# Assessment of Mutations Induced by Cold Atmospheric Plasma Jet Treatment Relative to Known Mutagens in *Escherichia coli*

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## ABSTRACT

The main bactericidal components of cold atmospheric plasma (CAP) are thought to be reactive oxygen and nitrogen species (RONS) and UV radiation, both of which have the capacity to cause DNA damage and mutations. Here, the mutagenic effects of CAP on *Escherichia coli* were assessed in comparison to X- and UV-irradiation. DNA damage and mutagenesis were screened for using a diffusion-based DNA fragmentation assay and modified Ames test respectively. Mutant colonies obtained from the latter were quantitated and sequenced. CAP was found to elicit a similar mutation spectrum to X-irradiation, that did not resemble that for UV implying that CAP produced RONS are more likely the mutagenic component of CAP. CAP treatment was also shown to promote resistance to the antibiotic ciprofloxacin. Our data suggest that CAP treatment has mutagenic effects that may have important phenotypic consequences.

## KEYWORDS

Atmospheric plasma jet; bacterial mutagenicity test; ionising radiation; UV radiation; antimicrobial resistance; plasma medicine

## ABBREVIATIONS

AMR – antimicrobial resistance; CAP – cold atmospheric plasma; RONS – reactive oxygen and nitrogen species

## INTRODUCTION

Bacterial infection of a wound is a common complication and radically increases associated morbidity and mortality. The ability of bacteria to produce complex, dense communities known as "biofilms" [1] coupled with increasing antimicrobial resistance (AMR) [2], presents difficult challenges for effective wound treatment [3]. AMR is common within species frequently isolated from wounds, known collectively as the "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp) [4]. The World Health Organisation (WHO) has predicted that by 2050 10 million people per annum will die of AMR infections, making the need for new treatment options critical [5].

The field of plasma medicine, while relatively new, is rapidly growing [6]. Cold atmospheric plasma (CAP) jets are being developed for a range of medical applications such as cancer treatment and decontamination of wounds. CAP is formed through the ionisation of inert gases, usually either helium or argon, at room temperature which, on contact with air produces a "cocktail" of reactive oxygen and nitrogen species (RONS) together with other components, including UV photons [7,8].

CAP-produced RONS are able to interact with biological targets and have been demonstrated to have bactericidal efficacy against a range of bacterial species, including antibiotic resistant strains, in both free-living, planktonic and biofilm forms [9,10]. Short-lived RONS components with half-lives ranging from milliseconds to seconds include the hydroxyl radical, peroxynitrous acid and superoxide. These in turn decay to longer-lived secondary RONS components, including hydrogen peroxide and nitrite, that are thought to be particularly critical for plasma medicine applications [7].

The presence of UV within plasma jets depends on operating pressure, for example low pressure plasmas are known to have vaccum UV (VUV, < 200 nm) and UVC (200-280 nm) components capable of sterilisation [11]. Within atmospheric plasmas the presence and role of UV radiation in sterilisation is widely debated, with parameters such as gas composition and humidity being important determinants of the relative contribution of UV [12].

Importantly, many CAP components, including individual RONS and UV radiation, have been previously demonstrated to be able to cause damage to DNA, potentially resulting in mutation. Mutations play a key role in the adaptation and evolution of bacteria by enabling them to respond to their environment, a process which occurs naturally through the formation of spontaneous mutations. The rate of mutation varies between bacterial species; in the human commensal pathogen *Escherichia coli (E. coli),* the spontaneous mutation rate is ~$1x10^{-3}$ per

genome, per generation [13] but exposure to chemical or physical mutagens such as UV or ionising radiation can greatly increase the frequency of mutations [14–16]. In comparison to other known mutagens, there have been comparatively few reports on the mutagenicity of CAP systems in bacteria, with Zhang *et al* reporting CAP is able to induce mutations in *Salmonella typhimurum* [17] and, more recently, a report that repeated CAP treatments are able to induce bacterial resistance to certain antibiotics [18]. In contrast, there have been numerous reports as to whether CAP can induce mutations in mammalian models, as measured using the *hprt* mutation assay, with most models tested not resulting in an increase in mutation rate [19–21] although Boehm *et al.* have shown that plasma-treated foetal calf serum (a model for complex bio-fluids) is mutagenic [22].

The objective of this study is to elucidate the mutagenic effects of sublethal doses of CAP using strains of *E. coli*, one wild type and one with a DNA repair defect, in comparison to known mutagens: UV radiation and X-radiation. This was carried out by assessing the effects of CAP on a number of biological endpoints including measurement of DNA damage, characterisation of mutation spectrum and antibiotic resistance.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*E. coli* strains WP2 and WP2 *uvrA*$^{-}$ were purchased from the Yale *E. coli* Stock Centre. The strains were maintained as frozen stocks at -80°C on 15% glycerol, as required they were plated onto Luria-Bertani (LB) agar (Sigma-Aldrich, Dorset, UK) and incubated overnight at 37°C to obtain single colonies before storage at 4°C. Bacterial broth cultures were grown by inoculating a single colony into 10 mL of LB broth (Sigma-Aldrich, Dorset, UK). Cultures were then incubated at 37°C at 200 rpm for 18 hrs.

