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

Lu Han
Technological University Dublin, lu.han@dit.ie

Daniela Boehm
Dublin Institute of Technology, 452929@dit.ie

Sonal Patil
Dublin Institute of Technology, sonalpatil81@gmail.com

Patrick Cullen
Dublin Institute of Technology, pjcullen@dit.ie

Paula Bourke
Dublin Institute of Technology, paula.bourke@dit.ie

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

3 Han, L.[1], Boehm, D.[1], Patil, S.[1], Cullen, P.J.[1,2], & Bourke, P.[1]*

4

5 1. *School of Food Science and Environmental Health, Dublin Institute of Technology,*
6 *Ireland*
7 2. *School of Chemical Engineering, UNSW, Sydney, Australia*

8

9

10 *Corresponding Author

11 Paula Bourke

12 School of Food Science and Environmental Health

13 Dublin Institute of Technology

14 Dublin 1, Ireland

15 paula.bourke@dit.ie

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24 **Running title: Stress response to Atmospheric Cold Plasma**

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26

27 **Abstract**

28 Aim: This study investigated the effect of Atmospheric cold plasma (ACP) exposure induced
29 stress on microbial inactivation patterns and the regulation of genes involved in the microbial
30 stress response in conjunction with key processing parameters of exposure time and post
31 treatment storage time.
32 Methods and Results: Cell suspensions of *Escherichia coli* BW 25113 and its isogenic knock-
33 out mutants in *rpoS*, *soxR*, *soxS*, *oxyR* and *dnaK* genes were treated with high voltage ACP in
34 a sealed package for 1, 3 and 5 min followed by 0, 1 and 24 h post-treatment storage. ROS
35 densities and colony formation were determined. *ΔrpoS* strain showed higher microbial
36 reduction and greater cell permeability than other mutants, while *ΔoxyR* only showed this
37 effect after 5 min treatment. With increased post-treatment storage time, *ΔsoxS* and *ΔsoxR*
38 had increased sensitivity and resistance, respectively. *ΔdnaK* cell suspensions had much
39 higher ROS than other strains and showed increased sensitivity with 24 h post-treatment
40 storage.
41 Conclusions: *RpoS* and *oxyR* genes have both short-term and long-term regulatory effects
42 under plasma stress. However, knocking out *dnaK* gene had an immediate response on ROS
43 scavenging and long-term repairing mechanisms. *ΔsoxR* and *ΔsoxS* had different responses to
44 ACP treatment with the increase of post-treatment time in relation to clearance of reactive
45 species implying the different characteristics and functions as subunits.
46 Significance and Impact of study: By comparing the response of mutants under ACP
47 exposure to key processing parameters, the mechanism of microbial inactivation was partly
48 revealed with respect to cellular regulation and repairing genes.
49 Key words: Atmospheric cold plasma treatment, *Escherichia coli*, reactive oxygen species,
50 long-term reactive species, stress response, knock-out mutants

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53 **Introduction**

54 Atmospheric cold plasma (ACP) generates neutral ionised gases at room temperature, which
55 are composed of particles including free electrons, radicals, positive and negative ions,
56 quanta of electromagnetic radiation, excited and non-excited molecules (Misra et al. 2011).
57 Dielectric barrier discharges (DBD) combine the advantages of non-equilibrium plasma
58 properties with the ease of atmospheric-pressure operation and have been widely used for
59 industrial large scale application (Kogelschatz et al. 1997). Research on DBD-ACP system
60 development is rapidly progressing for surface modification and biological applications such
61 as tissue treatment or food sterilization (Cui and Brown 2002; Fridman et al. 2006; Ziuzina et
62 al. 2014).
63 During plasma discharge in air, the reactive factors generated could include reactive oxygen
64 species (ROS), reactive nitrogen species (RNS), ultraviolet (UV) radiation, energetic ions and
65 charged particles, where the reactive species generation with the DBD-ACP system used has
66 been characterised by Moiseev et al. (2014). The microbiocidal effect of these reactive
67 species has been studied by Laroussi and Leipold (2004), and ROS have been proved to play
68 the most crucial role in the inactivation of microbes (Joshi et al. 2011). The ROS, including
69 ozone, singlet oxygen, oxygen atom and hydroxyl radicals, have strong oxidative effects on
70 cell components, and can result in lipid peroxidation, DNA and protein damage, which lead
71 to cell death (Cabiscol et al. 2000; Joshi et al. 2011). Moreover, excited nitrogen can be easily
72 oxidized by oxygen in the system and form nitrogen oxides (NO, NO_2, NO_3, N_2O_3, N_2O_4,
73 N_2O_5), of which nitrites and nitrates are known to be long-lived antimicrobial species (Naïtali
74 et al. 2010), and generate peroxynitrites ($ONOO^-$, ONOOH) with secondary and synergistic
75 reactions, which all have strong antimicrobial effect by damaging proteins, lipids and DNA

