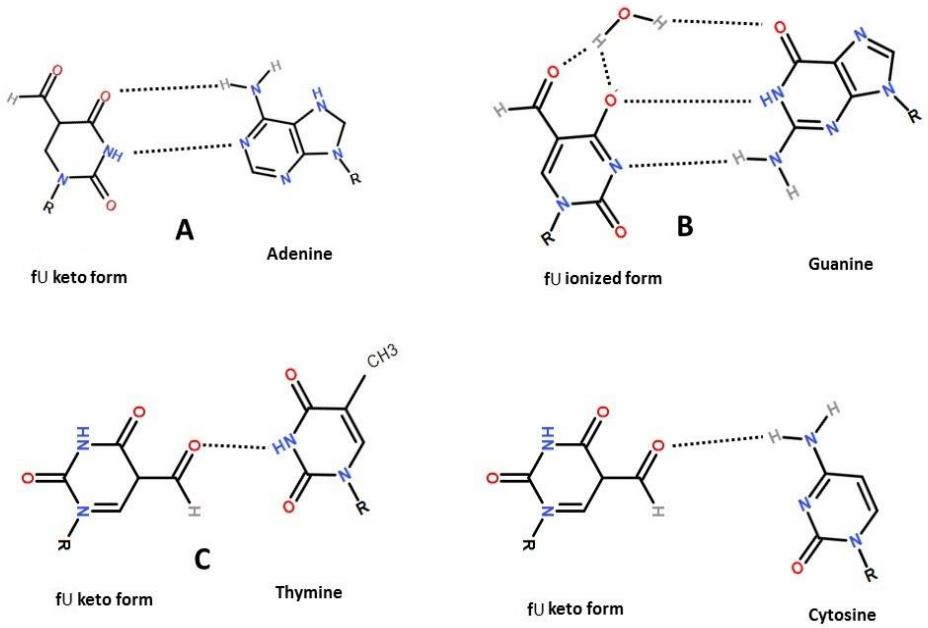


**Figure 4: Base pairing ability of fU with different bases in DNA. Adapted from [2].**

In *E. coli*, the forced incorporation of fdU triphosphate in the bacterial cell by heat shock treatment media induces mutation. The mutation frequency was found to increase with increased dose of damaged nucleotides [19]. In mammalian cells, fU and its nucleoside derivatives were found to cause toxicity and mutagenicity. In the latter case, fU and fdU promote mutagenicity at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus of CHF (Chinese hamster fibroblast) cells [20]. In COS-7 cells, fU was found to be weakly mutagenic causing the transversion mutations T $\rightarrow$ G and T$\rightarrow$ A, which suggest the formation of fU:C and fU:T mispairs [17].

## 1.2 DNA repair mechanism for oxidative damage

The living organisms have developed various DNA repair mechanisms to remove and correct DNA lesions, thus maintaining the integrity of the genome and cellular function [21] [13]. Various studies suggest that oxidative damages in DNA play a role in cancer, aging and many degenerative diseases in humans, if not properly repaired. So, it is crucial to study the repair pathways that repair each damage to understand how such disorders originate [13] [22]. Base excision repair (BER) and nucleotide excision repair (NER) are major DNA repair pathways for removal of DNA lesions. BER is the main pathway that repairs spontaneously arisen DNA damages including oxidised bases in DNA. NER eliminates a wide variety of lesions such as cyclobutane pyrimidine dimers and other photoproducts induced by UV light, as well as large chemical residues attached to DNA bases [23].

### 1.2.1 Base excision repair

The majority of DNA damages resulting from oxidation, deamination and methylation are repaired by the BER pathway. The first enzyme in BER was first discovered by Tomas Lindahl in 1974 with the identification of *E. coli* uracil-DNA glycosylase [24].

The BER pathway begins with cleavage of the N-glycosyl bond between the damaged base and the deoxyribose residue by a DNA glycosylase, thus releasing a free base and leaving behind an apurinic/apyrimidinic (AP) site in DNA [24]. Enzymes that have ability to only cleave the N-glycosidic bond are called mono-functional. While the other type of DNA glycosylase is called bi-functional, because it has an additional AP lyase activity capable of incising the phosphodiester backbone 3' to the AP site by a β- or β/δ-elimination reaction [25] [26].

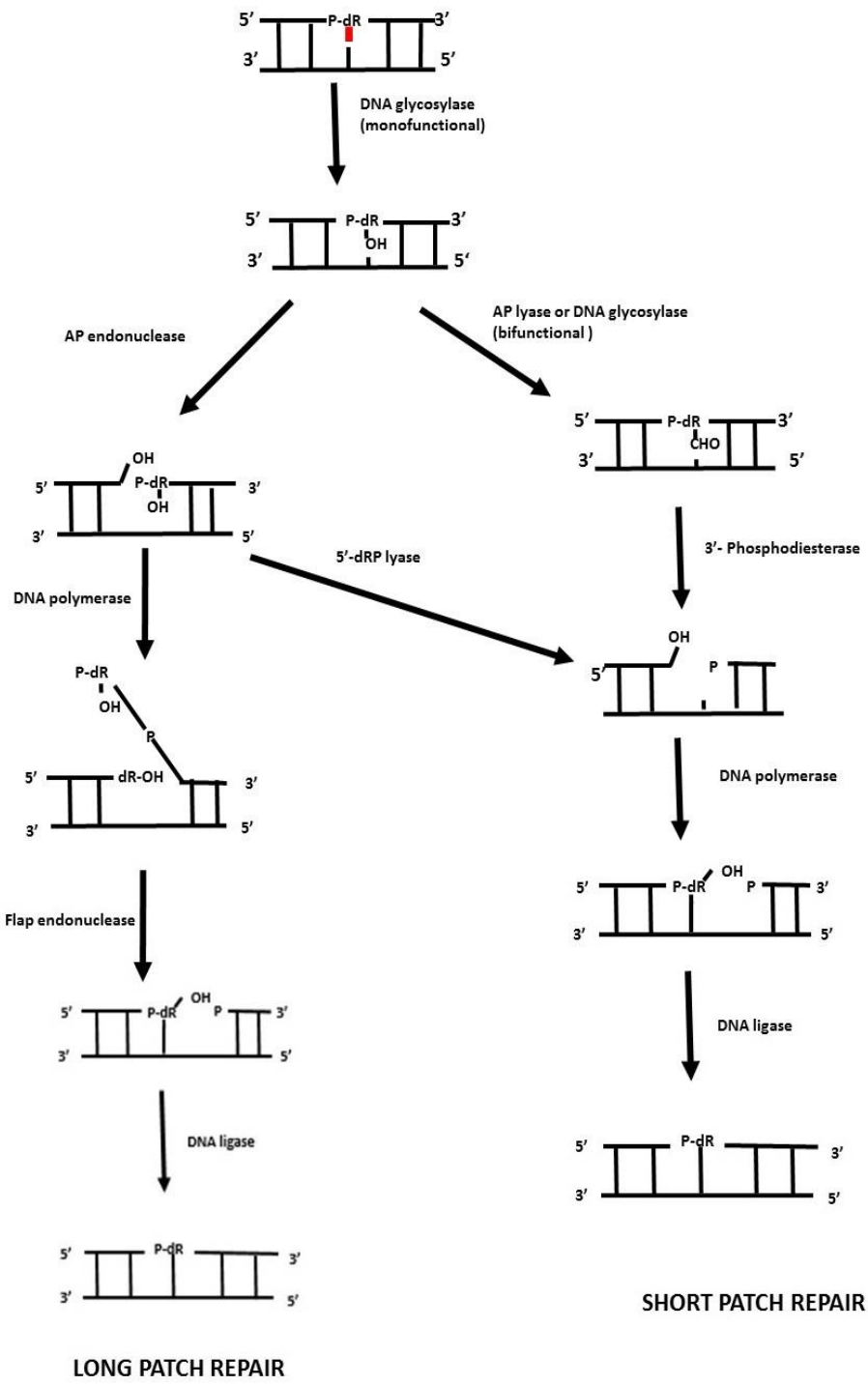


**Figure 5: Base excision repair pathway in prokaryotes. Adapted from [1]**

Further repair of the AP site is initiated by an AP endonuclease or an AP lyase. The AP endonuclease, which requires $Mg^{2+}$ for its activity, catalyses the cleavage of the phosphodiester bond 5' to the AP site producing a DNA strand with a 3'-hydroxyl group ready for single nucleotide insertion, and a 5'-deoxyribose phosphate (dRP) remnant which has to be removed. The AP lyase cleaves the phosphodiester bond 3' to the AP site leaving behind a 3'-saturated or unsaturated aldehyde remnant which has to be removed, and a 5'-phosphate group ready for ligation [27] [28] [29]

The BER system may continue using one of two sub-pathways, often called short-patch repair and long-patch repair. In the short-patch repair pathway, the 5'-dRP remnant left behind by an AP endonuclease is removed by a 5'-dRP activity that can be a function of several enzymes including bi-functional DNA glycosylases. The 3'-aldehyde remnant left behind by an AP lyase is removed by a 3'-phosphodiesterase activity which is a function of an AP endonuclease. The result of both these two alternatives to remove the AP site is a single nucleotide gap that is filled in by a DNA polymerase followed by ligation by a DNA ligase. If the efficiency to remove a 5'-dRP remnant is challenged, the DNA polymerase initiates and continues polymerization from the free 3'-OH group displacing the original DNA strand downstream, which means that the long-patch repair pathway has been started. After a while, the single stranded displaced original strand (the DNA flap) can be removed by a flap endonuclease followed by ligation as in short-patch repair [30] [11] [13].