### Viability

Bacterial overnight cultures were subcultured into fresh LB broth and grown to $2.5\times10^{8}$ CFU/mL or optical density at 600 nm [$OD_{600}$] of ~ 0.5. 100 μL was aliquoted on to LB agar plates in triplicate per treatment condition. Plates were incubated at 37°C for 18 hrs, following which the number of colony-forming units (CFU) per mL was enumerated and survival calculated relative to untreated control.

**CAP jet set-up**

CAP jet configuration has been previously described [23]. The plasma jet consists of a 150 mm long glass tube with non-tapered nozzle. The power was supplied to 15 mm long external copper electrode wound onto the glass tube. The jet assembly was used in a single electrode configuration (with no second grounded electrode). The flow rate of helium (He) was fixed at 2 standard litres per minute (SLPM) throughout. A sinusoidal driving high voltage of 10 $kV_{p-p}$ (peak-to-peak) at 25 kHz was applied to the external copper electrode with a PVM500 power supply. The distance between the plasma jet and the treatment substrate was 5 mm. Voltage and current waveforms were measured with a high voltage probe and current monitor.

**X-irradiation**

Cell-Rad X-irradiator was used as per manufacturer's instructions to administer varying dosages in Grays (Gy) of radiation. Bacterial overnight cultures were subcultured into fresh LB and grown to $2.5\times10^8$ CFU/mL or optical density at 600 nm [$OD_{600}$] of ~ 0.5. 100 µL was aliquoted and spread onto Glucose Minimal (GM) agar in triplicate per treatment condition. These were treated in tandem with LB agar survival plates (outlined previously). Treatment was administered according to manufacturer's instructions delivering 0.901 Gy/min.

**UV treatment**

UVC supplied by a germicidal fluorescent tube (Philips) with peak output at 254 nm was administered at an intensity of 1 $W/m^2$ for varying dosages. 125 µl of bacterial overnight culture was subcultured into 5 ml of fresh LB and grown to $2.5\times10^8$ CFU/ml or optical density at 600 nm [$OD_{600}$] of ~ 0.5. 100 µL was aliquoted on to GM agar in triplicate per treatment condition, these were treated in tandem with LB agar survival plates (outlined previously). Plates were exposed to varying dosages of UVC. Doses administered were 10, 20 and 30 $J/m^2$.

**CAP jet treatment**

Bacterial overnight culture was subcultured into fresh LB and grown to $2.5\times10^8$ CFU/mL or optical density at 600 nm [$OD_{600}$] of ~ 0.5. 350 µL of this culture was placed in a well on a standard 96-well plate and treated with CAP jet for varying treatment times of 0, 0.5, 1, 2, 3, 4, 5 & 10 minutes under the conditions described above. After CAP treatment, the culture was left for 30 minutes at room temperature and then 100 µL was spread onto GM agar in triplicate and in tandem with LB agar survival plates.

**DNA fragmentation assay**

*E. coli* WP2 were grown overnight and treated with CAP as described and incubated at 25°C for 30 mins. 2.5 μL of bacteria was then added to the immobilisation tube (containing 90 μL low-melting agarose, 5 μL of lysozyme (20 mg/mL), 1 μL of RNase (50 U/mL). This mixture was left to incubate at 37°C for 10 mins. 75 μL of the mix was then placed on a Comet Slide (obtained from Comet SCGE Assay kit, Enzo Life Sciences, Inc) and left to dry. Slides were then placed into a lysis solution and incubated at 4°C for 30 mins followed by immersion in alkaline solution (300 mM NaOH, 1 mM EDTA) and incubated for 20 mins in the dark at 25°C. Slides were then washed twice in 1X TBE. Slides were then rinsed in 70% ethanol for 5 mins and then stained with a 1 in 10,000 dilution of SYBR Gold dye concentrate (ThermoFisher) as per the manufacturer's instructions. Slides were then viewed using a Zeiss LSM 880 microscope.

**Mutagenesis assay**

100 μL of mutagen-exposed bacterial culture was added to glucose minimal (GM) agar (5.3 g/L dibasic potassium phosphate, 2g/L monobasic potassium phosphate, 1g/L ammonium sulphate, 0.5g/L sodium citrate, 16g/L agar, 4g/l D(+) glucose, 10% (w/v) magnesium sulphate and 0.2% (w/v) thiamine (B1) (all purchased from Sigma-Aldrich, Dorset, UK)) in triplicate per treatment condition. 100 μL of untreated bacterial culture was also added to GM agar as a control for spontaneous mutants. Plates were subsequently incubated statically at 37°C for 48 hours before counting of colonies.