76 (Shigenaga et al. 1997; Boxhammer et al. 2012; Machala et al. 2013; Cullen et al. 2014;
77 Lukes et al. 2014; Moiseev et al. 2014).
78 Our previous studies established that the inactivation efficacy of ACP is governed by system
79 and process variables including duration and mode of exposure due to associated cell damage
80 and recovery, where survival with obvious cell leakage and DNA damage was observed
81 suggesting the importance of cellular regulatory and repair systems under plasma stress (Han
82 et al. 2014). Extended treatment time has been associated with greater inactivation efficacy
83 by inducing higher oxidative stress thus overwhelming repair systems (Joshi et al. 2011;
84 Boxhammer et al. 2013). With regard to mode of exposure, magnitude field and energetic
85 ions can directly affect cells subjected to direct exposure (Dobrynin et al. 2009; Dobrynin et
86 al. 2011). Recombined or longer lived species may contribute to a diffusion effect leading to
87 different inactivation patterns in association with post treatment storage time (Ziuzina et al.
88 2013; Han et al. 2014), while different reactive species might be generated due to the
89 quenching effect in association with mode of exposure design (Moiseev et al. 2014). Some
90 regulatory and damage repair genes have been found upregulated under plasma stress
91 (Sharma et al. 2009). However, their roles in relation to ROS levels, DNA and membrane
92 damage which all result from plasma exposure and plasma stress have not been studied.
93 Therefore, five genes associated with stress regulation and responses were selected for this
94 study to investigate their role in providing protection against ACP or whether absence
95 confirms enhanced sensitivity towards ACP. RpoS is a well-known global regulator for
96 stationary phase and general stress (Matsuoka and Shimizu 2011). Two major transcriptional
97 regulators, SoxRS and OxyR, have been found to control bacterial genetic responses to
98 oxidative stress. SoxR can be induced by both ROS and RNS (Greenberg et al. 1990; Ding
99 and Demple 2000), and they trigger its regulation through *soxS* (Pomposiello et al. 2001).
100 OxyR is induced by hydrogen peroxide and controls the expression of a group of enzymes for

101 scavenging ROS species and the regulatory gene *oxyS* for further response (Christman et al.
102 1989; Mukhopadhyay and Schellhorn 1997). DnaK is one of the most abundant constitutively
103 expressed and stress inducible chaperones (Calloni et al. 2012) involved in cell repair.
104 Comparing knock out mutants of these genes with the parent strain, untreated controls as well
105 as plasma treated samples were investigated with respect to plasma process parameters of
106 treatment time, post-treatment storage time as well as mode of exposure. Inactivation
107 patterns, ROS density and membrane integrity were assessed. Therefore, the regulatory
108 effects and antioxidative functions of these genes were further elucidated.

109 **MATERIALS AND METHODS**

110 ***Bacterial Strains and Growth Conditions***

111 Parent strain *E. coli* BW 25113 and its 5 mutants *ΔrpoS* (*E. coli* JW 5437), *ΔsoxR* (*E. coli* JW
112 4024), *ΔsoxS* (*E. coli* JW 4023), *ΔoxyR* (*E. coli* JW 3933), *ΔdnaK* (*E. coli* JW 0013) were
113 used in this study (Baba et al. 2006; Patil et al. 2011) and are described in Table 1. All strains
114 were obtained from the National BioResource Project, Japan (NIG, Japan). Strains were
115 maintained as frozen stocks at -70 ºC in the form of protective beads, which were plated onto
116 tryptic soy agar (TSA, Scharlau Chemie, Barcelona, Spain) and incubated overnight at 37 ºC
117 to obtain single colonies before storage at 4 ºC.

118 ***Preparation of Bacterial Cell Suspensions***

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

126 ***ACP system configuration***

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

135 ***ACP treatment***

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

148 ***Microbiological Analysis***

149 To quantify the effects of plasma treatment, 1 ml of treated samples were serially diluted in
150 Maximum Recovery Diluent (MRD, Scharlau Chemie, Barcelona, Spain) and 0.1 ml aliquots

151 of appropriate dilutions were surface plated on TSA. In order to obtain low microbial
152 detection limits, 1 ml of the treated sample was spread onto TSA plates as described by EN
153 ISO 11290-2 method (ISO 11290-2, 1998), incubated at 37 ºC for 24 h and counted. Any
154 plates with no growth were incubated for up to 72 h and checked for the presence of colonies
155 every 24 h. Results are reported in Log_{10} CFU ml^{-1} units.

