

Ultrasound processing of liquid system(s) and its antimicrobial mechanism of action

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1 **Ultrasound processing of liquid system(s)**
2 **and its antimicrobial mechanism of action**

3
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13
14 **Running head:** Ultrasound processing of liquids

15
16 **SIGNIFICANCE AND IMPACT OF THE STUDY**

17 This study looks at the mechanism of action of ultrasound technology for the
18 disinfection of wastewater. Different mutants with deleted genes were used to study
19 the respective sensitivity or resistance to this treatment. This is essential to
20 characterise changes at the molecular level, which might be occurring during
21 treatment, resulting in bacterial adaptation.

22
23 **EXTENDED ABSTRACT**

24 Ultrasound creates cavitation phenomena, resulting in the formation of several free
25 radicals, namely OH• and H•, due to the breakdown of the H₂O molecule. These

26 radicals affect the cellular integrity of the bacteria, causing the inactivation of several
27 processes, and thus it is important to unravel the mechanism of action of this
28 technology. This research looks into the application and mechanism of action of
29 ultrasound technology as a means of disinfection by acoustic cavitation. Sterile water
30 and synthetic waste water were inoculated with different mutants of *E. coli* K12 strains
31 containing deletions in genes affecting specific functional properties of *E. coli*. These
32 were: *dnak soxR*, *soxS*, *oxyR*, *rpoS*, *gadA/gadB*, *gadC* and *yneL*. *E. coli* K-12 Δ *oxyR*,
33 appeared to be more resistant to the treatment together with *gadW*, *gadX*, *gabT* and
34 *gabD*, whereas the mutant K-12 Δ *dnak* was more sensitive with approximately 2.5 log
35 (CFU/mL) reduction in comparison to their isogenic wild type *E. coli* K-12. This
36 indicates that the *dnak* gene participates in general stress response and more
37 specifically to hyperosmotic stress. The other *E. coli* deleted genes tested (*soxS*, *rpoS*,
38 *gadB*, *gadC*, *yneL*) did not appear to be involved in protection of microbial cells against
39 ultrasound.

40

41 **Keywords:** ultrasound, *E. coli* K12, ultrasound, mutant cells, mechanism of action,
42 GABA, GAD system

43

44 INTRODUCTION

45

46 Europe has extensive water resources compared to other regions of the world, and
47 water has long been considered an inexhaustible public commodity. However, this
48 position has been challenged in the last decades by growing water stress, both in terms
49 of water scarcity and water quality deterioration. Indeed, in recent years, approximately
50 half of the European countries, representing almost 70% of the population, have been
51 facing water stress issues (Wintgens *et al.* 2006). Treatment of wastewater, has been
52 a decade long practice for many European countries. Before 2011, most of the raw

53 sewage was discharged back into the sea, without being treated, which is against the
54 current EU Urban Waste Water directive (91/271/EEC). A study published in 2006 by
55 Bixio et al. (2006), summarising the European water reuse practices and set out the
56 map of the water reclamation technologies and reuse applications concluding that
57 almost 70% of the population were facing water stress.

58

59 The quality requirements for wastewater reuse are predominantly oriented towards the
60 planned usage and they are regulated in norms and legal provisions specific to each
61 country. Besides the residual concentration of inorganic nutrients, total suspended
62 solids and dissolved organic matter, the microbiological contamination of wastewater
63 is an important criterion for its safe reuse (Haaken *et al.* 2014). Indeed, several
64 pathogenic microorganisms and parasites are commonly found in domestic
65 wastewater and in effluents from wastewater treatment plants. Three categories of
66 pathogens are encountered in the environment: bacterial pathogens, including
67 indigenous aquatic bacteria, viral pathogens and protozoan parasites. Wastewater
68 bacteria have been characterized and belong to the following groups: Gram-negative
69 facultatively anaerobic bacteria (e.g. *Aeromonas*, *Vibrio*, *Enterobacter*, *Escherichia*,
70 *Klebsiella*, *Shigella*), Gram-negative aerobic bacteria (e.g. *Pseudomonas*, *Alcaligenes*,
71 *Flavobacterium*, *Acinetobacter*), Gram-positive spore forming bacteria (e.g. *Bacillus*
72 spp) and nonspore-forming Gram-positive bacteria (e.g. *Arthrobacter*,
73 *Corynebacterium*, *Rhodococcus*) (Bitton, 2005; Machnicka, 2014). *Escherichia coli* is
74 one of the main indicators for assessing the quality of wastewater.