***TrpE* and *glnU/glnW* sequencing**

Mutant colonies isolated from the mutagenesis assay were suspended in phosphate buffer saline (PBS) and subjected to colony PCR; cultures were also grown from each colony for storage as glycerol stocks for future analysis. For colony PCR, 350bp of the *trpE* gene of *E. coli* was amplified for analysis of mutations. Oligonucleotide primers used were *TrpE*For (5' CTTCCTGAAACGGGCAGTGT 3') and *TrpE*Rev (5' AGGGCGTTATCCAGTAGTGC 3') (Sigma). Colony PCR was carried out using GoTAQ reaction mix (Promega) in 50 μl reactions containing 100 μM primers. The amplification program consisted of an initial denaturation step of 94°C for 30 s, followed by 30 cycles of 30 s at 94°C (denaturation) and 30 s at 63 °C (annealing) 60 s at 68 °C (extension) and then a final extension at 68 °C for 5 min. PCR products were checked using 2% agarose gel electrophoresis and successful amplification was

indicated by a band at 350 bp. Amplified products were purified using GeneJET PCR purification kit (ThermoFisher) and sent for Sanger sequencing (Eurofins). For analysis of *glnU/glnW* suppressor mutations, DNA from cultures prepared from glycerol stocks taken from clones identified as lacking a reverting *trpE* mutation was amplified by colony PCR using appropriate forward (5'CGAAATTGGTAGACGCACCAG 3') and reverse (5' CATTCAGAATTTGGTGGCTAC 3') primers, complementary to sequences within the *leuW* and within and adjacent to the *metU* genes respectively. PCR reactions and downstream processing and sequence analysis were carried out using the conditions described above.

**Antibiotic susceptibility assay**

*E. coli* BW25113 was grown and treated with CAP (0-10 mins) as above, and left to incubate for 30 mins at 25°C. After treatment, 100 mL of bacterial suspension was added to LB agar supplemented with above minimum inhibitory concentration (MIC) of ciprofloxacin (0.03 mg/mL) and spread across the surface evenly. 100 mL of untreated subculture was also added to ciprofloxacin-supplemented agar to enumerate spontaneously resistant mutants. CAP treated bacteria were serially diluted in PBS and plated on LB agar to calculate number of surviving bacteria post CAP treatment.

## RESULTS

### CAP induces fragmentation of bacterial genomic DNA

To analyse the effects of CAP treatment on the integrity of bacterial DNA, a diffusion-based DNA fragmentation assay developed for use in bacteria [24] was carried out to assess DNA damage *in situ.* This assay operates on similar principles to the Comet assay that has been used extensively for the study of *in situ* DNA damage in eukaryotic cells but reflects the substantially smaller size (by several orders of magnitude) of bacterial genomes. In the assay, cells with fragmented DNA, arising as a consequence of DNA stand breaks, produce a peripheral halo of passively diffused DNA, detectable with the highly sensitive DNA dye, SYBR Gold (**Figure 2**). It is known the CAP produces substantial amount of RONS, and RONS have been shown to result in a variety of base damages and DNA strand breaks [25].

After exposure to 5 minutes of CAP treatment, nucleoids from the *E. coli* WP2 strain exhibited a peripheral halo of fragmented DNA more frequently than those from untreated cells (**Figure 2)**. These results are similar to the observations of Fernandez *et al*. [24], who observed increased numbers of fragmented nucleoids in *E. coli* cells upon treatment with hydrogen

peroxide, and are consistent with a model of CAP-induced RONS causing oxidative damage to cellular biomolecules like DNA.

**CAP-mediated DNA damage causes mutations at sublethal doses**

Having established that CAP treatment of 5 mins was sufficient to induce detectable levels of DNA damage in *E. coli* WP2 cells, CAP's ability to induce mutations was then tested. The WP2 strain is noteworthy owing to having an ochre stop codon at the fourth codon of the tryptophan biosynthesis gene *trpE*, removing its ability to produce tryptophan [26,27]. On introduction of a mutation at this codon, reversion will occur and a colony will grow on media without tryptophan. This strain can therefore be used to test physical and chemical agents for mutagenicity, analogous to reversion to histidine auxotrophy in the Ames *Salmonella* assay [28]. According to Ames *et al*. if the number of revertant mutants from auxotrophy to prototrophy is more than two-fold higher than the rate of spontaneous mutations and shows a dose-dependent increase then the chemical or physical agent under test can be considered mutagenic [27,28]. Reversion rate was calculated by number of revertant colonies divided by the surviving bacterial count (CFU in 100 µl aliquot) and compared to the untreated control. Our observed spontaneous revertant rates were of the range 1.8-2.8 x $10^{-7}$, consistent with reports elsewhere (e.g. Hamel et al. report rates of 2-5 x $10^{-7}$ for the WP2 strain [29]).