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

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

168 ***Cell membrane integrity***

169 Membrane integrity was examined using propidium iodide (PI) fluorescence staining.
170 After ACP treatment, cells were incubated with PI (Sigma-Aldrich, USA) at a final
171 concentration of 3×10^{-3} mol/m^3 in PBS for 15 min at 37 °C. Aliquots of 200 μL from each
172 sample were transferred into 96 well fluorescence microplate wells and measured by
173 Synergy™ HT Multi-Mode Microplate Reader at excitation and emission wave lengths of
174 485 and 590 nm respectively.

175 Membrane integrity was alternatively examined by determination of the release of material
176 absorbing at 260 and 280 nm (Virto *et al.* 2005). The UV absorbance at 260 nm and 280 nm
177 (A_{260} and A_{280}) has been widely used to indicate the effect on cell membrane integrity.
178 Untreated (bacterial cells in PBS) and ACP-treated samples were centrifuged at 13,200 g for
179 10 min. Supernatant of each sample (200 μL) was transferred into microtitre plate wells and
180 measured by Synergy^{TM} HT Multi-Mode Microplate Reader at 260 nm and 280 nm.

181 ***Statistical Analysis***

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

186 **Results**

187 Intracellular ROS levels were detected using fluorescence probe DCFH-DA and are
188 represented in Figure 2. The ROS signal was generally decreased with post-treatment storage
189 time, due to their oxidative reaction with cell components. Figure 2 a and b shows an increase
190 of ROS levels in tandem with increasing treatment times in samples with no post-treatment
191 storage, while in-package ozone concentrations showed the same trend in Table 2. *ΔdnaK*
192 with both direct and indirect exposure had much higher signal than all other mutant strains
193 ($p<0.05$). However, when samples were stored for 1 h post treatment, *ΔsoxS* with indirect
194 exposure had the highest ROS signal followed by *ΔoxyR* and *ΔdnaK* (Figure 2 c and d,
195 $p<0.05$). After 24 h post-treatment storage, higher remaining ROS levels were observed from
196 *ΔsoxS* than other mutants (Figure 2 e and f, $p<0.05$).

197 In-package Ozone levels strongly increased with extended treatment time ($p<0.05$), where 5
198 min (both direct and indirect) exposure to ACP generated around 4000 ppm ozone (Table 2).
199 Similar to ROS results obtained from DCFH DA assay, the detected ozone levels dropped

200 significantly in 1 h ($p<0.05$), while 24 h post-treatment storage decreased the concentrations
201 under the detection limit.
202 The membrane integrity was tested using PI after 24 h post-treatment storage only (Figure 3).
203 PI staining indicates the cell membrane integrity by binding with double strand of DNA,
204 where the breakage of ds DNA in severely damaged cells will decrease the fluorescence
205 signal. *ΔrpoS* mutant strain samples had a signal strength twice that of the control, while the
206 signal of *ΔoxyR* mutants was 1.5 fold higher than the control ($p<0.05$). However, as ACP
207 treatment causes both cell envelope damage and intracellular DNA damage in bacteria (Han
208 et al. 2014), the detected levels of PI staining may be a combination of both effects.
209 Therefore, Figure 4 represents the membrane integrity by an alternative measurement,
210 absorbance at 260 nm based on the release of DNA by damaged cells (Virto et al. 2005). The
211 results obtained at 280 nm (protein) were similar (data not shown). *ΔrpoS* samples had
212 significantly higher absorbance than other mutant strains ($p<0.05$), while *ΔrpoS* strains
213 exposed to indirect treatment showed greater leakage than those exposed to direct treatment.
214 However, *ΔoxyR* had an absorbance equivalent to the other strains (except *ΔrpoS*) ($p>0.05$).
215 This suggests that membrane integrity had been compromised to allow PI to enter but cell
216 damage was not severe enough to result in DNA leakage from the cell.
217 Figure 5 shows inactivation patterns for *E. coli* mutants over different ACP treatment times
218 and post-treatment storage times. When no post-treatment storage time was employed, the
219 immediate effect of the reactive species generated was assessed. All mutant strains surviving
220 numbers were similarly affected by direct and indirect exposure to plasma for 1 min ($p>0.05$),
221 with some difference in response noted after 3 min exposure. However, obvious separation of
222 effect corresponding to the mutant strain characteristics was noted after 5 min ($p<0.05$).
223 *ΔrpoS* was reduced below the detection limit after 5 min treatment with indirect exposure,
224 while direct exposure reduced cell concentration to 1.38 ± 0.25 Log_{10} CFU ml^{-1}. Both wild

