Assessing Stress Responses to Atmospheric Cold Plasma Exposure Using Escherichia Coli Knock Out Mutants

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Assessing stress responses to atmospheric cold plasma exposure using *Escherichia coli* knock out mutants

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Running title: Stress response to Atmospheric Cold Plasma
Abstract

Aim: This study investigated the effect of Atmospheric cold plasma (ACP) exposure induced stress on microbial inactivation patterns and the regulation of genes involved in the microbial stress response in conjunction with key processing parameters of exposure time and post treatment storage time.

Methods and Results: Cell suspensions of Escherichia coli BW 25113 and its isogenic knock-out mutants in rpoS, soxR, soxS, oxyR and dnaK genes were treated with high voltage ACP in a sealed package for 1, 3 and 5 min followed by 0, 1 and 24 h post-treatment storage. ROS densities and colony formation were determined. ΔrpoS strain showed higher microbial reduction and greater cell permeability than other mutants, while ΔoxyR only showed this effect after 5 min treatment. With increased post-treatment storage time, ΔsoxS and ΔsoxR had increased sensitivity and resistance, respectively. ΔdnaK cell suspensions had much higher ROS than other strains and showed increased sensitivity with 24 h post-treatment storage.

Conclusions: RpoS and oxyR genes have both short-term and long-term regulatory effects under plasma stress. However, knocking out dnaK gene had an immediate response on ROS scavenging and long-term repairing mechanisms. ΔsoxR and ΔsoxS had different responses to ACP treatment with the increase of post-treatment time in relation to clearance of reactive species implying the different characteristics and functions as subunits.

Significance and Impact of study: By comparing the response of mutants under ACP exposure to key processing parameters, the mechanism of microbial inactivation was partly revealed with respect to cellular regulation and repairing genes.

Key words: Atmospheric cold plasma treatment, Escherichia coli, reactive oxygen species, long-term reactive species, stress response, knock-out mutants
Introduction

Atmospheric cold plasma (ACP) generates neutral ionised gases at room temperature, which are composed of particles including free electrons, radicals, positive and negative ions, quanta of electromagnetic radiation, excited and non-excited molecules (Misra et al. 2011). Dielectric barrier discharges (DBD) combine the advantages of non-equilibrium plasma properties with the ease of atmospheric-pressure operation and have been widely used for industrial large scale application (Kogelschatz et al. 1997). Research on DBD-ACP system development is rapidly progressing for surface modification and biological applications such as tissue treatment or food sterilization (Cui and Brown 2002; Fridman et al. 2006; Ziuzina et al. 2014).

During plasma discharge in air, the reactive factors generated could include reactive oxygen species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation, energetic ions and charged particles, where the reactive species generation with the DBD-ACP system used has been characterised by Moiseev et al. (2014). The microbiocidal effect of these reactive species has been studied by Laroussi and Leipold (2004), and ROS have been proved to play the most crucial role in the inactivation of microbes (Joshi et al. 2011). The ROS, including ozone, singlet oxygen, oxygen atom and hydroxyl radicals, have strong oxidative effects on cell components, and can result in lipid peroxidation, DNA and protein damage, which lead to cell death (Cabisco et al. 2000; Joshi et al. 2011). Moreover, excited nitrogen can be easily oxidized by oxygen in the system and form nitrogen oxides (NO, NO₂, NO₃, N₂O₃, N₂O₄, N₂O₅), of which nitrites and nitrates are known to be long-lived antimicrobial species (Naïtali et al. 2010), and generate peroxynitrites (ONOO⁻, ONOOH) with secondary and synergistic reactions, which all have strong antimicrobial effect by damaging proteins, lipids and DNA.
Our previous studies established that the inactivation efficacy of ACP is governed by system and process variables including duration and mode of exposure due to associated cell damage and recovery, where survival with obvious cell leakage and DNA damage was observed suggesting the importance of cellular regulatory and repair systems under plasma stress (Han et al. 2014). Extended treatment time has been associated with greater inactivation efficacy by inducing higher oxidative stress thus overwhelming repair systems (Joshi et al. 2011; Boxhammer et al. 2013). With regard to mode of exposure, magnitude field and energetic ions can directly affect cells subjected to direct exposure (Dobrynin et al. 2009; Dobrynin et al. 2011). Recombined or longer lived species may contribute to a diffusion effect leading to different inactivation patterns in association with post treatment storage time (Ziuzina et al. 2013; Han et al. 2014), while different reactive species might be generated due to the quenching effect in association with mode of exposure design (Moiseev et al. 2014). Some regulatory and damage repair genes have been found upregulated under plasma stress (Sharma et al. 2009). However, their roles in relation to ROS levels, DNA and membrane damage which all result from plasma exposure and plasma stress have not been studied. Therefore, five genes associated with stress regulation and responses were selected for this study to investigate their role in providing protection against ACP or whether absence confirms enhanced sensitivity towards ACP. RpoS is a well-known global regulator for stationary phase and general stress (Matsuoka and Shimizu 2011). Two major transcriptional regulators, SoxRS and OxyR, have been found to control bacterial genetic responses to oxidative stress. SoxR can be induced by both ROS and RNS (Greenberg et al. 1990; Ding and Demple 2000), and they trigger its regulation through soxS (Pomposiello et al. 2001). OxyR is induced by hydrogen peroxide and controls the expression of a group of enzymes for
scavenging ROS species and the regulatory gene \textit{oxyS} for further response (Christman et al. 1989; Mukhopadhyay and Schellhorn 1997). DnaK is one of the most abundant constitutively expressed and stress inducible chaperones (Calloni et al. 2012) involved in cell repair. Comparing knock out mutants of these genes with the parent strain, untreated controls as well as plasma treated samples were investigated with respect to plasma process parameters of treatment time, post-treatment storage time as well as mode of exposure. Inactivation patterns, ROS density and membrane integrity were assessed. Therefore, the regulatory effects and antioxidative functions of these genes were further elucidated.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