75

76 As of recently, the application of ultrasonic technology has received wide attention in
77 water and wastewater treatment and environmental remediation areas, including the
78 application for disinfection purposes (Chen, 2012; Han *et al.*, 2013; Cesaro and
79 Belgiorno, 2016). Ultrasound generates elastic vibrations and waves whose frequency
80 is over 15-20 kHz. Whilst ultrasound can stimulate the activity and growth of

81 microorganisms at low intensities and small influence durations, at greater intensities
82 it kills and inactivates microorganisms. Long term water treatment by ultrasound of 20
83 – 100 kHz with a sound intensity of between 10 and 1,000 W/cm² can achieve
84 disinfection (Vasilyak 2011).

85

86 The disinfection capacity of sonication in water is due to the phenomenon of acoustic
87 cavitation, which is the formation and collapse of micro-bubbles occurring in
88 milliseconds, producing extreme temperature and pressure gradients (Drakopoulou *et*
89 *al.* 2009; Sango *et al.* 2014). Indeed, the collapse of these micro-bubbles leads to
90 extremely high local temperatures and pressures. These conditions have shown to
91 result in the generation of highly reactive radicals. Ultrasound is therefore able to
92 inactivate bacteria and de-agglomerate bacterial clusters through a number of
93 physical, mechanical, and chemical effects caused by acoustic cavitation (Antoniadis
94 *et al.* 2007; Broekman *et al.* 2010; Vasilyak, 2011). Nevertheless, to the knowledge of
95 the authors, there are no studies focusing on identifying the major effects of sonication
96 stress, and particularly the characterisation of mechanisms of microbiological
97 responses of wastewater microorganisms under ultrasound treatment. Several similar
98 studies on the mode of action has been carried out on other novel disinfection
99 technologies such as plasma, ozone and nanomaterials (Laroussi 1996; Mahapatra *et*
100 *al.* 2005; Perni *et al.* 2007; Nath *et al.* 2014). Unravelling the mode of action of
101 ultrasound would be essential for fully understanding the microbial responses of *E. coli*
102 and thus its efficient use in industrial applications.

103

104 The aim of this study is to assess the antimicrobial mechanisms of action of ultrasound
105 on *E. coli* by performing a comparative study between wild type bacteria and selected
106 mutants that have important general stress tolerance genes deleted. The outcome
107 aims to address the role of several knock-out genes in the protection or sensitivity
108 against ultrasound generated radicals.

109 RESULTS AND DISCUSSION

110

111 In this experiment, the medium effect on free radical formation during ultrasound
112 treatments was studied. Results indicate that the only significant difference between
113 the different media was observed in the *dnaK* mutant. It should be emphasized that in
114 this case, the *dnaK* mutant was mostly affected by temperature. Table 2 illustrates the
115 behaviour of all the mutant strains in comparison to their isogenic wild type *E. coli* K-
116 12. It appears clearly that the mutant $\Delta oxyR$ was more resistant to the treatment
117 (reduction of 0.60 log) whereas $\Delta dnaK$ was nearly as sensitive as the wild type after 3
118 minutes of continuous treatment, even though temperature was controlled. For all
119 other mutants, the reduction was similar to that of *E. coli* K-12 wild type. On average,
120 most of the mutants, similarly to the wild type, showed a 1 log reduction.