225 type and *ΔoxyR* were reduced below the detection limit after 5 min treatment with both
226 modes of exposure. Similar trends of 4 Log_{10} CFU ml^{-1} surviving populations were observed
227 from other mutants, *ΔsoxR*, *ΔsoxS* and *ΔdnaK* (Table 1 and Figure 5 a and b, $p>0.05$).
228 After 1 h post-treatment storage, 1 min ACP treated *ΔsoxS* with direct exposure had a slightly
229 lower survival level than all other strains ($p<0.05$). But with 3 min treatment, both *ΔsoxR* and
230 *ΔoxyR* mutants exhibited the greatest resistance to plasma effects ($p\leq0.05$), while wild type
231 with indirect exposure and *ΔsoxS* samples exposed to either mode of exposure were reduced
232 below the detection limit. Further extending the treatment time to 5 min, *ΔsoxR* levels were
233 maintained at 3.22±0.20 and 4.38±0.10 Log_{10} CFU ml^{-1} with direct and indirect exposure
234 respectively, whilst the *ΔdnaK* mutant was still detectable at 1.98±0.21 Log_{10} CFU ml^{-1}
235 following direct exposure only. However, all other mutant strains were reduced below the
236 detection limit regardless of mode of exposure (Figure 5 c and d).
237 By extending post treatment storage time to 24 h, only *ΔsoxR* samples had populations
238 surviving above the detection limit with either 3 or 5 min ACP treatment duration. With the
239 short treatment time of 1 minute, minimal further mutant strain differentiation was apparent,
240 where *ΔrpoS* had lowest survival level of 4.88±0.10 Log_{10} CFU ml^{-1} with direct exposure
241 only ($p<0.05$).

242 **Discussion**

243 The cellular response of all strains to ACP exposure stress are discussed below with respect
244 to process and system parameters.

245 ***Effect of treatment time***

246 Treatment time, which dominates the plasma exposure doses, had a positive effect on
247 inactivation efficacy for all strains, while similar results have been widely reported by many
248 researchers (Ghomi et al. 2009; Kvam et al. 2012; Liu et al. 2013; Han et al. 2014). The in-
249 package ROS generation was increased with respect to extension of ACP treatment time, as

250 represented by ozone level and ROS in Table 2 and Figure 2. The overall greater amount of
251 reactive species led to the increasing of cell membrane permeability in Figure 3 and 4, and
252 resulted in lower survival level (Figure 5).

253 ***Effect of post-treatment storage time***

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

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

268 With the increase of post-treatment storage time, *ΔsoxR* and *ΔsoxS* had significantly different
269 performance ($p<0.05$). *ΔsoxR* mutant was the most resistant strain with 1 and 24 h post-
270 treatment storage, while *ΔsoxS* showed increased sensitivity. Initially, similar ROS levels
271 were detected, but much higher levels of ROS remained in *ΔsoxS* over prolonged storage
272 after extended treatment times, which indicated the importance of SoxS for ROS scavenging.
273 *SoxRS* has been reported as an important regulon of *E. coli* under superoxide and nitric oxide
274 stress (Greenberg et al. 1990; Perni et al. 2007). The SoxS stimulates genes for resistance to

275 oxidative stress and antibiotics, while SoxR removes intracellular nitric oxide by its [2Fe-2S]
276 centres (Demple 1996; Ding and Demple 2000). The transcriptional initiation of *soxS* is
277 known to be induced by SoxR, whereas it is also regulated proteolytically by the Lon
278 protease (Blanchard et al. 2007). The difference of ROS levels observed from this study
279 implied that *soxS* is the crucial subunit in the regulon under plasma stress rather than *soxR*.
280 The alternative mechanism of activating *soxS* without *soxR* is still unclear where Gaudu et al.
281 (1997) discussed the hypothesis but it has not been verified.
282 The importance of *dnaK* gene increased gradually with extended post-treatment storage time,
283 indicating the damage of DNA and demand of the DNA repairing system to be a long-term
284 effect of ACP treatment rather than an immediate reaction. . Additionally, the highest initial
285 ROS densities observed after ACP treatment without post-treatment storage time (Figure 2,
286 $p<0.05$), could be attributed to deficiencies in correlated cell components synthesis without
287 DnaK protein, which plays an important role in the chaperone network and in the initiation of
288 DNA replication (Rockabrand et al. 1995; Calloni et al. 2012) (Skowyra et al. 1990). The
289 absence of *dnaK* has been reported to increase the cell sensitivity under both thermal and
290 oxidative stress (Delaney 1990). However, it has been found to be down regulated with
291 several other chaperones after H_2O_2 treatment (Chang et al. 2002).
292 ***Effect of mode of exposure***
293 As another important system parameter, indirect mode of plasma exposure has been studied
294 with distanced treatment or additional mesh (Okubo et al. 2004; Fridman et al. 2007;
295 Dobrynin et al. 2009). Long-lived and recombined reactive species (such as peroxynitrites)
296 led to microbiocidal effects with indirect exposure, instead of short-lived species and free
297 radicals in discharging area with direct exposure (Dobrynin et al. 2009; Han et al. 2014).
298 Obvious differences of ROS generation were observed, where indirect exposure led to much
299 higher ROS densities than direct exposure (Figure 1). During direct exposure treatment,

300 discharging occurred in both gas and liquid phase, where ozone formation rates are quenched
301 by OH radical generation and higher dissociation energy is required (Fridman 2008; Moiseev
302 et al. 2014). Whereas, indirect exposure offered discharging in air, where higher ozone
303 concentrations were generated. Hence, higher ROS concentration could be generated inside
304 the solution of indirectly exposed samples by following secondary reactions. Among the
305 products of secondary reactions, peroxynitrites are major antimicrobial agents (Machala et al.
306 2013; Lukes et al. 2014). A higher cell leakage level was observed from *ΔrpoS* samples using
307 indirect exposure comparing with direct exposure (Figure 3 and 4), which could be the result
308 of ROS level according to modes of exposure.