*E. coli* WP2 was grown to log phase and then exposed to varying doses of three mutagens: UV, X-irradiation and CAP, to assess and compare their effects on bacterial viability and mutation. UV doses of 10-30 J/$m^2$ were chosen as these were similar to the UV doses used in previous studies [30,31]. X-irradiation doses were also chosen based on precedent in the literature and are cited in Gray (Gy) [32,33]. CAP dose is presented as function of treatment time. Reversion rates adjusted for survival were calculated as in the treat and plate methodology described by Brusick *et al*. [34]. A dose-dependent increase in reversion rate was observed for UV, CAP and X-irradiation, with highest overall rates seen with the highest dose of UV (30 J/$m^2$) (**Figure 3 A-C**). The mutageneic threshold is the dose of mutagen found to produce a significant increase in mutation rate compared to the untreated control. For CAP treatment this was found to be between 4-5 minutes of treatment, for X-irradiation the mutagenic threshold was between 40-60 Gy and for UV between 20-30 J/$m^2$. The low level of reversion rate observed after CAP and X-irradiation compared to UV indicates that the UV component of CAP is having little effect.

As can be seen from **Figure 3D**, CAP had little significant effect on WP2 strain viability even at the extended exposure time of 10 minutes' treatment. This effect was comparable to ionising

X-irradiation, which also had minimal effect on the viability of WP2 at the doses tested (**Figure 3E**). This is consistent with previous work demonstrating that this B/r derivative is resistant to ionising radiation [28] due to combined deficiencies in Lon protease and the cell division inhibitor, SulA protein, which results in a loss of SOS-mediated cell division arrest [35]. We have also observed that the WP2 strain is more resistant to CAP than other common strains of *E. coli*. For example, in *E. coli* strain BW25113, a K-12 derivative [36], between a 2-3 log reduction in viable bacterial cells was observed for the same treatment times (data not shown). These data collectively support the idea that whilst the CAP RONS component plays an important role in inducing bacterial cell death [10], CAP, like X-irradiation, is mutagenic even at the sublethal doses used.

In contrast, UV treatment resulted in a 1-log reduction in *E. coli* WP2 cells even at the lowest dose used (**Figure 3F**). This suggests that the biological effects of the UV component of the CAP jet even at the longest treatment time are less than those induced by 10 J/m$^2$ of 254 nm radiation; this is seen by CAP's limited effect on bacterial viability at the highest dose tested.

To further assess whether the UV component of CAP has any significant mutagenic or bactericidal effects, CAP treatment was applied to *E. coli* WP2 and its *uvrA* derivative. This derivative is deficient in nucleotide excision repair (NER). As shown in **Figure 4,** there is a significant increase in the mutation rate post CAP treatment for *E. coli* WP2 *uvrA* when compared to *E. coli* WP2, with a 5-10-fold increase relative to the NER competent strain. However, this 5-10 fold increase is only slightly greater than that reported in a previous study comparing the sensitivity of *uvrA* mutant and wildtype WP2 strains to oxidative mutagens (and it is worth noting that in that in this earlier study only absolute numbers of mutants were counted with no adjustment made for survival) [37]. If UV were playing a truly significant role we would expect that the production of significant quantities of bulky lesions eg. UV-mediated pyrimidine dimers (that are repaired by NER) would have resulted in an increase in sensitivity of several orders of magnitude in scale [38]. The *uvrA* results, taken with the results in **Figure 3**, suggest that the observed CAP-induced mutagenesis has occurred predominantly through the effects of RONS.

**Point Mutation Analysis**

To further understand the observations, revertant colonies were sequenced to analyse the specific mutation behind tryptophan reversion. Ohta et al had previously identified the *trpE65* ochre site as codon 4, which usually encodes glutamine, and confirmed that all seven possible point mutations within the ochre codon that can result in an amino acid codon are able to cause

reversion (**Supplemental Table 1**) [27]. Alternatively, suppressor mutations in genes encoding tRNAs can also arise, enabling the ochre codon to be read through by the ribosome. There are a number of suppressor mutations that can suppress ochre mutations: anticodon changes including UUG→UUA in *glnU*, UUU→UUA in *lysT*, and GUA→UUA in *tyrT* and *tyrU* (termed *supB*, *supL*, *supC* and *supM* respectively) [39].

To assess which point mutations were more frequently induced by known mutagens (UV and X-irradiation) and provide a basis for comparison, the *trpE* gene of revertant colonies was sequenced and the ochre mutations observed compared to those induced by CAP (**Figure 5**). Notably, only six of the seven possible ochre mutations were observed in this experiment, with TAA to TAT being absent under all conditions tested. The mutation spectra induced in a WP2 strain by a range of mutagens have previously been reported [27] but the types of reversion mutations induced by X-rays, UV or CAP in the WP2 system have not been characterised until now.

As can be seen in **Figure 5A**, there is a distinct difference in the spectrum of point mutations induced by X-rays compared to UV. The most notable effect is that TAA to TCA and TAA to CAA mutations are entirely absent from the UV-induced mutation spectrum. It is important to note that *trpE* ochre reversions can not occur via the classic UV signature of a C to T mutation in a dipyrimidine context. Two of the ochre mutations observed after UV are due to a A:T to T:A transversion mutation – this type of mutation was also observed by Miller in his definitive study of *lacI* mutations induced by UV [40]. The same study also showed that A:T to G:C transition mutations, such as would be required for the absent TAA to CAA reversion mutation, were more under-represented and only occurred in a dipyrimidine context. As the ochre codon is present in the sequence context CATAAAA, these conditions do not apply for the TAA to CAA reversion.