121 The temperature profiles obtained show that from the three different treatments, all
122 showed a significant difference on the heating rate between the three different set-ups.
123 The controlled temperature treatment resulted in 0.1029°C/s and a final temperature
124 39.5°C, non-temperature controlled treatment with a heating rate of 0.2008°C/s and a
125 final temperature of 58.3°C and with just cold water 0.1209°C/s with a maximum
126 temperature of 44.5°C. Thus, it is evident that in some of the mutants, the log reduction
127 observed, is related to ultrasound activity rather than the temperature as shown in table
128 2. In fact, according to Patil et al. (2011), the *soxR*, *soxS*, *oxyR*, *rpoS* and *dnaK* genes
129 have been reported to play an important role in the protection against reactive oxygen
130 radicals. As explained previously, one of the phenomena induced by cavitation is the
131 formation of radicals H• and OH• and of H₂O₂ (Joyce et al. 2003), which are known to
132 provoke oxidative stress in bacteria. The experimental results show that not all mutants
133 were affected in the same way by the ultrasonic treatment.

134

135 Two of the most affected mutants were found to be $\Delta oxyR$ and $\Delta dnaK$ (temperature)
136 mutants. The OxyR subunit of RNA polymerase is the master regulator of hydrogen

137 peroxide genes in *E. coli* as it positively regulates the production of surface proteins
138 that control the colony morphology and auto-aggregation ability. The DnaK protein is,
139 among other, essential for growth at high temperatures and plays a role in the
140 regulation of the heat shock response. The heat shock response is an inducible cellular
141 response to a variety of stresses such as heat, exposure to ethanol, oxidants, and
142 DNA-damaging agents, production of abnormal proteins, viral infections, and
143 starvation for nutrients (Bukau and Walker 1989). The deletion of the *dnaK* gene can
144 explain the sensitivity of the corresponding mutant was particularly sensitive to heat in
145 the ultrasound experiments where the temperature during the treatment was not
146 controlled. It can also be an explanation to the fact that this mutant which was more
147 sensitive to the ultrasonic treatment than the K-12 wild type of *E. coli*, as ultrasounds
148 lead to an oxidative stress on bacteria. Deletion of *dnaK* resulted in a sensitive
149 phenotype, to ultrasound, although the bacterial populations were not completely
150 inactivated with the applied treatment. This *dnaK* gene would therefore play a role in
151 the protection against ultrasound treatment of the bacteria.

152

153 Under the conditions tested, the mutant K-12 $\Delta oxyR$ appeared to be more resistant to
154 the treatment whereas the K-12 $\Delta dnaK$ was more sensitive in comparison with the wild
155 type strain (Table 2). The *dnaK* would therefore play a role in the protection against
156 ultrasound treatment of the bacteria, and the corresponding mutant also shows a great
157 sensitivity to the heat generated during the ultrasonic treatment. An interesting
158 observation that needs to be noted is that involving $\Delta oxyR$. The *oxyR* controls the
159 expression of a set of genes that constitute the *oxyR* regulon. The OxyR protein is
160 produced constitutively and is oxidized by H₂O₂. The oxidized form of OxyR binds to
161 promoter regions of target genes and activates transcription by protein–protein contact
162 with RNA polymerase. The OxyR-activated genes have direct and indirect antioxidant
163 functions in the defence of the cell, such as removal of H₂O₂ by catalase and the
164 protection of DNA from oxidative attack by the Dps protein (Pomposiello and Demple

165 2001). The current results show that this mutant was more resistant to ultrasound
166 indicating that the produced H₂O₂ during ultrasound treatments is not stable.

167

168 Furthermore, we also assessed mutants in genes associated with the GAD system
169 (Table 2) and found a possible role in ultrasound treatment. The GAD system is known
170 to play an important role in acid tolerance of bacteria (Smith *et al.* 1992, C. Feehily and
171 Karatzas 2013; Paudyal and Karatzas 2016) but it has been shown to play a role in
172 oxidative stress only in *Saccharomyces cerevisiae* (Coleman *et al.* 2001) and
173 *Francisella tularensis* (Ramond *et al.* 2014) but not in other organisms. This is the first
174 report showing a possible role for the GAD system in oxidative stress in *E. coli*. Here
175 we show that absence of the decarboxylase *gadB* did not affect survival (Table 2).
176 However, absence of *gadA* and *gadC* resulted in sensitivity when treatment occurred
177 without cold water and in resistance in synthetic wastewater. This might suggest a
178 differential role of the GAD system in different temperatures/conditions, or the
179 upregulation of alternative mechanisms that protect against oxidative stress under
180 specific conditions (e.g. synthetic wastewater).