Parent strain \textit{E. coli} BW 25113 and its 5 mutants \textit{ΔrpoS} (\textit{E. coli} JW 5437), \textit{ΔsoxR} (\textit{E. coli} JW 4024), \textit{ΔsoxS} (\textit{E. coli} JW 4023), \textit{ΔoxyR} (\textit{E. coli} JW 3933), \textit{ΔdnaK} (\textit{E. coli} JW 0013) were used in this study (Baba et al. 2006; Patil et al. 2011) and are described in Table 1. All strains were obtained from the National BioResource Project, Japan (NIG, Japan). Strains were maintained as frozen stocks at -70 °C in the form of protective beads, which were plated onto tryptic soy agar (TSA, Scharlau Chemie, Barcelona, Spain) and incubated overnight at 37 °C to obtain single colonies before storage at 4 °C.

**Preparation of Bacterial Cell Suspensions**

Cells were grown overnight (18 h) by inoculating isolated single colony of respective bacteria in tryptic soy broth without glucose (TSB-G, Scharlau Chemie, Barcelona, Spain) at 37 °C. Cells were harvested by centrifugation at 8,720 g for 10 min, and washed twice with sterile phosphate buffered saline (PBS, Oxoid LTD, UK). The pellet was re-suspended in PBS and the bacterial density was determined by measuring absorbance at 550 nm using McFarland standard (BioMérieux, Marcy-l'Étoile, France). Finally, cell suspensions with concentration of $10^8$ CFU ml$^{-1}$ were prepared in PBS.
ACP system configuration

The dielectric-barrier discharge (DBD) ACP system used in this study consists of a high voltage transformer (with input voltage 230 V at 50 Hz), and a voltage variac (output voltage controlled within 0~120 kV) (Figure 1). ACP discharge was generated between two 15-cm diameter aluminium electrodes separated by two perspex dielectric layers (10 mm and 1mm thickness). The system was operated at high voltage level of 70 kV_{RMS} under atmospheric pressure. A polypropylene container, which acted as a sample holder and another dielectric barrier, was placed between the two perspex dielectric layers. The overall distance between the two electrodes was kept constant (3 cm) for all experiments.

ACP treatment

For direct plasma treatment, 10 ml of bacterial cell suspensions in PBS were aseptically transferred to a sterile plastic petri dish, which was placed in the centre of the container, between the electrodes. For indirect plasma treatment, a separate container was used, where the sample petri dish was placed on the upper left corner of the container, outside the plasma discharge. Each container was sealed in a high barrier polypropylene bag (B2630; Cryovac Sealed Air Ltd, Dunkan, SC, USA) using atmospheric air as a working gas for ACP generation. Bacterial samples were then treated with ACP at 70 kV_{RMS} for 1, 3 and 5 min. After ACP treatment, samples were opened immediately or subsequently stored at room temperature for 1 or 24 h (Han et al. 2014). Ozone concentrations generated were measured using GASTEC gas tube detectors (Product # 18M, Gastec Corporation, Kanagawa, Japan) immediately after treatment and also after 1 or 24 h storage as indicated in Figure 1. All experiments were carried out in duplicate and replicated twice.