### 1.2.2 DNA glycosylases for repair of lesions induced by oxidation

The major DNA glycosylase activities for repair of lesions induced by oxidation in *E. coli* are functions of the Fpg, Nth, Nei and MutY proteins [5] (Table 1). Fpg (formamidopyrimidine-DNA glycosylase) was first discovered in *E. coli* as an activity that removes the ring-opened degradation product (2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine) of $N^7$-methylguanine, the major lesion induced by methylation of DNA, and the enzyme has associated AP lyase activity [31]. Later it was shown that it primarily recognises and removes base lesions formed from oxidation of guanine, preferably 8-oxoG [32]. This also applies to MutY protein, which is without AP lyase activity. However, in this case it is the mispaired adenine inserted opposite 8-oxoG in DNA that is removed, hence MutY is called adenine-DNA glycosylase. Nth and Nei are DNA glycosylases with AP lyase activity, which is the reason that they are called endonuclease III and endonuclease VIII, respectively. Although both Nth and Nei exhibit overlapping substrate specificity with Fpg, they are primarily involved in recognising and removing oxidised pyrimidines from DNA. The protein sequence of Nei shows significant homology with Fpg [33] [34].

**Table 1: DNA glycosylases involved in the repair of lesions induced by oxidation in *E. coli*. Adapted from [26] [5].**

| | Glycosylase | Name | Function |
|---|---|---|---|
| Monofunctional | Tag | 3-Methyladenine-DNA glycosylase I | Remove alkylated bases from DNA, through hydrolysis. |
| | AlkA | 3-Methyladenine-DNA glycosylase II | Remove alkylated bases from DNA, through hydrolysis. |
| | Mug | Mismatch-specific uracil-DNA glycosylase | Removes mismatched thymine from DNA. |
| | Ung | Uracil-DNA glycosylase | Remove uracil from ssDNA or dsDNA. |
| | MutY | Adenine-DNA glycosylase | Remove oxidized guanine from DNA |
| Bifunctional | Nei | Endonuclease VIII | Remove damaged pyrimidines from ds DNA. Leaves behind an AP-site |
| | Fpg | Formamidopyrimidine- DNA glycosylase | Removes oxidized purines from DNA. Leaves behind an AP-site |
| | Nth | Endonuclease III | Remove damaged pyrimidines from ds DNA. Leaves behind an AP-site. |

### 1.2.3 5-Formyluracil-DNA glycosylases

The oxidised base damage fU can be removed by *E. coli* AlkA protein (3-methyladenine DNA glycosylase II) [35]. Other *E.coli* proteins shown to be involved in the repair of fU *in vitro* are Nth, Nei and Fpg [36].

AlkA is a protein of 31-kDa containing 282 amino acid residues, and has broad substrate specificity compared to other DNA glycosylases. The expression of

the *alkA* gene in *E. coli* is controlled by the so-called adaptive response to alkylation and *ada* regulon. When cells are exposed to a sub-lethal dose of alkylating agent, the *ada* regulon induces transcription of the *alkA* gene [37, 38]. Mutation in the *ada* gene results in a block of induction of *alkA* [13].

In addition to AlkA, Fpg, Nth and Nei, which should be able to remove fU from DNA opposite all normal bases, fU opposite G is recognised by the MutS mismatch repair protein of *E. coli* indicating that the methyl-directed mismatch repair system is a back-up for fU repair *in vivo* [39] [40].

The human endonuclease III homolog hNTH1 exhibits DNA glycosylase activity that recognises and removes fU from DNA [41]. However, its kinetic parameters for fU-removal shows that it is 20 times less efficient than hSMUG1, which is regarded as the primary enzyme in removing fU from mammalian DNA [42] [43]. hSMUG1 was first characterised and named as a single stranded selective mono-functional uracil glycosylase, but has also been called fU-DNA glycosylase (FDG). FDG recognises fU in both single and double stranded DNA. In addition to uracil and fU, the enzyme shows glycosylase activity for 5-hydroxyuracil and 5-(hydroxymethyl)uracil in single and double stranded DNA [42]. The results hitherto obtained suggest that hSMUG1 is a primary repair enzyme for a group of oxidised pyrimidines like fU, 5-hydroxyuracil and 5-(hydroxymethyl)uracil as well as being a backup enzyme for hUNG [43].

### 1.2.4 Nucleotide excision repair

The NER pathway represents a complex DNA repair system in all living organisms, especially mammalian cells where it consists of many proteins, carrying out damage recognition, damage verification, incision, excision, repair synthesis and DNA ligation [44] [13].

In *E. coli* and other prokaryotic organisms, UvrA, UvrB and UvrC are the three proteins that play a main role in the NER system. The NER system in prokaryotes starts with the formation of complex containing one UvrB protein and two UvrA proteins (A2B1 complex). This complex recognises the damage on the DNA by tracking along the DNA backbone and binds it to form unstable A2B1-DNA complex. Now, the activation of UvrB-dependent helicase causes unwinding and bending of DNA through an ATP-dependent reaction facilitating further recognition of the damaged strand by UvrB. UvrB forms a tighter complex at a lesion site and UvrA dissociates. UvrC binds to the UvrB-DNA complex which activates UvrC and initiates the 3'incision. After few seconds of 3' incision, UvrC is activated and initiates 5' incision. The binding of UvrD (helicase II) releases the excised oligomer and UvrC, leaving behind a stable UvrB-gapped DNA complex. DNA polymerase I (*POL* I) repairs the excision gap and releases the UvrB [13] [3].

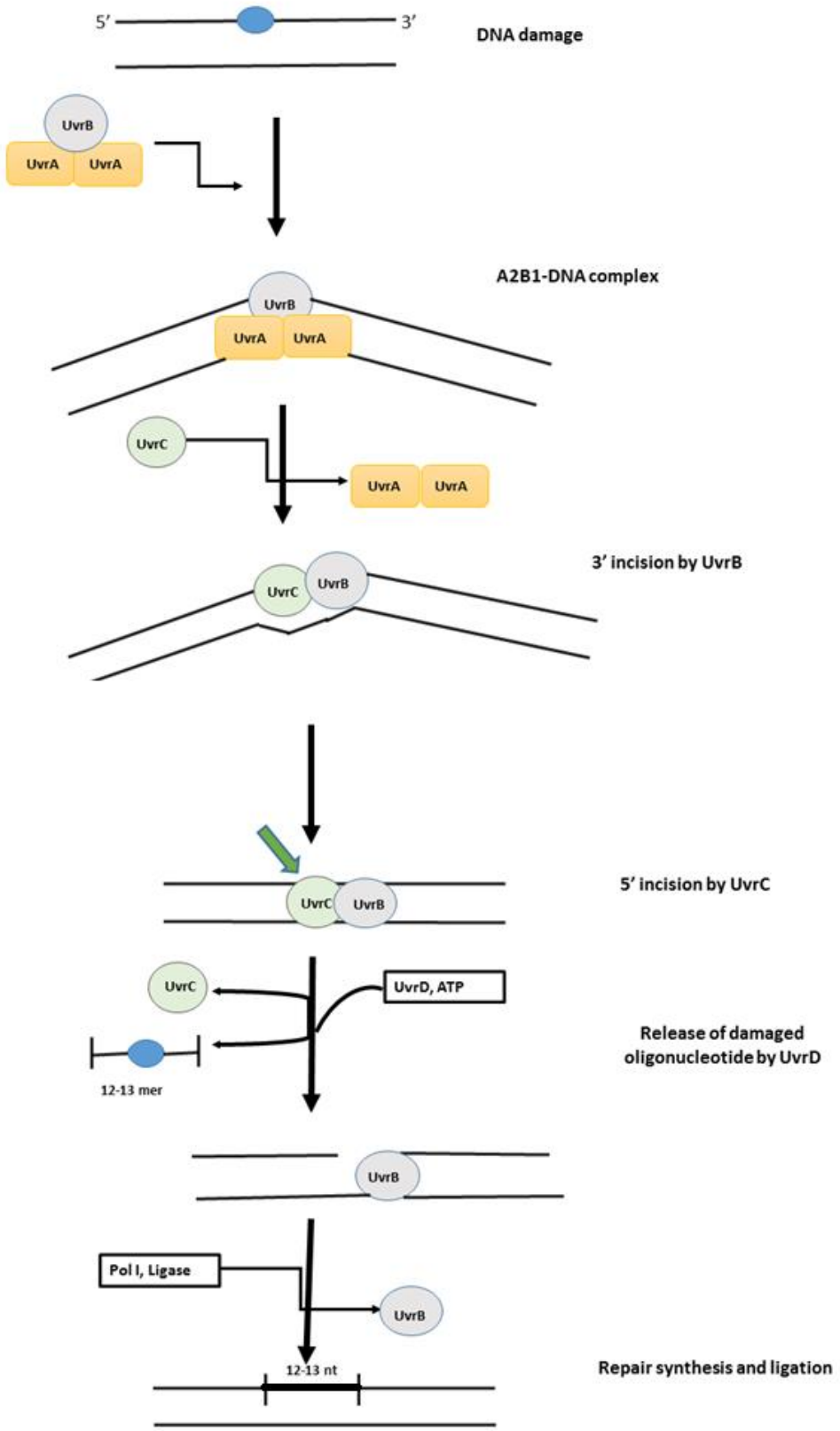


**Figure 6: Nucleotide excision repair pathway in prokaryotes. Adapted from [3]**

### 1.2.5 The SOS response

The existence of the SOS response was first described 40 years ago [45], and is regulated by the LexA repressor and RecA protein in *E. coli*. An inducing signal is generated when DNA replication is inhibited by DNA damages resulting in accumulation of single-stranded regions in DNA. RecA molecules attach to these regions, which stimulates LexA to cleave itself by autocatalysis thus activating SOS genes (Figure book page no 518) [46] including *uvrA* and *uvrB*. In contrast, *uvrC* is not under such control [47] [48]. When the DNA is repaired the inducing signal is eliminated and RecA proteins can no longer activate the self-cleavage activity of LexA [46].