While there are clear distinctions in the frequency of individual point mutations between the three treatment groups, the mutagenic signature of CAP seems to resemble that of the X-irradiated group more closely than the UV exposed group. The spectra induced by both CAP and X-irradiation are also far more similar to each other than either is to any induced by the range of mutagens tested by Ohta *et al* [27]. Taken together these data indicate that CAP mutagenesis is similar to X-irradiation mutagenesis and therefore likely involves oxidative stress via the RONS component [41].

For all three mutagens tested, suppressor mutations comprised a substantial proportion of the revertants tested (**Figure 5A**). UAA is the most commonly used stop codon in *E. coli* and ochre suppressors are therefore associated with a considerable fitness cost. Moreover, different tRNA

anticodon mutants have different suppressor efficiencies and will therefore impose differing selective pressures [42]. Therefore, interpretation of the distribution of point mutations in tRNA anticodons relative either to each other or to point mutations in the *trpE65* ochre codon would be challenging. However, among tRNA gene mutation-based ochre suppressors, the one that targets the *glnU* (and its co-located duplicate, *glnW*) anticodon-encoding triplet results from a C to T transition mutation in a bipyrimidine context (i.e. TCAA to TTAA). Given that this is the signature mutation associated with UVC exposure, it might be expected that *glnU/glnW* suppressor mutations would be over-represented amongst UVC suppressor revertants compared to CAP. We therefore sequenced the *glnU/glnW* locus of revertants that had been identified as having a suppressor mutation (**Figure 5B**). As expected, and consistent with previous studies , the majority (80 %) of suppressor mutations in the UVC-treated bacteria occurred at either *glnU* or *glnW*, while only a minority (22 %) of suppressor mutations in CAP-treated bacteria occurred at this site. These data complement our findings on the sensitivity of the WP2 *uvrA* strain shown in **Figure 4**, further suggesting that UV does not play a substantial role in CAP mutagenesis.

**CAP induced ciprofloxacin resistance**

Resistance to ciprofloxacin, a widely used fluoroquinolone antibiotic, often occurs as a result of single point mutations. Fluoroquinolone resistance is primarily caused by mutational alterations in the target genes of fluoroquinolones, DNA gyrase (at codons 83 or 87 of *gyrA*) and/or topoisomerase IV (at codons 80 or 84 of *parC*) [43]. For example, in a recent report Hamed *et al*. demonstrated that all tested fluoroquinolone resistant bacterial isolates from cancer patients had point mutations in *gyrA*, with 93.3% having an additional mutation in *parC*. In contrast, the presence of plasmid-mediated quinolone resistance genes was detected less frequently [44].

We therefore investigated whether the mutagenic effects of CAP would be able to induce ciprofloxacin resistance in a treated population of *E. coli*. After CAP treatment for various times, *E. coli* suspensions were challenged with plates supplemented with ciprofloxacin at above MIC concentrations (0.03 μg/ml). As CAP treatment time increased, the number of mutant colonies capable of growing in the presence of ciprofloxacin also increased (**Figure 6**), further indicating that CAP induces point mutations, which can have important phenotypic effects. Interestingly, the mutation rate is non-linear, with no detectable increase in ciprofloxacin resistance after the shortest treatment time, suggesting that there is a threshold effect which might arise through overwhelming of antioxidant defences at higher doses.

## DISCUSSION

In this study, CAP jet treatment under sub-lethal conditions was found to have a mutagenic effect on *E. coli* in a dose-dependent manner, with CAP treatment of above 5 minutes inducing a significant increase in mutations (**Figure 3**). Although it is known that CAP jets produce some UV radiation, our observations of the absence of a dramatically increased mutation rate or decreased survival rate in a *uvrA* strain, together with differing mutagenic spectra for Trp reversion, strongly suggest that CAP-produced UV is not the dominant mutagen in our system. The mutation spectrum induced by CAP appears to be more similar in pattern to that found in X-irradiated samples cf. UV treatment (**Figure 4**). These findings point to CAP-produced RONS being the dominant mutagenic factor in our treatment conditions, as a consequence of inducing DNA damage (**Figure 2**), although the possibility of adaptive mutagenesis can not be wholly discounted.

This is consistent with previous studies that have separated out the particle and UV components of plasma to demonstrate that the UV component makes a comparatively minor contribution to decontamination when $O_2$ is present [12,45] and a recent report that *E. coli* relies on genes associated with detoxification of RONS for resistance to CAP [46].