Microbiological Analysis

To quantify the effects of plasma treatment, 1 ml of treated samples were serially diluted in Maximum Recovery Diluent (MRD, Scharlau Chemie, Barcelona, Spain) and 0.1 ml aliquots
of appropriate dilutions were surface plated on TSA. In order to obtain low microbial detection limits, 1 ml of the treated sample was spread onto TSA plates as described by EN ISO 11290-2 method (ISO 11290-2, 1998), incubated at 37 °C for 24 h and counted. Any plates with no growth were incubated for up to 72 h and checked for the presence of colonies every 24 h. Results are reported in Log_{10} CFU ml^{-1} units.

**Detection of reactive oxygen species after plasma treatment**

The cellular assay probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used, which is widely used for fluorescence based assays in microtitre plate reader, flow cytometry and fluorescence microscopy. After ACP treatment and appropriate storage, treated cells were incubated with DCFH-DA (Sigma-Aldrich, USA) at a final concentration of 5 × 10^{-3} mol/m^3 in PBS for 15 min at 37 °C. Aliquots of 200 µL from each sample were transferred into 96 well fluorescence microplate wells (Fisher Scientific, UK) and intracellular ROS was measured using Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments Inc.) at excitation and emission wave lengths of 485 and 525 nm respectively. However, ACP generated reactive species in liquid can convert DCFH-DA to DCFH and further to fluorescent DCF, which has interference on this assay with an extracellular ROS signal (Gomes et al. 2005).

**Cell membrane integrity**

Membrane integrity was examined using propidium iodide (PI) fluorescence staining. After ACP treatment, cells were incubated with PI (Sigma-Aldrich, USA) at a final concentration of 3 × 10^{-3} mol/m^3 in PBS for 15 min at 37 °C. Aliquots of 200 µL from each sample were transferred into 96 well fluorescence microplate wells and measured by Synergy™ HT Multi-Mode Microplate Reader at excitation and emission wave lengths of 485 and 590 nm respectively.
Membrane integrity was alternatively examined by determination of the release of material absorbing at 260 and 280 nm (Virto et al. 2005). The UV absorbance at 260 nm and 280 nm ($A_{260}$ and $A_{280}$) has been widely used to indicate the effect on cell membrane integrity.

Untreated (bacterial cells in PBS) and ACP-treated samples were centrifuged at 13,200 g for 10 min. Supernatant of each sample (200 µL) was transferred into microtitre plate wells and measured by Synergy™ HT Multi-Mode Microplate Reader at 260 nm and 280 nm.

**Statistical Analysis**

Statistical analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, U.S.A.). Data represent the means of experiments performed in duplicate and replicated at least twice. Means were compared using analysis of variance (ANOVA) using Fisher’s Least Significant Difference-LSD at the 0.05 level.

**Results**

Intracellular ROS levels were detected using fluorescence probe DCFH-DA and are represented in Figure 2. The ROS signal was generally decreased with post-treatment storage time, due to their oxidative reaction with cell components. Figure 2 a and b shows an increase of ROS levels in tandem with increasing treatment times in samples with no post-treatment storage, while in-package ozone concentrations showed the same trend in Table 2. $\Delta$dn$\alpha$K with both direct and indirect exposure had much higher signal than all other mutant strains (p<0.05). However, when samples were stored for 1 h post treatment, $\Delta$soxS with indirect exposure had the highest ROS signal followed by $\Delta$sxyR and $\Delta$naK (Figure 2 c and d, p<0.05). After 24 h post-treatment storage, higher remaining ROS levels were observed from $\Delta$saxS than other mutants (Figure 2 e and f, p<0.05).

In-package Ozone levels strongly increased with extended treatment time (p<0.05), where 5 min (both direct and indirect) exposure to ACP generated around 4000 ppm ozone (Table 2). Similar to ROS results obtained from DCFH DA assay, the detected ozone levels dropped...
significantly in 1 h (p<0.05), while 24 h post-treatment storage decreased the concentrations under the detection limit.