### 1.2.6 Nucleotide excision repair of 5-formyluracil

The mammalian NER system has been reported to exhibit activity for repair of fU [49]. No such evidence exists for bacterial NER [48].

# 2. Aim of the study

It is known that the DNA base lesion fU can be repaired by BER in *E. coli* initiated by the AlkA, Fpg, Nth or Nei DNA glycosylase, where the MMR system is regarded as a back-up [5]. In mammalian cells the SMUG protein is believed to be the major fU-DNA glycosylase [42]. Interestingly, *in vitro* evidence has also suggested a role of mammalian NER in fU repair [49]. To obtain a more complete picture of fU repair in *E. coli* it is thus reasonable to study the role of NER in repair of fU.

Consequently, our research group at University of Stavanger started doing research on the *in vivo* consequences of inactivated *E. coli* NER genes, first *uvrA*, in fdU-induced mutagenesis, to possibly indicate any role of the UvrA protein in repair. The study on UvrA-deficient cells showed that UvrA promotes fdU-mediated mutagenesis. The *uvrA* cells exhibit altered distribution of the fdU induced base substitutions compared to wild-type, which indicates that it somehow participates in fU repair resulting in mutation induction [50]. A similar study on UvrB-deficient cells was also conducted in our laboratory.

The aim of the study presented was to investigate the effect of the UvrC protein on the fdU-mediated mutagenesis, to contribute to the understanding of this protein and the whole UvrABC complex in fdU-mediated mutagenesis and fU repair in *E. coli*. Specifically, mutagenesis was monitored on *uvrC* cells grown in a culture medium with 0.1 mM of fdU added, where the mutation rate and base substitutions induced were determined.

# 3. Materials and Methods

## 3.1 Mutagenesis fdU

The mutagenesis assay was performed as described in [51] and [52]:

### 1. Overnight cultures

A single colony of the bacterial strain (AB1884; *uvrC*) was isolated with a pipet tip and inoculated into 2 ml minimal A-medium [A buffer with 1 mM $MgSO_4$, 0.2 % (w/v) glucose, 0.04 mg/ml L-amino acids (Thr, Arg, Pro, Leu, His) and vitamin $B_1$ (5 µg/ml)]. For each assay, six bacterial cultures and one control (no bacterial colony) were prepared with 2 ml A-medium. The bacterial cultures were grown overnight at 37°C for 18–24 h. The tubes were allowed to lay down to increase surface area for oxygenation.

### 2. Mutagenesis

The number of bacteria/ml after overnight culture was measured by a spectrophotometer using $OD_{600} = 1 = 5 \times 10^8$ bacteria/ml. The bacterial cultures with an $OD_{600}$ between 0.4–0.8 were selected for mutagenesis due to their supposed exponential growing. Overnight cultures were diluted in 1× A-buffer [$K_2HPO_4$ (10.5 g/l), $KH_2PO_4$ (4.5 g/l), $(NH_4)_2SO_4$ (1 g/l), $C_6H_5Na_3O_7 \times 2H_2O$ (0.5 g/l)] to approximately 200,000 bacteria/ml. The diluted cultures were incubated in A-medium at 37°C with shaking (240 rpm) with a start concentration of 9000 bacteria/ml. After 2 h 0.1 mM fdU was added to the cultures (except controls). Bacterial cultures were incubated for 45–48 h at 37°C with shaking (240 rpm).

### 3. Growing the mutated cultures

After 48 h of incubation, the bacterial culture tubes were cooled on ice to terminate mutagenesis. The bacterial cultures were transferred to eppendorf tubes and centrifuged for 4 min at 5000 rpm and 4°C. The pellet was washed in 2 ml 1× A-buffer under the previous condition and finally re-suspended in 1 ml 1× A-buffer.

The bacterial cultures were diluted as described in the table:

| Dilution | | Bacterial culture | 1× A-buffer (µl) | Total Volume (µl) |
|---|---|---|---|---|
| $10^{-2}$ | 1:100 | 10 µl concentrated | 990 | 1000 |
| $10^{-4}$ | 1:10 000 | 10 µl 1:100 dilution | 990 | 1000 |
| $10^{-5}$ | 1:100 000 | 100 µl 1:10 000 dilution | 900 | 1000 |
| $10^{-6}$ | 1:1 000 000 | 100 µl 1:100 000 dilution | 900 | 1000 |
| 10-$^{7}$ | 1:10 000 000 | 100 µl 1:1 000 000 dilution | 900 | 1000 |

100 µl of bacteria diluted to $10^{-5}$, $10^{-6}$ and $10^{-7}$ was spread on the minimal agar plates with glucose (one plate for $10^{-5}$ dilution, two plates for $10^{-6}$ dilution and two plates for $10^{-7}$ dilution) using a loop sterilized by exposure to 96 % ethanol and flame. 100 µl of the concentrated bacteria were spread on the minimal agar plates with glucose containing 150 µg/l of rifampicin. Plates without rifampicin were incubated for 48 h at 37°C and plates with rifampicin were incubated for 96 h at 37°C.

4. **Analysis of the mutants**:

**Culture of the mutants**

Colonies of mutants from agar plates containing rifampicin were inoculated into tubes containing 2 ml LB media with 150 µg/ml rifampicin (a single colony per culture was analysed). The tubes with mutants were incubated at 37°C for 5–7 days with shaking (240 rpm).

**Preparing soluble DNA template (DNA extraction)**

5 µl of the mutants grown on 2 ml LB media containing 150 µg/ml rifampicin was mixed with 100 µl of sterile $H_2O$, boiled at 100°C for 5 min and immediately cooled on ice in order to break the bacterial wall by "temperature" shock. The material was centrifuged at 13,000 rpm for 3 min and 80 µl of the supernatant containing DNA was collected in a new tube and stored at -20°C.

**PCR reaction**

The $rif^R$ region was amplified by PCR using the forward primer 5'-GCCAAGCCGATTTCC-3' (F-1021) and the reverse primer 5'-GTATTCGTTAGTCTG-3' (R-1022). The PCR reaction was prepared as described in the table below:

| **PCR reagent** | **1× reaction (µl)** | **Final concentration** |
|---|---|---|
| 5× GoTaq Flexi buffer | 10 | 1× |
| 25 mM $MgCl_2$ | 3 | 1.5 mM |
| dNTP mix (each) | 1 | 200 µM each |
| Primer 1021 (10 pmol/µl) | 1 | 0.2 pmol/µl |
| Primer 1022 (10 pmol/µl) | 1 | 0.2 pmol/µl |
| GoTaq® HotStart DNA Polymerase, Promega (5 U/µl), | 0.25 | 1.25 U |
| Sterile $H_2O$ | 27.75 | |
| Total | 45 | |

45 µl of PCR reaction mix was mixed with 5 µl DNA template (from DNA extraction). A negative control was mixed with sterile $H_2O$. The PCR was run using the following parameters:

**PCR program:**

| Step | Time (min) | Tempera-ture °C | Cycles |
|---|---|---|---|
| Initial denaturation | 4 min | 94 | ×1 |
| Denaturation | 1 min | 94 | ×34 |
| Annealing | 1 min | 50 | |
| Extension | 20 s | 72 | |
| Final extension | 5 min | 72 | ×1 |
| Storage | ∞ | 4 | |

**Agarose gel electrophoresis**

1 % agarose gel was prepared with 5000× GelRed in 1× TAE buffer and allowed to dry for 20–30 min. 2 µl of low mass ladder and 5 µl of each PCR product were mixed with 6× loading dye solution and loaded to the wells of the gel. Electrophoresis was run for 40 min at 100 V. Bands of amplified DNA were visualized under UV light (Image Quant 350, GE Healthcare, program Image Analysis Software 7.0). Bands identified as the correct PCR product (300 base pairs) were purified using Nucleospin® GEL and sequenced using F-1021 as primer by GATC Biotech, Cologne, Germany (with Applied Biosystems 3730xl DNA analyzer).

Purification of PCR-product (NucleoSpin® Gel and PCR clean-up)

45 µl of the PCR products was mixed with 90 µl of Buffer NT1 and the mixture was loaded on the NucleoSpin® Gel and PCR Clean-up column placed into a collection tube and centrifuged for 30 s at 11,000 × g (DNA binging). The flow-through was discarded and the column was placed back into the collection tube. 700 µl of the Buffer NT3 was added to the column and centrifuged for 30 s at

11,000 × g (DNA washing). The flow through was discarded and the washing was repeated. The column was again centrifuged for 1 min at 11,000 × g to remove excess Buffer NT3 (drying silica membrane). The column was placed into a new DNase-free 1.5 ml tube and the DNA was eluted by addition of 15 µl Buffer NE, followed by incubation for 1 min at room temperature. The purified DNA was collected by centrifugation for 1 min at 11,000 × g.

## 3.2 PREPARATION OF CULTURE MEDIA AND CHEMICAL REAGENTS:

**1.** 1 M $MgSO_4 \times 7H_2O$

22.85 g of $MgSO_4$ (Merck: 1.05886.0500; MW 246.48 g/mol) was diluted in 100 ml distilled water and autoclaved.

**2.** 20 % glucose (w/v)

40 g of glucose (Merck: 1.08337.1000; MW 180.16 g/mol) was diluted in 200 ml distilled water and autoclaved.

**3.** 5 mg/ml vitamin $B_1$ (thiamine)

0.05 g of thiamine (SIGMA: T4625-10G; MW 337.27 g/mol) was diluted in 10 ml distilled water and autoclaved.

**4.** 4 mg/ml L-amino acids:

1 g L-threonine (SIGMA: T-8441; MW 119.1 g/mol), 1 g L-arginine (SIGMA: A-5131; MW 210.7 g/mol), 1 g L-proline (SIGMA: P-0380; MW 115.1 g/mol), 1 g L-leucine (SIGMA: L-8125; MW 209.6 g/mol) and 1 g L-histidine (SIGMA: H-8125; MW 209.6 g/mol) were diluted in 250 ml distilled water and autoclaved.