181

182 We also assessed the role of other genes associated with the regulation of the GAD
183 system and the GABA shunt. Deletion of the GAD system regulators *gadW*, *gadX*, (
184 Tramonti *et al.* 2006; Sayed *et al.* 2007) resulted in resistance to ultrasound in sterile
185 water strengthening the role of the GAD system in oxidative stress. Similarly deletion
186 of *gabT* and *gabD* that encode for the GABA shunt that catabolise intracellular GABA
187 pools produced by the intracellular GAD system (Feehily *et al.* 2013), resulted in
188 resistance in sterile water but not in wastewater. It has been suggested that as the
189 GAD system coupled with the GABA shunt feed into the TCA cycle affecting the levels
190 of succinate and oxoglutarate that have anti-oxidant properties and can confer
191 resistance to oxidant species (Ramond *et al.* 2014) that might be produced during

192 ultrasound treatment. However, further work is required to identify the above
193 hypothesis and other possible links between the GAD system and oxidative stress.

194

195 In conclusion, this research looked into the application and mechanism of action of
196 ultrasound technology as a means of disinfection by acoustic cavitation. Sterile water
197 and synthetic waste water were inoculated with different mutants of *E. coli* K12 strains
198 containing deletions in genes affecting specific functional properties of *E. coli*. *E. coli*
199 K-12 $\Delta oxyR$, appeared to be more resistant to the treatment together with *gadW*, *gadX*,
200 *gabT* and *gabD*, whereas the mutant K-12 $\Delta dnaK$ was more sensitive with
201 approximately 2.5 log (CFU/mL) reduction in comparison to their isogenic wild type *E.*
202 *coli* K-12. This indicated that the *dnaK* gene participates in general stress response
203 and more specifically to hyperosmotic stress. The other *E. coli* deleted genes tested
204 (*soxS*, *rpoS*, *gadB*, *gadC*, *yneL*) did not appear to be involved in protection of microbial
205 cells against ultrasound. Furthermore, we also showed for the first time here a possible
206 role of the GAD system in ultrasound treatment and oxidative stress that requires
207 further investigation, as these have shown that they are essentially crucial in the
208 protection from oxidative stress.

209

210 In the context of the wastewater recycling and reuse, the aim is to find a treatment
211 ensuring to remove or significantly reduce all the pathogens to minimize contamination
212 of the receiving waters and to provide public health protection. Ultrasound treatments
213 can be a potential technology for this type of treatments.

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220 **Materials and Methods**

221

222 **Bacterial strains and preparation of inoculum**

223

224 In this study, the bacterial strains used were *E. coli* K-12 wild type, and its isogenic
225 mutants $\Delta dnaK$, $\Delta soxS$, $\Delta soxR$, $\Delta oxyR$, $\Delta rpoS$, $\Delta gadA$ (Jkl 3485), $\Delta gadB$ (Jkl 1488)
226 $\Delta gadC$ (Jkl 1487) and $\Delta yneL$ (Jkl 5247), all obtained from the National Bio-Resource
227 Project, Japan (NIG, Japan). A description of the mutants and their proteins' functions
228 is given in Table 1.

229 The pure cultures of strains were stored in vials at -80°C . Before any experiment, pure
230 cultures with isolated colonies were prepared. Under aseptic conditions, a loop from
231 the frozen vial was streaked on Tryptone Soya Agar (TSA; Oxoid, United Kingdom)
232 plates for *E. coli*. Following overnight incubation at 37°C , these pure culture plates
233 were stored at 5°C , and kept for 3 to 4 weeks the most until further use.

234 Experiments were performed in two types of liquid systems: (i) sterile water (SW) and
235 (ii) synthetic wastewater (SyW). The working solution to be treated was prepared by
236 diluting 2 mL of the working culture in 298 mL in SW or SyW in a 500 mL sterile beaker.
237 The SyW was prepared as described by Antionadis et al. (2007) and Ayyildiz et al.
238 (2011), i.e., peptone 64.0g/L; Meat Extract 44.0g/L; Urea 12.0g/L; K_2HPO_4 11.2g/L;
239 NaCl 2.8g/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.6g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.8g/L).