The membrane integrity was tested using PI after 24 h post-treatment storage only (Figure 3). PI staining indicates the cell membrane integrity by binding with double strand of DNA, where the breakage of ds DNA in severely damaged cells will decrease the fluorescence signal. AǫpoS mutant strain samples had a signal strength twice that of the control, while the signal of AǫxyR mutants was 1.5 fold higher than the control (p<0.05). However, as ACP treatment causes both cell envelope damage and intracellular DNA damage in bacteria (Han et al. 2014), the detected levels of PI staining may be a combination of both effects. Therefore, Figure 4 represents the membrane integrity by an alternative measurement, absorbance at 260 nm based on the release of DNA by damaged cells (Virto et al. 2005). The results obtained at 280 nm (protein) were similar (data not shown). AǫpoS samples had significantly higher absorbance than other mutant strains (p<0.05), while AǫpoS strains exposed to indirect treatment showed greater leakage than those exposed to direct treatment. However, AǫxyR had an absorbance equivalent to the other strains (except AǫpoS) (p>0.05). This suggests that membrane integrity had been compromised to allow PI to enter but cell damage was not severe enough to result in DNA leakage from the cell.

Figure 5 shows inactivation patterns for E. coli mutants over different ACP treatment times and post-treatment storage times. When no post-treatment storage time was employed, the immediate effect of the reactive species generated was assessed. All mutant strains surviving numbers were similarly affected by direct and indirect exposure to plasma for 1 min (p>0.05), with some difference in response noted after 3 min exposure. However, obvious separation of effect corresponding to the mutant strain characteristics was noted after 5 min (p<0.05). AǫpoS was reduced below the detection limit after 5 min treatment with indirect exposure, while direct exposure reduced cell concentration to 1.38±0.25 Log_{10} CFU ml^{-1}. Both wild
type and ΔoxyR were reduced below the detection limit after 5 min treatment with both
d modes of exposure. Similar trends of 4 Log_{10} CFU ml^{-1} surviving populations were observed
from other mutants, ΔsoxR, ΔsoxS and ΔdnaK (Table 1 and Figure 5 a and b, p>0.05).
After 1 h post-treatment storage, 1 min ACP treated ΔsoxS with direct exposure had a slightly
lower survival level than all other strains (p<0.05). But with 3 min treatment, both ΔsoxR and
ΔoxyR mutants exhibited the greatest resistance to plasma effects (p<0.05), while wild type
with indirect exposure and ΔsoxS samples exposed to either mode of exposure were reduced
below the detection limit. Further extending the treatment time to 5 min, ΔsoxR levels were
maintained at 3.22±0.20 and 4.38±0.10 Log_{10} CFU ml^{-1} with direct and indirect exposure
respectively, whilst the ΔdnaK mutant was still detectable at 1.98±0.21 Log_{10} CFU ml^{-1}
following direct exposure only. However, all other mutant strains were reduced below the
detection limit regardless of mode of exposure (Figure 5 c and d).
By extending post treatment storage time to 24 h, only ΔsoxR samples had populations
surviving above the detection limit with either 3 or 5 min ACP treatment duration. With the
short treatment time of 1 minute, minimal further mutant strain differentiation was apparent,
where ΔrpoS had lowest survival level of 4.88±0.10 Log_{10} CFU ml^{-1} with direct exposure
only (p<0.05).
Discussion
The cellular response of all strains to ACP exposure stress are discussed below with respect
to process and system parameters.
Effect of treatment time
Treatment time, which dominates the plasma exposure doses, had a positive effect on
inactivation efficacy for all strains, while similar results have been widely reported by many
researchers (Ghomi et al. 2009; Kvam et al. 2012; Liu et al. 2013; Han et al. 2014). The in-
package ROS generation was increased with respect to extension of ACP treatment time, as
represented by ozone level and ROS in Table 2 and Figure 2. The overall greater amount of reactive species led to the increasing of cell membrane permeability in Figure 3 and 4, and resulted in lower survival level (Figure 5).

**Effect of post-treatment storage time**

A post-treatment storage time facilitated prolonged and contained exposure to a range of longer lived reactive species (Figure 5 e and f) and increased inactivation efficacy, by providing extended reaction time for cell lethal species thus exposing a clearer differentiation of inactivation patterns between mutant strains.

With all post treatment storage times, the absence of RpoS increased the sensitivity of cells to ACP treatment. Comparing with wild type, a slightly lower capability for ROS clearance of Δ*rpoS* was only observed following 1 h post-treatment storage (Figure 2, p<0.05), while its cell membrane permeability was significantly increased (Figure 3, p<0.05). *RpoS* is known as encoding a crucial regulator and 11% of the genes it regulates are correlated to stress management (Weber et al. 2005) *katE* is regulated by *rpoS* and participates in the antioxidant defence mechanism (Jung and Kim 2003). *SurA* is another gene regulated by *rpoS* and involved in the folding of membrane proteins (Lazar and Kolter 1996). Thus, the absence of *rpoS* gene could have resulted in the incorrect folding of some membrane proteins, which further affected the cell permeability.