**5.** 10× A-buffer

105 g $K_2HPO_4$ (Merck: 1.05104.1000; MW 174.18 g/mol), 45 g $KH_2PO_4$ (Merck: 1.04873.1000; MW 136.09 g/mol), 10 g $(NH_4)_2SO_4$ (Merck: 1.01217.1000; MW 132.10 g/mol) and 5 g $C_6H_5Na_3O_7 \times 2H_2O$ (Merck: 1.06448.1000; MW 294.10 g/mol) were diluted in 1000 ml distilled water and autoclaved.

**6.** A-medium (liquid)

20 ml 10× A-buffer, 0.2 ml 1 M $MgSO_4$, 2 ml 20 % glucose, 2 ml amino acids (4 g/l) and 0.2 ml $B_1$ vitamin (5 mg/ml) were diluted in 200 ml distilled water, transferred to 15 and 27 ml bottles and autoclaved.

**7.** Minimal agar plates with glucose

12 g agar-agar (Merck: 1.01614.1000), 1 ml 1 M $MgSO_4$ (Riedel-de-Haën: 31420; MW 228.46 g/mol), 2 g glucose (Merck: 1.08337.1000; MW 180.16 g/mol), 10 ml amino acids (4 g/l), 1 ml vitamin $B_1$ (5 mg/ml) and 100 ml 10× A-buffer were mixed with 1000 ml distilled water, autoclaved, and 25–30 ml was transferred to sterile petri dishes to polymerize.

**8.** Minimal agar plates with glucose + rifampicin dishes

The media was prepared as for the minimal agar plates with glucose, and 5 ml of 30 mg/ml rifampicin was added to the media precooled to 55°C, where 25–30 ml were transferred to sterile petri dishes.

**9.** 30 mg/ml rifampicin

0.150 g rifampicin (SIGMA: R-3501; MW 823.00 g/mol) was diluted in 5 ml methanol.

**10.** 10 mM fdU

0.026 g fdU (from Prof. A. Matsuda, Japan; MW 256.18 g/mol) was diluted in 10 ml distilled water, sterile filtrated and stored at -20°C.

**11.** LB-medium (overnight cultures)

25 g LB (Merck: 1.10285.0500) was diluted in 1000 ml distilled water and autoclaved.

**12.** 0.5 M EDTA

37.22 g EDTA (SIGMA: ED2SS; MW 372.2 g/mol) was dissolved in 150 ml distilled water and pH was adjusted to 8.0, filled up to a total volume of 200 ml with distilled water and autoclaved.

**13.** 50× TAE

48.4 g Tris base (SIGMA: T6066; MW 121.14 g/mol), 11.42 ml acetic acid (Merck: 1.00063.2500) and 20 ml 0.5 M EDTA (pH 8.0) were diluted in 200 ml distilled water.

**14.** 1× TAE

20 ml of 50× TAE was diluted in 980 ml distilled water.

# 4. Result

The addition of 0.1 mM fdU to the exponentially growing *E. coli uvrC* bacteria does not affect the relative growth rate significantly. This confirms that fU is a mutagenic rather than cytotoxic lesion [5]. The spontaneous mutation rate was measured to be $1.84 \times 10^{-9}$ while the mutation rate caused by addition of 0.1 mM fdU was found to be $1.99 \times 10^{-9}$, i.e., addition of fdU to the culture medium resulted in no or a minimal increases in mutagenesis (Table 2).

**Table 2: Mutation rates for Rif$^R$ resistance in exponentially growing *uvrC* cells of *E. coli* without and in the presence of 0.1 mM fdU. Number of experiments is indicated in parenthesis.**

| **fdU (mM)** | ***uvrC*** | |
|---|---|---|
| | **Mutation rate ($\times 10^{-9}$)** | **Fold** |
| 0 | 1.84 (55) | 1 |
| 1 | 1.99 (74) | 1.1 |

The distribution of base substitutions among the Rif$^R$ mutants arisen spontaneously and induced by addition of 0.1 mM fdU to exponentially growing *uvrC* cells of *E. coli* is listed in Table 2. The GC → AT transition was found to be the most predominant in both cases accounting for ~70 % of the spontaneous mutations and 60 % of the fdU-induced mutations. The transversions AT → CG, GC → CG and AT → TA were either not recorded, as for the two former, or only a single mutant was recorded, as for the latter. This contrasts with the GC → TA transversion, which was the second most abundant mutation accounting for 25 % of the spontaneous mutations and 36 % of the fdU-induced mutations. Surprisingly, the AT → GC transition appeared with the same low abundance of 3–4 % whether fdU was supplemented or not.

**Table 3: Distribution of base substitutions among the Rif$^R$ mutants arisen spontaneously and induced by addition of 0.1 mM fdU to exponentially growing *uvrC* cells of *E. coli*. Number of mutants recorded is indicated in parenthesis.**

| | ***uvrC*** | |
|---|---|---|
| | **Spontaneous** | **0.1 mM fdU** |
| AT → CG | 0 | 0 |
| GC → AT | 69 (22) | 60 (27) |
| GC → CG | 0 | 0 |
| GC → TA | 25 (8) | 36 (16) |
| AT → TA | 3 (1) | 0 |
| AT → GC | 3 (1) | 4 (2) |
| Total | 100 (32) | 100 (45) |

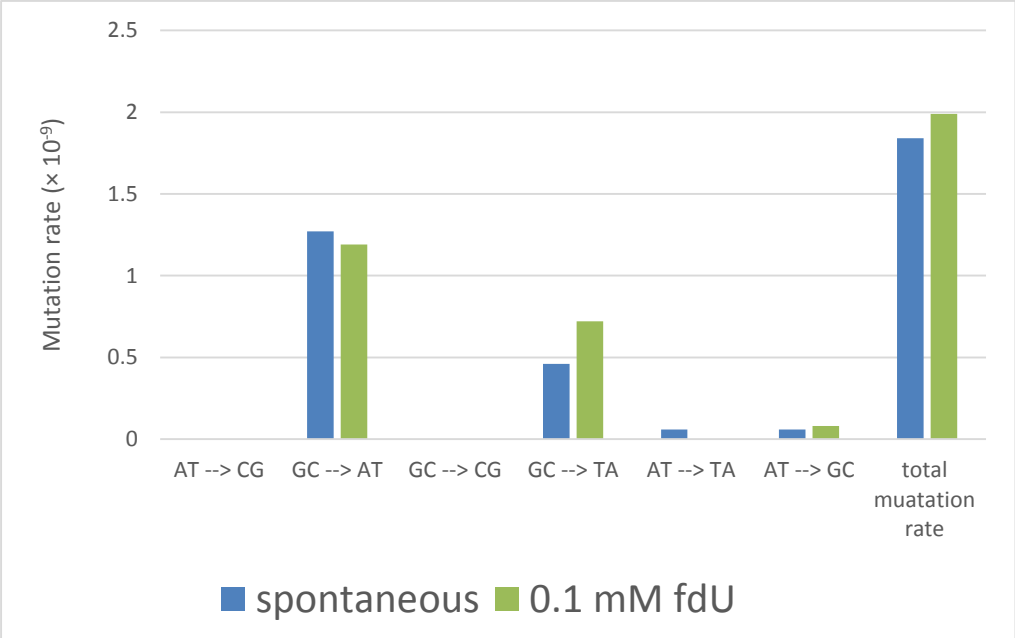


**Figure 7: Bar diagram showing the contribution of each base substitution to spontaneous and fdU-induced mutagenesis in *uvrC* cells of *E. coli***

When the mutation rates (Table 2) are allocated to each base substitution we see that the very small increase in rate caused by fdU is entirely due to a 1.5 fold increase of the GC → TA transversion rate (Figure 7). Apart from this, the mutation rates of the other base substitutions were almost equal no matter whether fdU is supplied or not.