309 ***Survival of ΔoxyR mutants***

310 *ΔoxyR* was found to be resistant with high surviving populations after 1 and 3 min treatment
311 followed by 0 and 1 h post-treatment storage or 24 h storage following 1 min treatment. But,
312 extended treatment time could dramatically reduce it under the detection limit. This indicated
313 that *oxyR* gene could have crucial effects on ROS scavenging under high oxidative stress,
314 which is shown by its high ROS signal after 1 h post-treatment storage in Figure 1d and a
315 higher cell permeability of *ΔoxyR* samples after 24 h post-treatment storage, which could be a
316 result of the prolonged ROS damaging effect on the cell membrane.. Moreover, long lived
317 species are generated during extended storage time, such as H_2O_2, O_3 and HNO_x (Moiseev et
318 al. 2014), where H_2O_2 dominates the activation of *ΔoxyR*. OxyR is activated by the
319 generation of disulfide bonds resulting from oxidative species such as hydrogen peroxide,
320 and further acts as a transcription activator and induces a series of antioxidative enzymes
321 (Christman et al. 1989; Mukhopadhyay and Schellhorn 1997; Cabiscol et al. 2000). Without
322 the presence of *oxyR*, *hemH* might be an alternative mode of response under oxidative stress
323 by encoding ferrochelatase (Zheng et al. 2001), which could explain the survival of *ΔoxyR*
324 mutants.

325 ***Overview***

326 Stress response system studies in *E. coli* have been extensively reported but information on
327 the cellular response to cold plasma is still limited. Sharma et al. (2009) studied the cold
328 plasma response of *E. coli* on transcription level using micro-array. Some genes related to
329 SOS response, oxidative repair, regulation were up-regulated significantly, which contributed
330 to the repair of DNA damage and redox of reactive species. These mechanisms involving
331 oxidative stress response were further elucidated in our study based on the different reactions
332 to ACP of *E. coli* mutants deficient in key stress responsive genes, which also demonstrated
333 different sensitivities to ozone treatment in a previous study (Patil et al. 2011).
334 Overall, among the investigated process and system parameters, distinctive responses were
335 observed with respect to increasing the post-treatment storage time, while treatment time and
336 mode of exposure showed similar effects on all strains. A proposed response model of
337 mutants under ACP exposure stress is therefore presented in figure 6 based on our
338 observations. The absence of *rpoS* and *oxyR* genes led to reduced cell envelope recovery and
339 ROS scavenging effects, regardless of post-treatment storage time, while some genes varied
340 in impact during the storage period, suggesting their different roles in the stress response.
341 *SoxS* gene became important for cell survival by clearance of reactive species with both 1 and
342 24 h storage, which was not observed from *ΔsoxR*, implying their different characteristics as
343 subunits. *DnaK* showed its repairing function correlated to cell survival with 24 h storage.
344 Besides general regulatory and resistance functions, the cell response against plasma
345 generated oxidative stress could be divided into short term and long term, dominated by
346 oxidative stress response genes and damage repair genes, respectively. The uncovered
347 emergency response relating to reactive species clearance and further mechanisms of cell
348 repair provides an explanation of cell survival under plasma stress and offers promising
349 insights for optimising ACP applications.

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353 **Conflict of Interest**