CAP induced mutations were found to impact *E. coli*'s susceptibility to a commonly used antibiotic, ciprofloxacin (**Figure 6**). Our data are consistent with a very recent report that repeated treatments with CAP can lead to AMR in a range of bacteria, partly ascribed by the authors to adaptive mutagenesis [18]. It is worth noting that in our study we were able to isolate ciprofloxacin-resistant colonies after just a single treatment. Moreover, the authors of the earlier study did not observe acquired ciprofloxacin resistance in any of the four *E. coli* strains they tested but did in other organisms including *Listeria monocytogenes* and *Salmonella typhimurium* [18].

These observations of CAP-induced mutagenesis have important ramifications for its medical use. An immediate implication to consider is that CAP treatment is intended for decontamination of bacterial infected wounds, including chronic wounds, where patients will often be on antibiotic treatments in tandem with CAP treatment. These findings would suggest some risk of CAP treatment increasing antibiotic tolerance within bacteria, as such, greater caution should be exercised in the use of CAP treatment for the decontamination of infected chronic wounds until more is known.

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**FIGURES**

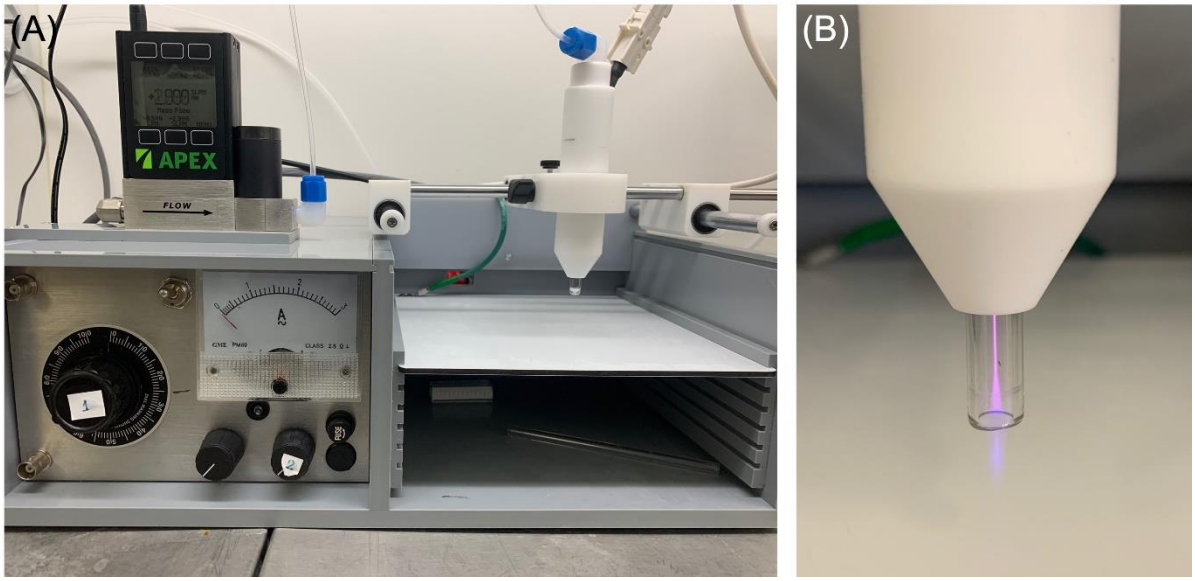


**Figure 1: The cold atmospheric plasma source used in this study.** (A) Configuration of helium plasma jet (b) plume of non-tapered plasma jet at 2 SLPM

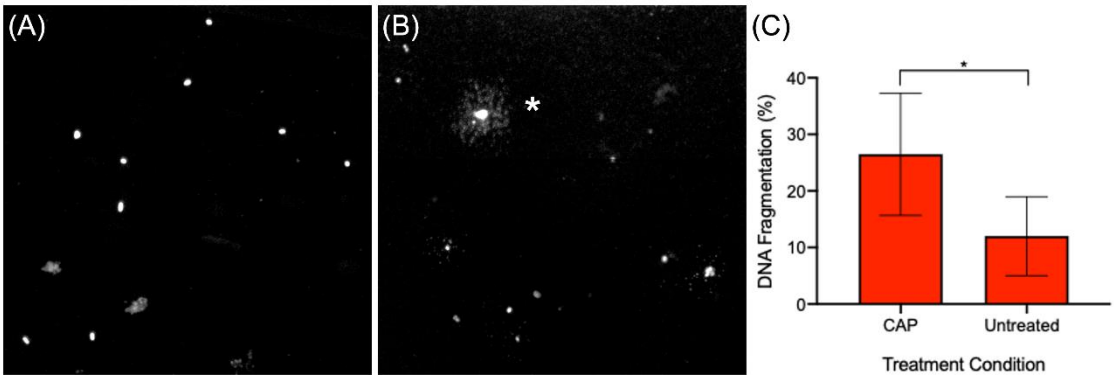


**Figure 2: Diffusion-based DNA fragmentation assay performed on *E. coli* WP2 cells to assess DNA damage.** (A) Untreated *E. coli* exhibit bright, dense nucleoids. (B) After 5 min of CAP treatment evidence of DNA damage is observed, seen as speckling around the nucleoid (*). (C) DNA fragmentation (%) of *E. coli* WP2 after 5 minutes treatment with CAP jet compared to untreated control. The percentage of nucleoids with detectable fragmentation relative to intact nucleoids was calculated. A total of 138 nucleoids for CAP-treated and 157 nucleoids for untreated were analysed from 3 biological replicates. Error bars show standard deviation of the mean. Data was plotted using GraphPad 8.0, Students t-test was performed p= 0.0361