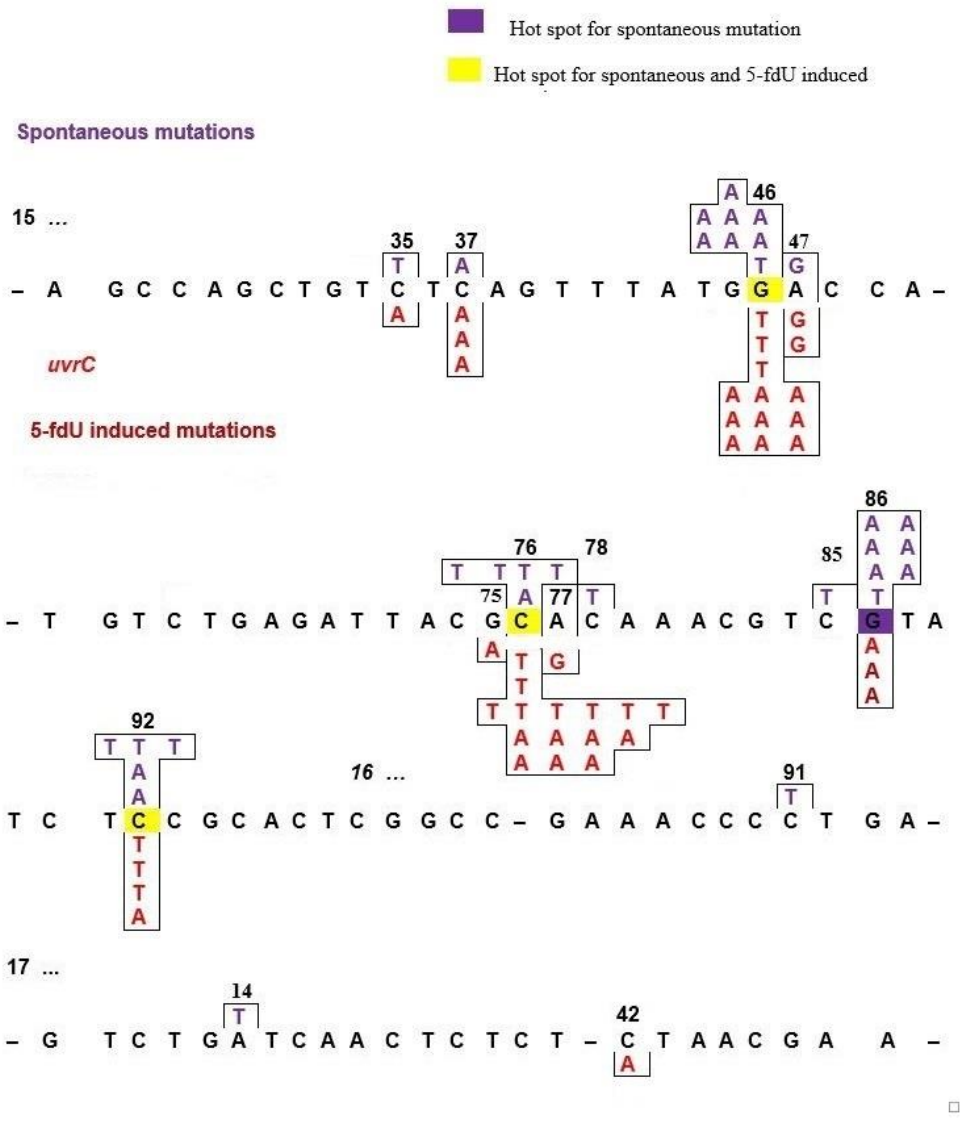


**Figure 8: Base substitution spectra obtained by sequencing rif$^R$ region of the *rpoB* gene of *uvrC* cells of *E. coli* resistant to rifampicin. Spontaneous mutations detected in violet and mutations recorded in cells grown in the presence of 0.1 mM fdU in red.**

Base substitutions were detected at 14 different sites in the $Rif^R$ region of the *rpoB* gene (Figure 8). By defining a site as a mutational hot spot if harbouring ~10 % or more of the total mutations detected in each bacterial cell type, four such sites were identified. Three of these, i.e. positions 1546, 1576 and 1592 are common for spontaneous and fdU-induced mutations, while site 1586 is a hot spot for spontaneous mutations.

# 5. Discussion

To reach a conclusion regarding the effect of the UvrC protein, or maybe the whole UvrABC complex, on fdU-mediated mutagenesis in *E. coli*, the results presented here must be compared to results obtained following supplement of 0.1 mM fdU to wild-type and other repair-deficient cells grown under identical conditions. Thus, the results for the *uvrC* cells will be compared with results gathered from similar studies of wild-type, *alkA*, *uvrA* and *uvrB* cells as discussed below.

**Table 4: Mutation rates for Rif$^R$ resistance in exponentially growing cells of wild-type, *alkA*, *uvrA*, *uvrB* and *uvrC* cells of *E. coli* without and in the presence of 0.1 mM fdU. Number of experiments is indicated in parenthesis. Mr, mutation rate (× $10^{-9}$) [Data for wild-type, *alkA*, *uvrA*, *uvrB* *E.coli* strain obtained from [53] [54]].**

| fdU | Wild-type | | *alkA* | | *uvrA* | | *uvrB* | | *uvrC* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Mr** | **Fold** | **Mr** | **Fold** | **Mr** | **Fold** | **Mr** | **Fold** | **Mr** | **Fold** |
| 0 | 1.338 (294) | 1.0 | 1.389 (241) | 1.0 | 1.151 (115) | 1 | 1.050 | 1.0 | 1.84 (55) | 1 |
| 0.1 | 2.491 (2.491) | 1.9 | 2.821 (92) | 2.0 | 1.366 (163) | 1.2 | 2.068 | 2.0 | 1.99 (74) | 1.1 |

The total mutation rates for Rif$^R$ resistance in exponentially growing *E. coli* cells of wild-type, *alkA*, *uvrA*, *uvrB* and *uvrC* without and in the presence of 0.1 mM fdU are listed in Table 4. We can observe that the total spontaneous mutation rates for wild-type and *alkA* are nearly the same and slightly higher than for *uvrA* and *uvrB*. In contrast, the total spontaneous mutation rate for *uvrC* is significantly higher than for the other cell types. Taking into account that the values calculated for *uvrB* and *uvrC* are based on less data than for the other cell types,

the different "repair" types can be grouped into two categories regarding the effect of fdU: 1) wild-type, *alkA* and *uvrB* doubled the mutation rate, while the mutation rate of 2) *uvrA* and *uvrC* only increased slightly, by addition of fdU. This indicates that mutagenesis caused by 0.1 mM fdU is highly dependent on the UvrA and UvrC proteins and is, at most, only slightly affected by the presence or absence of AlkA or UvrB protein. This is surprising, since AlkA is regarded as the most effective DNA glycosylase to excise fU from DNA *in vitro* [35, 36] and thus probably is the most important glycosylase *in vivo*. The results furthermore implicate the NER complex, especially UvrA and UvrC, in fdU-mediated mutagenesis.

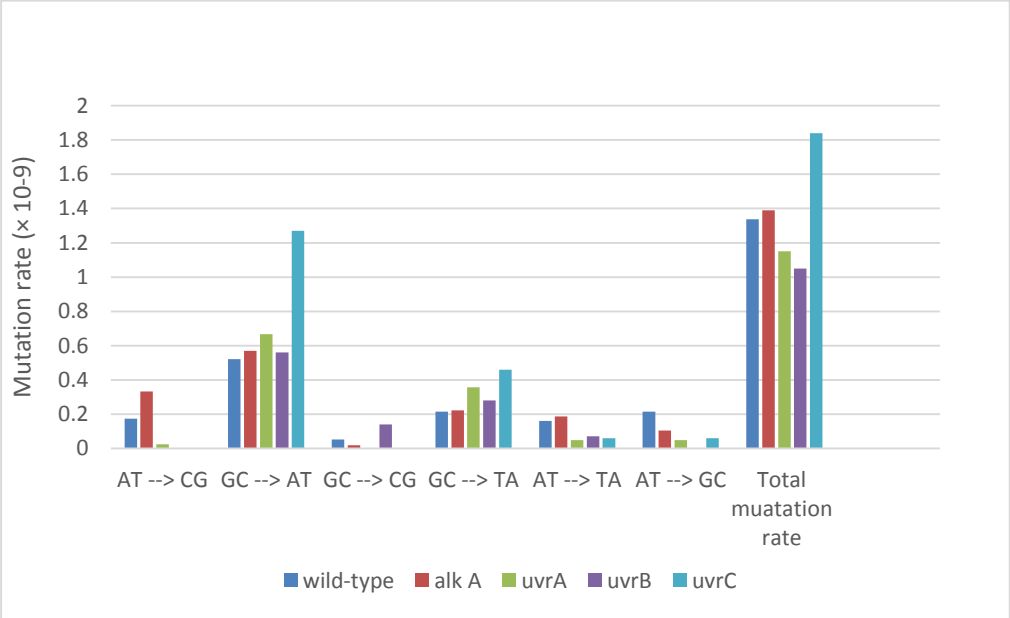


**Figure 9: Bar diagram showing the contribution of each base substitution to the spontaneous mutation rates in wild-type, *alkA*, *uvrA*, *uvrB* and *uvrC* cells of *E. coli*.**

When we compare the mutation rates for each spontaneously arisen base substitution (Figure 9) of wild-type, *alkA*, *uvrA*, *uvrB* and *uvrC*, the most striking result is that the GC → AT rate for *uvrC* is twice of the other cell types and accounts for most of the increased total spontaneous mutation rate observed for

*uvrC*, although GC → TA contributes slightly. To our knowledge, an explanation for this is lacking. Other "unexplainable" observations are the slower spontaneous generation of AT → CG, AT → TA and AT → GC in the NER-deficient cells as compared to wild-type and *alkA*.

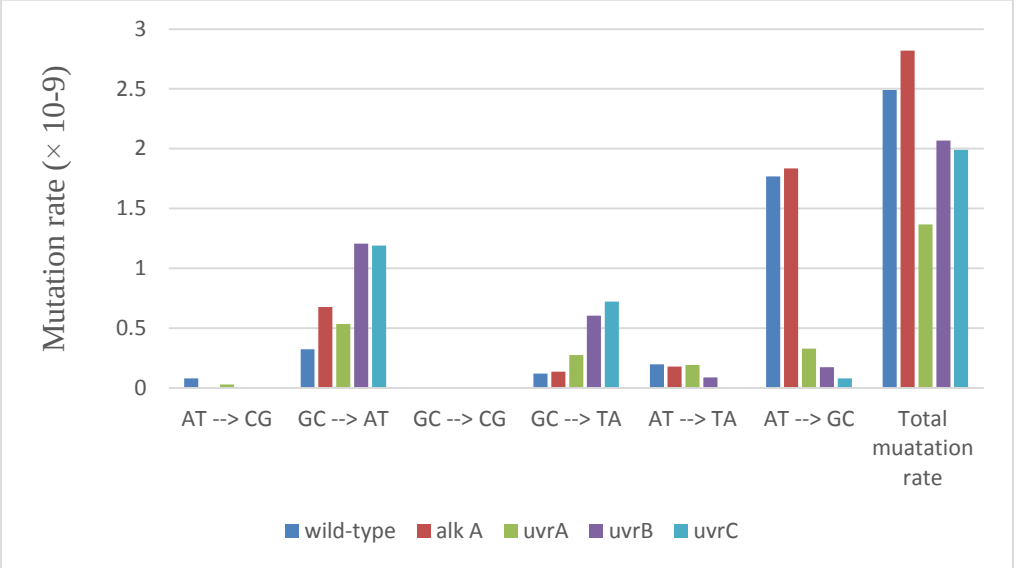


**Figure 10: Bar diagram showing the contribution of each base substitution to the mutation rates when 0.1 mM fdU was supplemented to the culture medium of wild-type, *alkA*, *uvrA*, *uvrB* and *uvrC* cells of *E. coli*.**

When we compare the mutation rates for each base substitution induced by 0.1 mM fdU for all cell types (Figure 10), we find significant differences. Strikingly, the most common and virtually only mutation generated by fdU in wild-type and *alkA*, AT → GC, is almost lacking in all NER-deficient cells, and thus seems to depend on a functional UvrABC complex to be formed. This is an unexpected result since this base substitution is caused by insertion of G opposite fU in DNA (Figure 4B), and it seems difficult to clarify at the molecular level how UvrABC endonuclease itself, or in collaboration with BER glycosylase(s), may promote mutagenesis. A bit similar, the GC → TA rate doubles in

*uvrB* by supplement of fdU, also indicating a role of UvrB in mutation promotion. This contrasts somewhat with GC → AT, where UvrB (but not UvrA or UvrC) seems to counteract mutation induction by fdU (Figures 9 and 10).