354 There is no conflict of interest to declare.

355

356

357 **References:**

358 Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M.,
359 Wanner, B.L. and Mori, H. (2006) Construction of *Escherichia coli* K‐12 in‐frame, single‐gene
360 knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 1-11.
361 Blanchard, J.L., Wholey, W.-Y., Conlon, E.M. and Pomposiello, P.J. (2007) Rapid changes in gene
362 expression dynamics in response to superoxide reveal SoxRS-dependent and independent
363 transcriptional networks. *PLoS One* **2**, e1186.
364 Boxhammer, V., Li, Y., Köritzer, J., Shimizu, T., Maisch, T., Thomas, H., Schlegel, J., Morfill, G. and
365 Zimmermann, J. (2013) Investigation of the mutagenic potential of cold atmospheric plasma at
366 bactericidal dosages. *MUTAT RES-GEN TOX EN* **753**, 23-28.
367 Boxhammer, V., Morfill, G.E., Jokipii, J.R., Shimizu, T., Klämpfl, T., Li, Y.F., Köritzer, J., Schlegel, J. and
368 Zimmermann, J.L. (2012) Bactericidal action of cold atmospheric plasma in solution. *New J Phys* **14**,
369 113042.
370 Cabiscol, E., Tamarit, J. and Ros, J. (2000) Oxidative stress in bacteria and protein damage by reactive
371 oxygen species. *J Biol Chem* **275**, 27393-27398.
372 Calloni, G., Chen, T., Schermann, S.M., Chang, H.-c., Genevaux, P., Agostini, F., Tartaglia, G.G., Hayer-
373 Hartl, M. and Hartl, F.U. (2012) DnaK functions as a central hub in the *E. coli* chaperone network. *Cell*
374 *reports* **1**, 251-264.
375 Chang, D.E., Smalley, D.J. and Conway, T. (2002) Gene expression profiling of *Escherichia coli* growth
376 transitions: an expanded stringent response model. *Mol Microbiol* **45**, 289-306.
377 Cheville, A.M., Arnold, K.W., Buchrieser, C., Cheng, C.M. and Kaspar, C.W. (1996) RpoS regulation of
378 acid, heat, and salt tolerance in *Escherichia coli* O157: H7. *Applied and Environmental Microbiology*
379 **62**, 1822-1824.
380 Christman, M.F., Storz, G. and Ames, B.N. (1989) OxyR, a positive regulator of hydrogen peroxide-
381 inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of
382 bacterial regulatory proteins. *Proc Natl Acad Sci* **86**, 3484-3488.
383 Cui, N.-Y. and Brown, N. (2002) Modification of the surface properties of a polypropylene (PP) film
384 using an air dielectric barrier discharge plasma. *Appl Surf Sci* **189**, 31-38.
385 Cullen, P.J., Misra, N., Han, L., Bourke, P., Keener, K., O'Donnell, C., Moiseev, T., Mosnier, J.P. and
386 Milosavljevic, V. (2014) Inducing a Dielectric Barrier Discharge Plasma Within a Package. *IEEE Trans*
387 *Plasma Sci* **42**, 2368-2369.
388 Datsenko, K.A. and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia*
389 *coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences* **97**, 6640-6645.
390 Delaney, J.M. (1990) Requirement of the *Escherichia coli dnaK* gene for thermotolerance and
391 protection against H_2O_2. *J Gen Microbiol* **136**, 2113-2118.
392 Demple, B. (1996) Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress
393 regulon—a review. *Gene* **179**, 53-57.
394 Ding, H. and Demple, B. (2000) Direct nitric oxide signal transduction via nitrosylation of iron-sulfur
395 centers in the SoxR transcription activator. *Proc Natl Acad Sci* **97**, 5146-5150.
396 Dobrynin, D., Fridman, G., Friedman, G. and Fridman, A. (2009) Physical and biological mechanisms
397 of direct plasma interaction with living tissue. *New J Phys* **11**, 115020.
398 Dobrynin, D., Friedman, G., Fridman, A. and Starikovskiy, A. (2011) Inactivation of bacteria using dc
399 corona discharge: role of ions and humidity. *New J Phys* **13**, 103033.
400 Fridman, A.A. (2008) *Plasma chemistry*. Cambridge ; New York: Cambridge University Press.
401 Fridman, G., Brooks, A.D., Balasubramanian, M., Fridman, A., Gutsol, A., Vasilets, V.N., Ayan, H. and
402 Friedman, G. (2007) Comparison of direct and indirect effects of non- thermal atmospheric- pressure
403 plasma on bacteria. *Plasma Process Polym* **4**, 370-375.