With the increase of post-treatment storage time, Δ*soxR* and Δ*soxS* had significantly different performance (p<0.05). Δ*soxR* mutant was the most resistant strain with 1 and 24 h post-treatment storage, while Δ*soxS* showed increased sensitivity. Initially, similar ROS levels were detected, but much higher levels of ROS remained in Δ*soxS* over prolonged storage after extended treatment times, which indicated the importance of SoxS for ROS scavenging. *SoxRS* has been reported as an important regulon of *E. coli* under superoxide and nitric oxide stress (Greenberg et al. 1990; Perni et al. 2007). The SoxS stimulates genes for resistance to...
oxidative stress and antibiotics, while SoxR removes intracellular nitric oxide by its [2Fe-2S] centres (Demple 1996; Ding and Demple 2000). The transcriptional initiation of soxS is known to be induced by SoxR, whereas it is also regulated proteolytically by the Lon protease (Blanchard et al. 2007). The difference of ROS levels observed from this study implied that soxS is the crucial subunit in the regulon under plasma stress rather than soxR.

The alternative mechanism of activating soxS without soxR is still unclear where Gaudu et al. (1997) discussed the hypothesis but it has not been verified.

The importance of dnaK gene increased gradually with extended post-treatment storage time, indicating the damage of DNA and demand of the DNA repairing system to be a long-term effect of ACP treatment rather than an immediate reaction. Additionally, the highest initial ROS densities observed after ACP treatment without post-treatment storage time (Figure 2, p<0.05), could be attributed to deficiencies in correlated cell components synthesis without DnaK protein, which plays an important role in the chaperone network and in the initiation of DNA replication (Rockabrand et al. 1995; Calloni et al. 2012) (Skowyra et al. 1990). The absence of dnaK has been reported to increase the cell sensitivity under both thermal and oxidative stress (Delaney 1990). However, it has been found to be down regulated with several other chaperones after H2O2 treatment (Chang et al. 2002).

Effect of mode of exposure

As another important system parameter, indirect mode of plasma exposure has been studied with distanced treatment or additional mesh (Okubo et al. 2004; Fridman et al. 2007; Dobrynin et al. 2009). Long-lived and recombined reactive species (such as peroxynitrites) led to microbiocidal effects with indirect exposure, instead of short-lived species and free radicals in discharging area with direct exposure (Dobrynin et al. 2009; Han et al. 2014). Obvious differences of ROS generation were observed, where indirect exposure led to much higher ROS densities than direct exposure (Figure 1). During direct exposure treatment,
13 discharging occurred in both gas and liquid phase, where ozone formation rates are quenched
by OH radical generation and higher dissociation energy is required (Fridman 2008; Moiseev
et al. 2014). Whereas, indirect exposure offered discharging in air, where higher ozone
concentrations were generated. Hence, higher ROS concentration could be generated inside
the solution of indirectly exposed samples by following secondary reactions. Among the
products of secondary reactions, peroxynitrites are major antimicrobial agents (Machala et al.
2013; Lukes et al. 2014). A higher cell leakage level was observed from ΔrpoS samples using
indirect exposure comparing with direct exposure (Figure 3 and 4), which could be the result
of ROS level according to modes of exposure.

**Survival of ΔoxyR mutants**

ΔoxyR was found to be resistant with high surviving populations after 1 and 3 min treatment
followed by 0 and 1 h post-treatment storage or 24 h storage following 1 min treatment. But,
extended treatment time could dramatically reduce it under the detection limit. This indicated
that oxyR gene could have crucial effects on ROS scavenging under high oxidative stress,
which is shown by its high ROS signal after 1 h post-treatment storage in Figure 1d and a
higher cell permeability of ΔoxyR samples after 24 h post-treatment storage, which could be a
result of the prolonged ROS damaging effect on the cell membrane. Moreover, long lived
species are generated during extended storage time, such as H_{2}O_{2}, O_{3} and HNO_{x} (Moiseev et
al. 2014), where H_{2}O_{2} dominates the activation of ΔoxyR. OxyR is activated by the
generation of disulfide bonds resulting from oxidative species such as hydrogen peroxide,
and further acts as a transcription activator and induces a series of antioxidative enzymes
(Christman et al. 1989; Mukhopadhyay and Schellhorn 1997; Cabiscol et al. 2000). Without
the presence of oxyR, hemH might be an alternative mode of response under oxidative stress
by encoding ferrochelatase (Zheng et al. 2001), which could explain the survival of ΔoxyR
mutants.
Overview