In conclusion, the present results from fdU-mediated mutagenesis in UvrC-deficient cells, together with results obtained from similar studies on UvrA- and UvrB-deficient cells, suggest novel molecular mechanisms participating in mutation induction in *E. coli*. However, these studies need to be supplemented with a larger amount of data and also the use of higher concentrations of fdU to more thoroughly establish what is going on *in vivo*. And importantly, they need to be accompanied by *in vitro* fU repair and molecular interaction studies to make final conclusions on how NER proteins, maybe in collaboration with BER proteins, participate in fU repair and mutagenesis.

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# 7. Appendix

## Appendix I:

**Table 5 : Detailed data to show the growing of *uvrC E.coli* strain bacteria in glucose media and rifampicin media without addition of 0.1mM 5-fdU (control).**

| | Number of bacteria colonies | | | | | | |
|---|---|---|---|---|---|---|---|
| **Experi-ment** | **10E-5*** | **10E-6** | **10E-6** | **10E-7** | **10E-7** | **Cells/100 µl (x10E+8)** | **Mu-tants/100 µl** |
| 2014/41 AK | 339 | 40 | 48 | 4 | 9 | 0.40 | 0.67 |
| 2014/41 DK | 0 | 0 | 0 | 0 | 0 | 0.00 | 0.00 |
| 2014/43 AK | 436 | 36 | 57 | 9 | 8 | 0.46 | 0.17 |
| 2014/43 BK | 250 | 30 | 45 | 3 | 3 | 0.32 | 4.17 |
| 2014/43 CK | 475 | 57 | 68 | 12 | 7 | 0.61 | 0.33 |
| 2014/43 DK | 497 | 92 | 76 | 8 | 6 | 0.72 | 0.33 |
| 2014/44 AK | 42 | 85 | 81 | 6 | 14 | 0.83 | 2.33 |
| 2014/44 BK | 800 | 138 | 81 | 6 | 14 | 1.10 | 1.83 |
| 2014/44 CK | 173 | 66 | 64 | 16 | 11 | 0.65 | 3.17 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2014/44 DK | 380 | 48 | 44 | 2 | 3 | 0.36 | 1.50 |
| 2014/45 AK | 539 | 118 | 85 | 12 | 4 | 1.08 | 1.83 |
| 2014/45 BK | >1000 | 194 | 178 | 17 | 8 | 1.81 | 1.60 |
| 2014/45 CK | >1000 | 202 | 158 | 32 | 34 | 2.55 | 1.20 |
| 2014/45 DK | 406 | 107 | 115 | 11 | 6 | 1.11 | 2.60 |
| 2014/46 AK | 7 | 0 | 0 | 0 | 0 | 0.00 | 0.50 |
| 2014/46 BK | 82 | 6 | 14 | 1 | 0 | 0.12 | 1.83 |
| 2014/46 CK | 65 | 5 | 0 | 2 | 0 | 0.28 | 0.50 |
| 2014/46 DK | 702 | 65 | 59 | 8 | 10 | 0.75 | 1.17 |
| 2015/48 AK I | 330 | 50 | 35 | 2 | 1 | 0.39 | 0.00 |
| 2015/48 AK II | 650 | 83 | 51 | 5 | 3 | 0.49 | 1.67 |
| 2015/48 BK I | 355 | 49 | 39 | 4 | 5 | 0.43 | 0.33 |
| 2015/48 BK II | 390 | 79 | 52 | 7 | 2 | 0.60 | 0.00 |
| 2015/48 CK I | 138 | 48 | 42 | 6 | 5 | 0.50 | 0.00 |
| 2015/48 CK II | 334 | 38 | 59 | 3 | 5 | 0.32 | 7.33 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2015/48 DK I | 248 | 13 | 17 | 1 | 3 | 0.32 | 0.17 |
| 2015/48 DK II | 93 | 17 | 10 | 0 | 1 | 0.12 | 0.00 |
| 2015/49 AK | 705 | 89 | 94 | 5 | 8 | 0.83 | 0.17 |
| 2015/49 BK | 426 | 83 | 57 | 4 | 7 | 0.52 | 0.17 |
| 2015/49 CK | 774 | 96 | 86 | 10 | 5 | 0.72 | 1.17 |
| 2015/49 DK | >1000 | 246 | 254 | 35 | 37 | 3.05 | 0.50 |
| 2015/410 AK I | 18 | 7 | 3 | 0 | 0 | 0.05 | 0.00 |
| 2015/410 AK II | 36 | 0 | 3 | 0 | 0 | 0.03 | 0.00 |
| 2015/410 BK I | 52 | 22 | 7 | 0 | 0 | 0.06 | 0.00 |
| 2015/410 BK II | 32 | 13 | 9 | 0 | 0 | 0.11 | 0.00 |
| 2015/411 AK | 64 | 16 | 15 | 2 | 2 | 0.18 | 0.00 |
| 2015/411 BK | 246 | 34 | 21 | 4 | 0 | 0.30 | 0.00 |
| 2015/411 CK | 111 | 6 | 6 | 0 | 3 | 0.06 | 0.00 |
| 2015/411 DK | 95 | 2 | 8 | 0 | 0 | 0.09 | 0.00 |
| 2015/412 AK | 136 | 8 | 8 | 3 | 1 | 0.14 | 0.17 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2015/412 BK | 28 | 11 | 0 | 0 | 0 | 0.07 | 0.17 |
| 2015/412 CK | 5 | 0 | 0 | 0 | 0 | 0.00 | 0.00 |
| 2015/412 DK | 0 | 0 | 0 | 0 | 0 | 0.00 | 0.00 |
| 2015/413 AK | 213 | 23 | 23 | 1 | 0 | 0.22 | 0.33 |
| 2015/413 BK | 74 | 20 | 24 | 3 | 1 | 0.19 | 0.00 |
| 2015/413 CK | 186 | 21 | 17 | 1 | 1 | 0.19 | 0.00 |
| 2015/413 DK | 3 | 0 | 0 | 1 | 1 | 0.10 | 0.00 |
| 2015/414 AK | 6 | 13 | 11 | 4 | 2 | 0.12 | 0.00 |
| 2015/414 BK | 18 | 5 | 10 | 1 | 1 | 0.08 | 0.17 |
| 2015/414 CK | 26 | 7 | 10 | 0 | 1 | 0.10 | 0.17 |
| 2015/414 DK | 1 | 3 | 6 | 2 | 0 | 0.05 | 0.00 |
| 2015/415 AK I | 276 | 37 | 35 | 2 | 2 | 0.24 | 0.00 |
| 2015/415 AK II | 251 | 19 | 27 | 1 | 5 | 0.16 | 0.00 |
| 2015/415 AK III | 204 | 21 | 26 | 3 | 1 | 0.20 | 0.17 |
| 2015/415 AK IV | 260 | 42 | 18 | 2 | 1 | 0.16 | 0.00 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2015/415 AK V | 298 | 32 | 30 | 5 | 3 | 0.34 | 0.00 |
| 2015/415 BK I | 174 | 5 | 32 | 3 | 3 | 0.31 | 0.00 |
| 2015/415 BK II | 314 | 38 | 25 | 1 | 1 | 0.23 | 0.00 |
| 2015/415 BK III | 196 | 29 | 21 | 4 | 1 | 0.48 | 0.50 |
| 2015/415 BK IV | 232 | 18 | 13 | 0 | 0 | 0.18 | 0.00 |
| 2015/415 BK V | 92 | 38 | 3 | 3 | 1 | 0.26 | 0.17 |