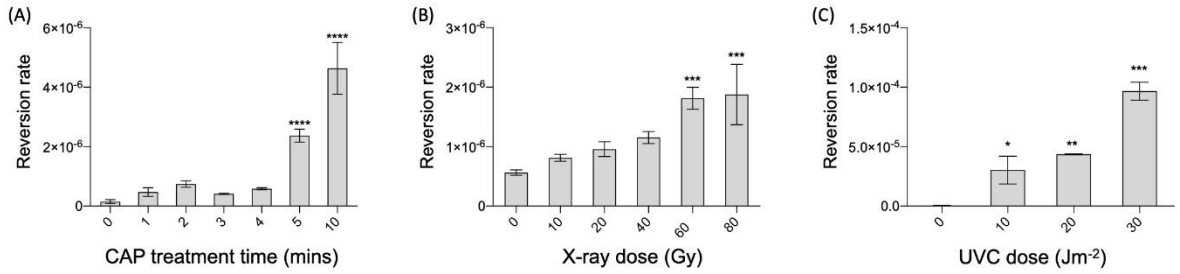


**Figure 3: CAP induces mutations at sublethal doses.** Graphs show the frequency of trpE revertant mutants for *E. coli* WP2 after exposure to varying dosage of: (A) CAP (* p=0.0288 & ***p=0.0002 respectively) (B) X-ionising (*** p=0.0002 for 60 Gy and p=0.0001 for 80 Gy) (C) UVC (** p=0.0011 and *** p= 0.0003 and 0.0001) and the effects on survival of *E. coli* WP2 strain of varying dosages of: (D) CAP, (E) X-irradiation and (F) UVC. Plated to measure survival as per materials and methods and plotted as surviving fraction relative to untreated control. Error bars in each case represent the standard deviation of the mean (n=3). Significance was analysed using Dunnett's t-test comparing to untreated control, according to Kirkland's recommendations [47].

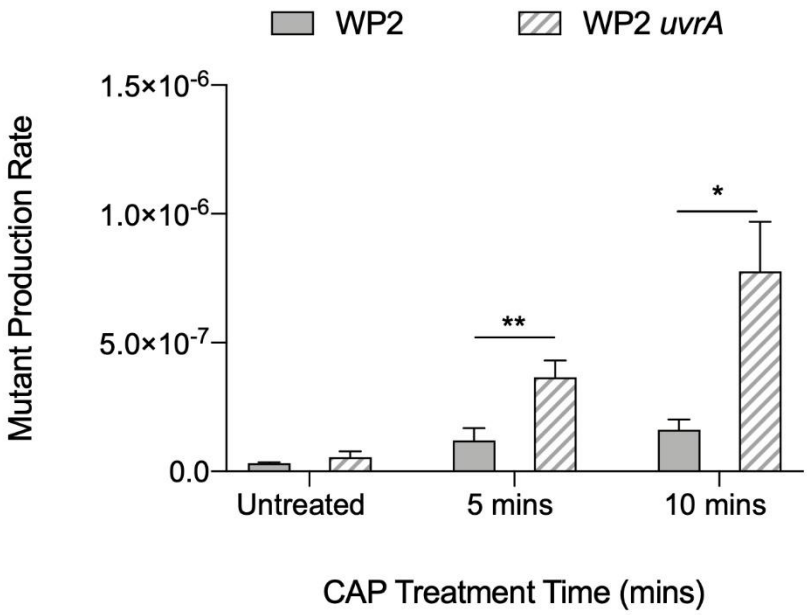


**Figure 4: A nucleotide excision repair deficient strain shows only a moderate increase in CAP-induced mutation rate.** The Trp reversion rate was measured for varying CAP treatment times in both *E. coli* WP2 and WP2 *uvrA* strains. (n=3) Error bars denote standard deviation of the mean. Data was plotted using GraphPad 8.0. Unpaired t test was used to assess statistical significance. ** $p < 0.01$ (0.0062 for 5 mins and 0.0056 for 10 mins).