404 Fridman, G., Peddinghaus, M., Balasubramanian, M., Ayan, H., Fridman, A., Gutsol, A. and Brooks, A.
405 (2006) Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier
406 discharge in air. *Plasma Chem Plasma Process* **26**, 425-442.
407 Gaudu, P., Moon, N. and Weiss, B. (1997) Regulation of the *soxRS* Oxidative Stress Regulon
408 Reversible Oxidation of the Fe-S Centers of SoxR In Vivo. *J Biol Chem* **272**, 5082-5086.
409 Ghomi, H., Safa, N.N., Ramezani, K. and Karimi, M. (2009) Bacterial inactivation by DBD plasma in
410 atmospheric pressure. In *ISPC19*.
411 Gomes, A., Fernandes, E. and Lima, J.L.F.C. (2005) Fluorescence probes used for detection of reactive
412 oxygen species. *J Biochem Biophys Methods* **65**, 45-80.
413 Greenberg, J.T., Monach, P., Chou, J.H., Josephy, P.D. and Demple, B. (1990) Positive control of a
414 global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli.
415 *Proc Natl Acad Sci* **87**, 6181-6185.
416 Han, L., Patil, S., Keener, K.M., Cullen, P.J. and Bourke, P. (2014) Bacterial inactivation by high‐
417 voltage atmospheric cold plasma: influence of process parameters and effects on cell leakage and
418 DNA damage. *J Appl Microbiol*, 784-794.
419 Joshi, S.G., Cooper, M., Yost, A., Paff, M., Ercan, U.K., Fridman, G., Friedman, G., Fridman, A. and
420 Brooks, A.D. (2011) Nonthermal dielectric-barrier discharge plasma-induced inactivation involves
421 oxidative DNA damage and membrane lipid peroxidation in *Escherichia coli. Antimicrob Agents*
422 *Chemother* **55**, 1053-1062.
423 Jung, I.L. and Kim, I.G. (2003) Transcription of ahpC, katG, and katE genes in *Escherichia coli* is
424 regulated by polyamines: polyamine-deficient mutant sensitive to H_2O_2-induced oxidative damage.
425 *Biochem Biophys Res Commun* **301**, 915-922.
426 Kogelschatz, U., Eliasson, B. and Egli, W. (1997) Dielectric-barrier discharges. Principle and
427 applications. *Journal de Physique IV* **7**.
428 Kvam, E., Davis, B., Mondello, F. and Garner, A.L. (2012) Nonthermal atmospheric plasma rapidly
429 disinfects multidrug-resistant microbes by inducing cell surface damage. *Antimicrob Agents*
430 *Chemother* **56**, 2028-2036.
431 Laroussi, M. and Leipold, F. (2004) Evaluation of the roles of reactive species, heat, and UV radiation
432 in the inactivation of bacterial cells by air plasmas at atmospheric pressure. *Int J Mass Spectrom* **233**,
433 81-86.
434 Lazar, S.W. and Kolter, R. (1996) SurA assists the folding of *Escherichia coli* outer membrane
435 proteins. *J Bacteriol* **178**, 1770-1773.
436 Liu, X., Hong, F., Guo, Y., Zhang, J. and Shi, J. (2013) Sterilization of *Staphylococcus Aureus* by an
437 atmospheric non-thermal plasma jet. *Plasma Sci Tech* **15**, 439.
438 Lukes, P., Dolezalova, E., Sisrova, I. and Clupek, M. (2014) Aqueous-phase chemistry and bactericidal
439 effects from an air discharge plasma in contact with water: evidence for the formation of
440 peroxynitrite through a pseudo-second-order post-discharge reaction of H2O2 and HNO2. *Plasma*
441 *Sources Sci Technol* **23**, 015019.
442 Machala, Z., Tarabova, B., Hensel, K., Spetlikova, E., Sikurova, L. and Lukes, P. (2013) Formation of
443 ROS and RNS in Water Electro‐Sprayed through Transient Spark Discharge in Air and their
444 Bactericidal Effects. *Plasma Processes and Polymers* **10**, 649-659.
445 Matsuoka, Y. and Shimizu, K. (2011) Metabolic regulation in Escherichia coli in response to culture
446 environments via global regulators. *Biotechnol J* **6**, 1330-1341.
447 Misra, N.N., Tiwari, B.K., Raghavarao, K.S.M.S. and Cullen, P.J. (2011) Nonthermal plasma
448 inactivation of food-borne pathogens. *IEEE Trans Plasma Sci* **30**, 1409-1415.
449 Moiseev, T., Misra, N., Patil, S., Cullen, P., Bourke, P., Keener, K. and Mosnier, J. (2014) Post-
450 discharge gas composition of a large-gap DBD in humid air by UV–Vis absorption spectroscopy.
451 *Plasma Sources Sci T* **23**, 065033.
452 Mukhopadhyay, S. and Schellhorn, H.E. (1997) Identification and characterization of hydrogen
453 peroxide-sensitive mutants of *Escherichia coli*: genes that require OxyR for expression. *J Bacteriol*
454 **179**, 330-338.