**Table 6 :Detailed data to show the growing of *uvrC E.coli* strain bacteria in glucose media and rifampicin media with addition of 0.1mM 5-fdU (test)**

| | Number of bacteria colonies | | | | | | |
|---|---|---|---|---|---|---|---|
| Experiment | **10E-5** | **10E-6** | **10E-6** | **10E-7** | **10E-7** | **Cells/100 µl (x10E+8)** | **Mu-tants/100 µl** |
| 2014/41 AF I | 380 | 50 | 52 | 4 | 2 | 0.45 | 0.50 |
| 2014/41 AF II | 328 | 56 | 41 | 7 | 3 | 0.35 | 2.50 |
| 2014/41 DF I | 608 | 63 | 70 | 4 | 4 | 0.55 | 2.33 |
| 2014/41 DF II | 331 | 53 | 38 | 8 | 6 | 0.41 | 8.67 |
| 2014/43 AF I | 233 | 42 | 28 | 4 | 7 | 0.33 | 0.00 |
| 2014/43 AF II | 349 | 32 | 58 | 3 | 2 | 0.32 | 0.83 |
| 2014/43 BF I | 540 | 52 | 59 | 5 | 8 | 0.54 | 2.00 |
| 2014/43 BF II | 402 | 45 | 54 | 8 | 7 | 0.46 | 0.83 |
| 2014/43 CF I | 380 | 62 | 64 | 7 | 1 | 0.63 | 1.17 |
| 2014/43 CF II | >1000 | 330 | 348 | 61 | 35 | 3.43 | 1.17 |
| 2014/43 DF I | 334 | 83 | 80 | 10 | 6 | 0.81 | 0.00 |
| 2014/43 DF II | 408 | 47 | 33 | 3 | 0 | 0.38 | 0.33 |
| 2014/44 AF I | 671 | 108 | 64 | 18 | 12 | 1.50 | 3.33 |
| 2014/44 AF II | 531 | 72 | 74 | 2 | 6 | 0.73 | 3.50 |
| 2014/44 BF I | 904 | 188 | 178 | 28 | 24 | 2.22 | 1.67 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2014/44 BF II | 716 | 72 | 121 | 12 | 18 | 0.96 | 12.67 |
| 2014/44 CF I | 420 | 79 | 68 | 12 | 1 | 0.74 | 1.67 |
| 2014/44 CF II | 510 | 53 | 39 | 13 | 9 | 0.48 | 1.33 |
| 2014/44 DF I | 508 | 40 | 48 | 1 | 13 | 0.46 | 0.33 |
| 2014/44 DF II | 9 | 12 | 15 | 2 | 4 | 0.14 | 0.50 |
| 2014/45 AF I | >1000 | 130 | 137 | 14 | 17 | 1.44 | 8.20 |
| 2014/45 AF II | >1000 | 127 | 132 | 11 | 12 | 1.22 | 5.60 |
| 2014/45 BF I | >1000 | 109 | 103 | 7 | 5 | 0.83 | 5.20 |
| 2014/45 BF II | >1000 | 145 | 75 | 9 | 18 | 1.03 | 3.40 |
| 2014/45 CF I | >1000 | 176 | 137 | 25 | 14 | 1.51 | 4.00 |
| 2014/45 CF II | 855 | 161 | 122 | 24 | 13 | 1.38 | 3.60 |
| 2014/45 DF I | 664 | 84 | 86 | 14 | 6 | 0.74 | 2.00 |
| 2014/45 DF II | 572 | 70 | 80 | 6 | 12 | 0.67 | 2.80 |
| 2014/46 AF I | 157 | 28 | 20 | 2 | 6 | 0.21 | 2.67 |
| 2014/46 AF II | 202 | 15 | 22 | 6 | 4 | 0.31 | 1.17 |
| 2014/46 BF I | 28 | 0 | 8 | 5 | 3 | 0.40 | 1.17 |
| 2014/46 BF II | 146 | 31 | 13 | 2 | 0 | 0.16 | 0.83 |
| 2014/46 CF I | 390 | 58 | 53 | 5 | 5 | 0.53 | 0.83 |
| 2014/46 CF II | >1000 | 95 | 106 | 10 | 7 | 0.93 | 0.50 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2014/46 DF I | 368 | 88 | 0 | 7 | 2 | 0.54 | 1.83 |
| 2014/46 DF II | 242 | 31 | 30 | 5 | 2 | 0.26 | 3.00 |
| 2015/48 AF | 290 | 60 | 65 | 4 | 9 | 0.64 | 1.33 |
| 2015/48 BF | 262 | 35 | 43 | 4 | 4 | 0.40 | 1.67 |
| 2015/48 CF | 224 | 49 | 63 | 7 | 3 | 0.53 | 0.17 |
| 2015/48 DF | 423 | 74 | 65 | 5 | 7 | 0.65 | 0.17 |
| 2015/49 AF I | 682 | 77 | 82 | 15 | 12 | 1.07 | 1.50 |
| 2015/49 AF II | 640 | 62 | 87 | 13 | 7 | 0.65 | 2.33 |
| 2015/49 BF I | 268 | 84 | 69 | 8 | 9 | 0.81 | 2.33 |
| 2015/49 BF II | 586 | 93 | 88 | 4 | 13 | 0.91 | 3.17 |
| 2015/49 CF I | 284 | 68 | 62 | 6 | 8 | 0.68 | 2.33 |
| 2015/49 CF II | 280 | 47 | 58 | 3 | 4 | 0.41 | 7.33 |
| 2015/49 DF I | 628 | 59 | 80 | 6 | 1 | 0.61 | 2.67 |
| 2015/49 DF II | 652 | 71 | 73 | 12 | 4 | 0.70 | 4.17 |
| 2015/410 AF I | 31 | 2 | 5 | 1 | 0 | 0.03 | 0.00 |
| 2015/410 AF II | 19 | 2 | 1 | 0 | 0 | 0.02 | 0.00 |
| 2015/410 BF I | 20 | 10 | 18 | 0 | 0 | 0.14 | 0.00 |
| 2015/410 BF II | 8 | 1 | 0 | 0 | 1 | 0.02 | 0.00 |
| 2015/411 AF I | 20 | 45 | 43 | 3 | 5 | 0.42 | 0.00 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2015/411 AF II | 4 | 13 | 7 | 1 | 1 | 0.17 | 0.00 |
| 2015/411 BF I | 208 | 27 | 20 | 2 | 4 | 0.26 | 0.00 |
| 2015/411 BF II | 263 | 8 | 37 | 2 | 0 | 0.28 | 0.00 |
| 2015/411 CF I | 189 | 17 | 30 | 5 | 1 | 0.25 | 0.00 |
| 2015/411 CF II | 4 | 19 | 37 | 1 | 4 | 0.27 | 0.00 |
| 2015/411 DF I | 25 | 11 | 25 | 0 | 0 | 0.18 | 0.00 |
| 2015/411 DF II | 13 | 15 | 19 | 0 | 1 | 0.34 | 0.00 |
| 2015/412 AF I | 18 | 8 | 0 | 0 | 0 | 0.05 | 0.00 |
| 2015/412 AF II | 23 | 12 | 0 | 0 | 0 | 0.07 | 1.00 |
| 2015/412 BF I | 125 | 10 | 19 | 1 | 0 | 0.13 | 0.17 |
| 2015/412 BF II | 189 | 17 | 18 | 1 | 0 | 0.16 | 0.17 |
| 2015/412 CF I | 6 | 0 | 0 | 0 | 0 | 0.00 | 0.00 |
| 2015/412 CF II | 3 | 0 | 0 | 0 | 1 | 0.02 | 0.17 |
| 2015/412 DF I | 35 | 0 | 0 | 1 | 0 | 0.04 | 0.17 |
| 2015/412 DF II | 11 | 0 | 0 | 0 | 0 | 0.00 | 0.00 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2015/413 AF I | 301 | 33 | 40 | 8 | 3 | 0.33 | 0.17 |
| 2015/413 AF II | 203 | 20 | 26 | 0 | 1 | 0.22 | 0.00 |
| 2015/413 BF I | 138 | 39 | 24 | 3 | 4 | 0.33 | 0.17 |
| 2015/413 BF II | 131 | 27 | 32 | 5 | 0 | 0.30 | 0.00 |
| 2015/413 CF I | 122 | 22 | 7 | 0 | 3 | 0.21 | 0.00 |
| 2015/413 CF II | 153 | 21 | 23 | 5 | 3 | 0.28 | 0.50 |
| 2015/413 DF I | 30 | 5 | 2 | 0 | 0 | 0.04 | 0.67 |
| 2015/413 DF II | 12 | 5 | 4 | 0 | 0 | 0.05 | 0.00 |
| 2015/414 AF I | 61 | 12 | 5 | 2 | 3 | 0.18 | 0.00 |
| 2015/414 AF II | 56 | 11 | 2 | 0 | 1 | 0.06 | 0.00 |
| 2015/414 BF I | 71 | 12 | 7 | 1 | 0 | 0.09 | 0.17 |
| 2015/414 BF II | 22 | 3 | 5 | 0 | 1 | 0.03 | 0.00 |
| 2015/414 CF I | 76 | 16 | 24 | 0 | 1 | 0.20 | 0.00 |
| 2015/414 CF II | 10 | 1 | 2 | 0 | 1 | 0.01 | 0.00 |
| 2015/414 DF I | 64 | 2 | 0 | 0 | 1 | 0.04 | 0.00 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2015/414 DF II | 0 | 4 | 0 | 0 | 0 | 0.01 | 0.00 |
| 2015/415 AF | 210 | 48 | 16 | 1 | 6 | 0.31 | 0.00 |
| 2015/415 BF | 61 | 16 | 20 | 0 | 3 | 0.22 | 0.00 |

**Appendix II:**

**Detection of mutation:**

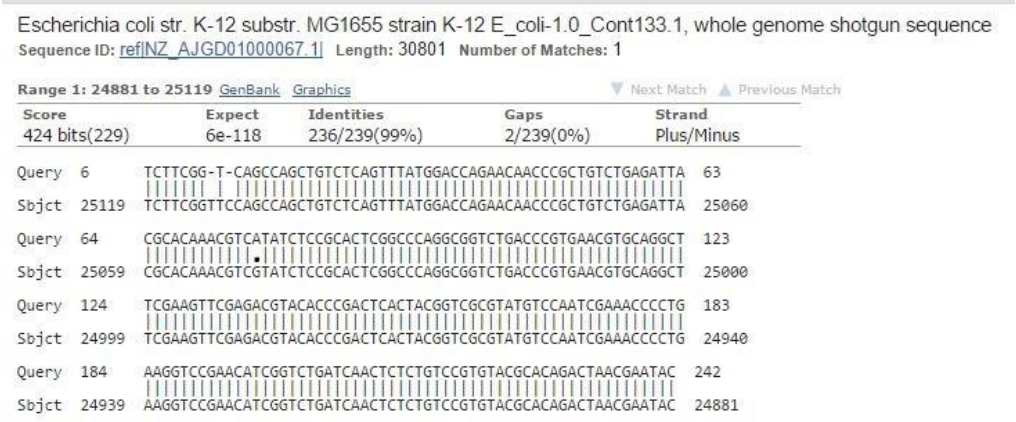
1. The sequence of the DNA are BLAST (Basic local alignment tool) for nucleotide blast. BLAST is an algorithm tool used to compare the query sequence with the database of sequences. Here, we compare the nucleotide sequence of DNA of our bacteria with reference genomic sequences of *Escherichia coli K-12(taxid: 83333)* to find the highly similar sequences (mega blast).
2. The mutation are detected on the blast result by looking for different sequences of our subject nucleotide sequences compared to nucleotide sequence of reference organism.
3. The mutation detected are confirmed by checking the sequences of our subject bacteria in the elution profile. The elution profile of the sequences are observed using the software Chromas LITE version 2.1.