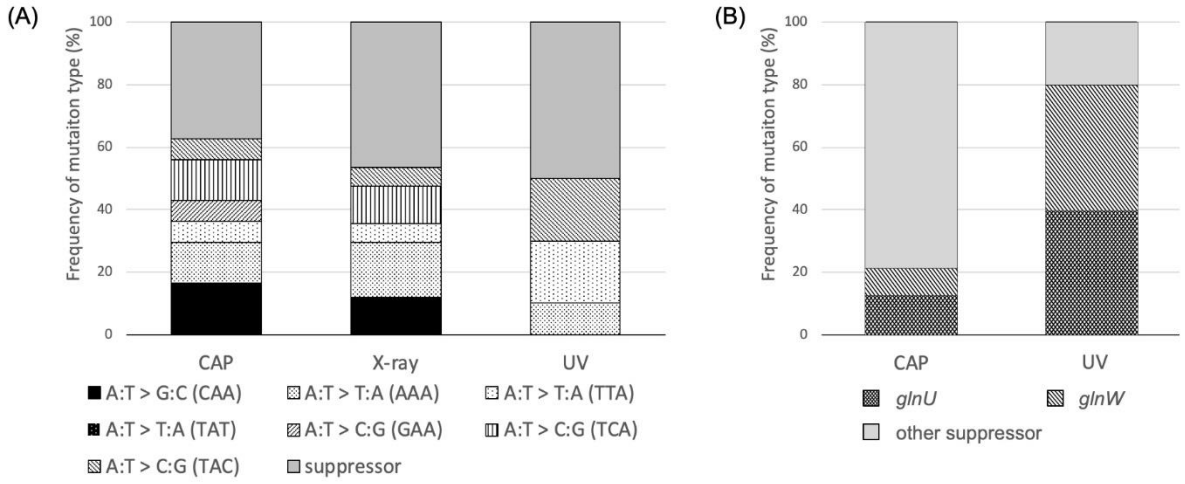


**Figure 5: Mutation spectra induced by CAP, X-irradiation and UV treatment.** (A) A section of the *trpE* gene covering the Ochre codon (TAA)-containing region was sequenced by the Sanger method and the type of reversion mutation assessed. The frequency of each mutation type was calculated as a percentage relative to the total number of samples sequenced per condition. Mutation types are coded by class: A:T to G:C transition is shown in black, A:T to T:A transversions are shown as spotted boxes and A:T to C:G transversions as striped. Suppressor mutations (i.e. where no ochre mutation was detected) are shown in grey. (B) The *glnU/glnW* locus from CAP and UV-induced revertants containing a suppressor mutation was amplified and sequenced. Sequences were analysed for the presence or absence of a CAA to TAA mutation in the anticodon templating strand of either *glnU* or *glnW*.

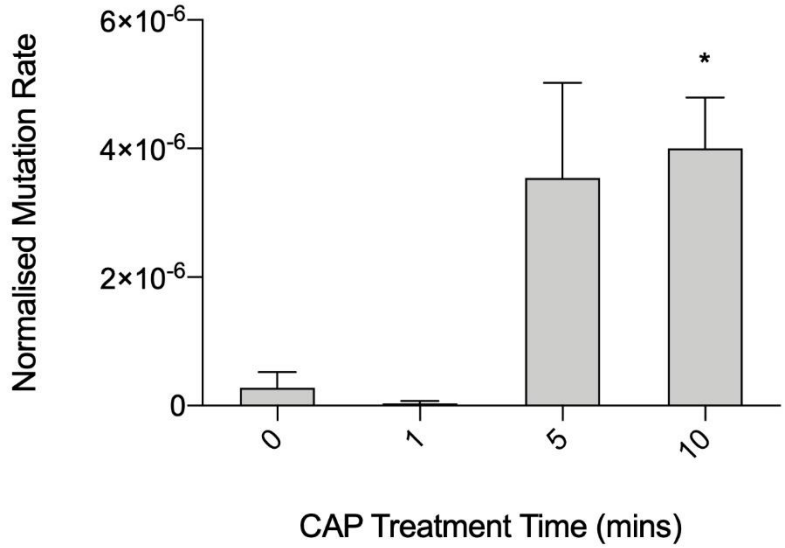


**Figure 6: Normalised mutation rate of *E. coli* BW25113 in the presence of ciprofloxacin (0.03 µg/mL) after CAP treatment for varying amounts of time.** Mutation rate is defined as number of ciprofloxacin-resistant *E. coli* cells per total surviving. Error bars represent standard deviation (n=3). Unpaired *t*-test performed to assess statistical significance * $p < 0.05$ (0.0387).

## SUPPLEMENTAL INFORMATION

Supplemental Table 1

**Table 1|** Within the ochre codon of the *TrpE* gene of *E. coli* there are known to be 7 possible point mutations which result in a change to an amino acid coding codon, these are outlined below [27].

| Mutation | Type of Mutation | Wild Type Codon | Point Mutation | Amino Acid Change |
|---|---|---|---|---|
| A•T → G•C | Transition | TAA | CAA | Glutamine (Gln) |
| A•T → T•A | Transversion | | AAA | Lysine (Lys) |
| A•T → T•A | Transversion | | TTA | Leucine (Leu) |
| A•T → T•A | Transversion | | TAT | Tyrosine (Tyr) |
| A•T → C•G | Transversion | | GAA | Glutamic Acid (Glu) |
| A•T → C•G | Transversion | | TCA | Serine (Ser) |
| A•T → C•G | Transversion | | TAC | Tyrosine (Tyr) |