455 Naïtali, M., Kamgang-Youbi, G., Herry, J.-M., Bellon-Fontaine, M.-N. and Brisset, J.-L. (2010)
456 Combined effects of long-living chemical species during microbial inactivation using atmospheric
457 plasma-treated water. *Appl Environ Microbiol* **76**, 7662-7664.
458 Nunoshiba, T., Hidalgo, E., Cuevas, C.F.A. and Demple, B. (1992) Two-stage control of an oxidative
459 stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS*
460 regulatory gene. *J Bacteriol* **174**, 6054-6060.
461 Okubo, M., Kuroki, T., Miyairi, Y. and Yamamoto, T. (2004) Low-temperature soot incineration of
462 diesel particulate filter using remote nonthermal plasma induced by a pulsed barrier discharge. *IEEE*
463 *Trans Ind Appl* **40**, 1504-1512.
464 Patil, S., Valdramidis, V.P., Karatzas, K.A.G., Cullen, P.J. and Bourke, P. (2011) Assessing the microbial
465 oxidative stress mechanism of ozone treatment through the responses of *Escherichia coli* mutants. *J*
466 *Appl Microbiol* **111**, 136-144.
467 Perni, S., Shama, G., Hobman, J., Lund, P., Kershaw, C., Hidalgo-Arroyo, G., Penn, C., Deng, X.T.,
468 Walsh, J.L. and Kong, M.G. (2007) Probing bactericidal mechanisms induced by cold atmospheric
469 plasmas with *Escherichia coli* mutants. *Appl Phys Lett* **90**, 073902.
470 Pomposiello, P.J., Bennik, M.H. and Demple, B. (2001) Genome-Wide Transcriptional Profiling of the
471 *Escherichia coli* Responses to Superoxide Stress and Sodium Salicylate. *J Bacteriol* **183**, 3890-3902.
472 Rockabrand, D., Arthur, T., Korinek, G., Livers, K. and Blum, P. (1995) An essential role for the
473 *Escherichia coli* DnaK protein in starvation-induced thermotolerance, H_2O_2 resistance, and reductive
474 division. *J Bacteriol* **177**, 3695-3703.
475 Sharma, A., Collins, G. and Pruden, A. (2009) Differential gene expression in *Escherichia coli* following
476 exposure to nonthermal atmospheric pressure plasma. *J Appl Microbiol* **107**, 1440-1449.
477 Shigenaga, M.K., Lee, H.H., Blount, B.C., Christen, S., Shigeno, E.T., Yip, H. and Ames, B.N. (1997)
478 Inflammation and NOx-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical
479 detection. *Proc Natl Acad Sci* **94**, 3211-3216.
480 Skowyra, D., Georgopoulos, C. and Zylicz, M. (1990) The *E. coli dnaK* gene product, the hsp70
481 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner.
482 *Cell* **62**, 939-944.
483 Virto, R., Manas, P., Alvarez, I., Condon, S. and Raso, J. (2005) Membrane damage and microbial
484 inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. *Appl*
485 *Environ Microbiol* **71**, 5022-5028.
486 Weber, H., Polen, T., Heuveling, J., Wendisch, V.F. and Hengge, R. (2005) Genome-wide analysis of
487 the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and sigma
488 factor selectivity. *J Bacteriol* **187**, 1591-1603.
489 Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., LaRossa, R.A. and Storz, G. (2001) DNA
490 microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide.
491 *J Bacteriol* **183**, 4562-4570.
492 Ziuzina, D., Patil, S., Cullen, P.J., Keener, K.M. and Bourke, P. (2013) Atmospheric cold plasma
493 inactivation of *Escherichia coli* in liquid media inside a sealed package. *J Appl Microbiol* **114**, 778-787.
494 Ziuzina, D., Patil, S., Cullen, P.J., keener, K.M. and Bourke, P. (2014) Atmospheric cold plasma
495 inactivation of *Escherichia coli, Salmonella enterica* serovar Typhimurium and *Listeria*
496 *monocytogenes* inoculated on fresh produce. *Food microbiol* **42**, 109-116.

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

Microorganisms	**Description**	**Gene information**
E. coli BW 25113	Parent strain	A derivative of the F-, λ-, *E. coli* K-12 strain (Datsenko and Wanner 2000)
E. coli JW 5437	Δ *rpoS*	Regulatory factor, influence on expression of genes for stationary phase or stress response (Cheville et al. 1996; Matsuoka and Shimizu 2011)
E. coli JW 4024	Δ *soxR*	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)
E. coli JW 4023	Δ *soxS*	Triggered by *soxR* under oxidative stress (Pomposiello et al. 2001), and stimulates genes for oxidative and antibiotic resistance (Demple 1996).
E. coli JW 3933	Δ *oxyR*	Required for the induction of a regulon of hydrogen peroxide-inducible genes (Christman et al. 1989)
E. coli JW 0013	Δ *dnaK*	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)

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503 Table 2. In-package ozone concentration after ACP treatment at 70 kV_{RMS} in response to

504 process parameters

Post-treatment storage time	Mode of exposure	Plasma treatment time					
		1min		3min		5min	
		Ozone (ppm)	SD*	Ozone (ppm)	SD*	Ozone (ppm)	SD*
0 h	Direct	1500	200	2600	231	3600	462
	Indirect	1950	191	2750	412	4650	191
1 h	Direct	100	8	180	28	288	55
	Indirect	130	26	258	43	288	63
24 h	Direct	ND*	-	ND*	-	ND*	-
	Indirect	ND*	-	ND*	-	ND*	-

505 ND*: non-detectable

506 SD*: standard deviation

507