Stress response system studies in *E. coli* have been extensively reported but information on the cellular response to cold plasma is still limited. Sharma et al. (2009) studied the cold plasma response of *E. coli* on transcription level using micro-array. Some genes related to SOS response, oxidative repair, regulation were up-regulated significantly, which contributed to the repair of DNA damage and redox of reactive species. These mechanisms involving oxidative stress response were further elucidated in our study based on the different reactions to ACP of *E. coli* mutants deficient in key stress responsive genes, which also demonstrated different sensitivities to ozone treatment in a previous study (Patil et al. 2011).

Overall, among the investigated process and system parameters, distinctive responses were observed with respect to increasing the post-treatment storage time, while treatment time and mode of exposure showed similar effects on all strains. A proposed response model of mutants under ACP exposure stress is therefore presented in figure 6 based on our observations. The absence of *rpoS* and *oxyR* genes led to reduced cell envelope recovery and ROS scavenging effects, regardless of post-treatment storage time, while some genes varied in impact during the storage period, suggesting their different roles in the stress response. *SoxS* gene became important for cell survival by clearance of reactive species with both 1 and 24 h storage, which was not observed from Δ*soxR*, implying their different characteristics as subunits. *DnaK* showed its repairing function correlated to cell survival with 24 h storage. Besides general regulatory and resistance functions, the cell response against plasma generated oxidative stress could be divided into short term and long term, dominated by oxidative stress response genes and damage repair genes, respectively. The uncovered emergency response relating to reactive species clearance and further mechanisms of cell repair provides an explanation of cell survival under plasma stress and offers promising insights for optimising ACP applications.
Acknowledgements

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Conflict of Interest

There is no conflict of interest to declare.
References:


Table 1: List of knock-out mutants and their characteristics

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Description</th>
<th>Gene information</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> BW 25113</td>
<td>Parent strain</td>
<td>A derivative of the F-, λ-, <em>E. coli</em> K-12 strain (Datsenko and Wanner 2000)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 5437</td>
<td>Δ <em>rpoS</em></td>
<td>Regulatory factor, influence on expression of genes for stationary phase or stress response (Cheville et al. 1996; Matsuoka and Shimizu 2011)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 4024</td>
<td>Δ <em>soxR</em></td>
<td>Correlated with oxidative damage repairing and antioxidative actions (Nunoshiba et al. 1992), can be induced by both ROS and RNS (Greenberg et al. 1990; Ding and Demple 2000)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 4023</td>
<td>Δ <em>soxS</em></td>
<td>Triggered by <em>soxR</em> under oxidative stress (Pomposiello et al. 2001), and stimulates genes for oxidative and antibiotic resistance (Demple 1996).</td>
</tr>
<tr>
<td><em>E. coli</em> JW 3933</td>
<td>Δ <em>oxyR</em></td>
<td>Required for the induction of a regulon of hydrogen peroxide-inducible genes (Christman et al. 1989)</td>
</tr>
<tr>
<td><em>E. coli</em> JW 0013</td>
<td>Δ <em>dnaK</em></td>
<td>Participating in initiation of DNA replication, which plays an important role in DNA repair, and is one of the most abundant constitutively expressed and stress inducible chaperones (Skowyra et al. 1990; Calloni et al. 2012)</td>
</tr>
</tbody>
</table>
Table 2. In-package ozone concentration after ACP treatment at 70 kV$_{RMS}$ in response to process parameters

<table>
<thead>
<tr>
<th>Post-treatment storage time</th>
<th>Mode of exposure</th>
<th>Plasma treatment time</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1min</td>
</tr>
<tr>
<td></td>
<td>Ozone (ppm)</td>
<td>SD*</td>
</tr>
<tr>
<td>0 h</td>
<td>Direct</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
<td>1950</td>
</tr>
<tr>
<td>1 h</td>
<td>Direct</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
<td>130</td>
</tr>
<tr>
<td>24 h</td>
<td>Direct</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>Indirect</td>
<td>ND*</td>
</tr>
</tbody>
</table>

ND*: non-detectable
SD*: standard deviation