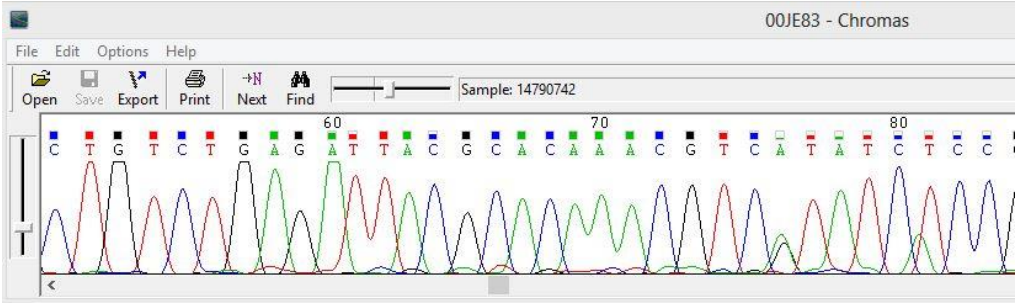
Some few examples to show the technique of mutation detection:

**Mutation analysis:**

**1. 2014/41 AFI**

Mutation detected: GC→AT



## 2. 2014/41 AF II

Mutation detected: GC→TA

Escherichia coli str. K-12 substr. MG1655 strain K-12 E_coli-1.0_Cont133.1, whole genome shotgun sequence

**Sequence ID:** ref|NZ_AJGD01000067.1| **Length:** 30801 **Number of Matches:** 1

**Range 1: 24881 to 25109** GenBank Graphics ▼ Next Match ▲ Previous Match

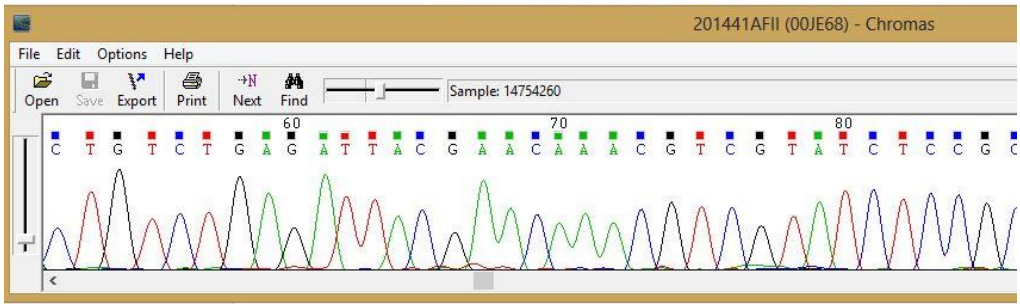
| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 418 bits(226) | 3e-116 | 228/229(99%) | 0/229(0%) | Plus/Minus |

```
Query  15     CAGCCAGCTGTCTCAGTTTATGGACCAGAACAACCCGCTGTCTGAGATTACGAACAAACG  74
              ||||||||||||||||||||||||||||||||||||||||||||||||||.|||||||
Sbjct  25109  CAGCCAGCTGTCTCAGTTTATGGACCAGAACAACCCGCTGTCTGAGATTACGCACAAACG  25050

Query  75     TCGTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCGAAGTTCG  134
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25049  TCGTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCGAAGTTCG  24990

Query  135    AGACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAGGTCCGAA  194
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24989  AGACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAGGTCCGAA  24930

Query  195    CATCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  243
              ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24929  CATCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  24881
```



### 3. 2014/41 AK

Mutation detected: GC→AT

Escherichia coli str. K-12 substr. MG1655 strain K-12 E_coli-1.0_Cont133.1, whole genome shotgun sequence

**Sequence ID:** ref|NZ_AJGD01000067.1| **Length:** 30801 **Number of Matches:** 1

**Range 1: 24881 to 25107** GenBank Graphics Next Match Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 414 bits(224) | 7e-115 | 226/227(99%) | 0/227(0%) | Plus/Minus |

```
Query  8      GCCAGCTGTCTCAGTTTATGGACCAGAACAACCCGCTGTCTGAGATTACGTACAAACGTC  67
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Sbjct  25107  GCCAGCTGTCTCAGTTTATGGACCAGAACAACCCGCTGTCTGAGATTACGCACAAACGTC  25048

Query  68     GTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCGAAGTTCGAG  127
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25047  GTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCGAAGTTCGAG  24988

Query  128    ACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAGGTCCGAACA  187
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24987  ACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAGGTCCGAACA  24928

Query  188    TCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  234
              ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24927  TCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  24881
```
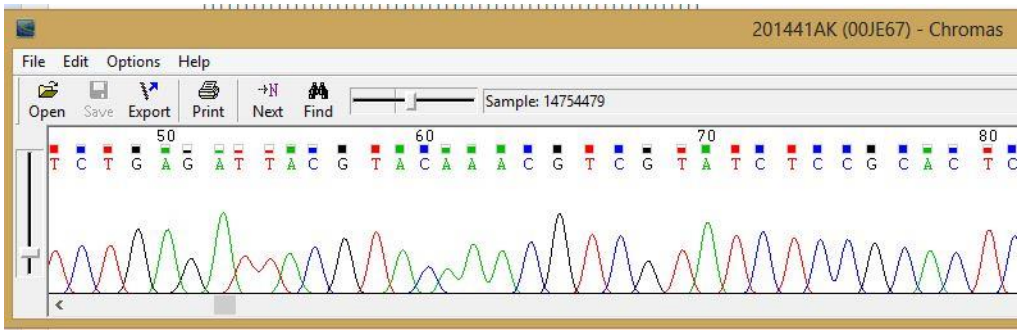



## 4. 2014/41 DF I

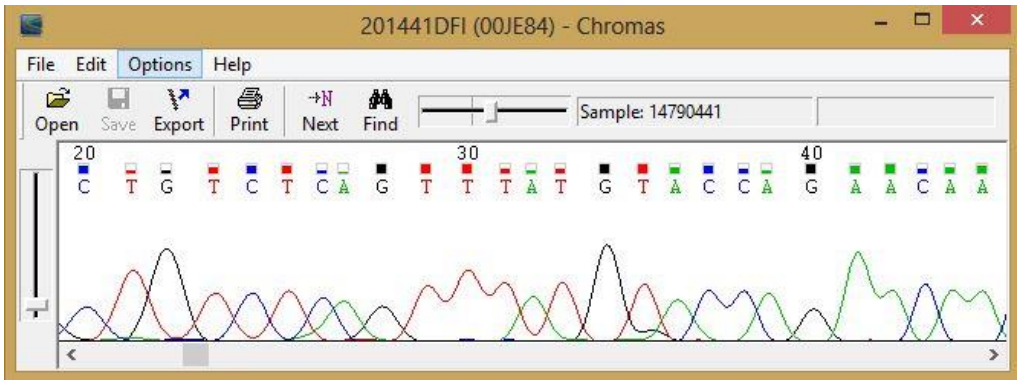
MUTATION DETECTED: GC→TA

Escherichia coli str. K-12 substr. MG1655 strain K-12 E_coli-1.0_Cont133.1, whole genome shotgun sequence
Sequence ID: ref|NZ_AJGD01000067.1| Length: 30801 Number of Matches: 1

Range 1: 24881 to 25116 GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 418 bits(226) | 2e-116 | 233/236(99%) | 1/236(0%) | Plus/Minus |

```
Query  7      TCGGAT-CAGCCAGCTGTCTCAGTTTATGTACCAGAACAACCCGCTGTCTGAGATTACGC  65
              |||| | |||||||||||||||||||||.||||||||||||||||||||||||||||||
Sbjct  25116  TCGGTTCCAGCCAGCTGTCTCAGTTTATGGACCAGAACAACCCGCTGTCTGAGATTACGC  25057

Query  66     ACAAACGTCGTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCG  125
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25056  ACAAACGTCGTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCG  24997

Query  126    AAGTTCGAGACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAG  185
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24996  AAGTTCGAGACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAG  24937

Query  186    GTCCGAACATCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  241
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24936  GTCCGAACATCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  24881
```



5. **2014/41 DF II**

MUTATION DETECTED: GC→ AT

Escherichia coli str. K-12 substr. MG1655 strain K-12 E_coli-1.0_Cont133.1, whole genome shotgun sequence

**Sequence ID:** ref|NZ_AJGD01000067.1| **Length:** 30801 **Number of Matches:** 1

**Range 1: 24881 to 25107** GenBank Graphics Next Match Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 407 bits(220) | 1e-112 | 225/227(99%) | 1/227(0%) | Plus/Minus |

```
Query  15     GCCAGCTGTCTCAGTTTATGGACCAGAA-AACCCGCTGTCTGAGATTACGTACAAACGTC  73
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Sbjct  25107  GCCAGCTGTCTCAGTTTATGGACCAGAACAACCCGCTGTCTGAGATTACGCACAAACGTC  25048

Query  74     GTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCGAAGTTCGAG  133
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25047  GTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGGCTTCGAAGTTCGAG  24988

Query  134    ACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAGGTCCGAACA  193
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24987  ACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCCTGAAGGTCCGAACA  24928

Query  194    TCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  240
              ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24927  TCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATAC  24881
```