240

241 **Ultrasound treatments**

242 The inoculated solution was transferred to a jacketed beaker, which was used to pass
243 cold water, to avoid temperature increase during ultrasound. The ultrasonic equipment
244 used was a UP200St (Hielscher, Germany) comprising an ultrasonic generator
245 UP200St-G (200 W, frequency 26 kHz), and a transducer UP200St-T that could be

246 integrated in a sound protection box. A temperature probe was connected to the
247 transducer and measured the temperature of the solution throughout the ultrasonic
248 treatment and that temperature profile was recorded on an integrated SD/USB
249 ComboCard. A 14 mm diameter sonotrode was used, and placed 2 cm deep in the
250 solution to be treated and was carefully cleaned between experiments with 70%
251 ethanol.

252

253 The first series of treatments were carried out applying an ultrasound treatment to the
254 working solutions of bacteria during 3 minutes in continuous mode, for all *E. coli* strains
255 using three conditions: (i) controlled temperature I (US-TI): Beaker was surrounded by
256 a cold water bath to keep the temperature lower than 45°C; (ii) non controlled
257 temperature (US): Beaker was not placed in cold water bath in order to study the effect
258 of ultrasound in combination with the generated heat; (iii) Controlled temperature II
259 (US-TII): SyW was placed in a jacketed beaker, which was used to control the
260 temperature preventing it from increasing above 37°C.

261 **Statistical analysis**

262 An F-test with 99.9% confidence level was used to check significance, within different
263 treatments, whilst a Bonferroni test correction was carried out to assess the
264 significance between each mutant.

265

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270

271 **Conflict of interest**

272 No conflict of interest declared.

273

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375 **Table 1: Information on the *E. coli* (strain K12) genes deleted for the mutants studied**
 376 **(adapted from (Patil *et al.* 2011); UniProt, 2014)**
 377

Gene	Protein encoded	Protein functions
<i>dnaK</i>	Chaperone protein DnaK	Essential role in the initiation of phage lambda DNA replication; involved in chromosomal DNA replication; participates actively in the response to hyperosmotic shock.
<i>soxR</i>	Redox-sensitive transcriptional activator SoxR	Activates the transcription of the <i>soxS</i> gene which itself controls the superoxide response regulons; contains a 2Fe-2S iron-sulfur cluster that may act as a redox sensor system that recognizes superoxide, the variable redox state of the Fe-S cluster is employed <i>in</i>

		<i>vivo</i> to modulate the transcriptional activity of SoxR in response to specific types of oxidative stress.
<i>soxS</i>	Regulatory protein SoxS	Transcriptional activator of the superoxide response regulon of <i>E.coli</i> that includes at least 10 genes such as <i>sodA</i> , <i>nfo</i> , <i>zwf</i> and <i>micF</i> ; facilitates the subsequent binding of RNA polymerase to the <i>micF</i> and the <i>nfo</i> promoters.
<i>oxyR</i>	Hydrogen peroxide-inducible genes activator	Hydrogen peroxide sensor; activates the expression of a regulon of hydrogen peroxide-inducible genes; positive regulatory effect on the production of surface proteins that control the colony morphology and auto-aggregation ability
<i>rpoS</i>	RNA polymerase sigma factor RpoS	Master transcriptional regulator of the stationary phase and the general stress response; controls positively or negatively the expression of several hundred genes which are mainly involved in metabolism, transport, regulation and stress management
<i>gadA</i> <i>gadB</i>	Glutamate decarboxylase alpha Glutamate decarboxylase beta	Convert glutamate to gamma-aminobutyrate (GABA); the <i>gad</i> system helps to maintain a near-neutral intracellular pH when cells are exposed to extremely acidic conditions.
<i>gadC</i>	Probable glutamate/gamma-aminobutyrate antiporter	Involved in glutamate-dependent acid resistance; imports glutamate inside the cell while simultaneously exporting to the periplasm the GABA produced by GadA and GadB.

Putative HTH-type
yneL transcriptional regulator YneL
 A predicted transcriptional regulator which controls the conversion of DNA to RNA and the gene activity.

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Table 2: Microbial log reduction of studied *E. coli* mutants under both controlled and freely increasing temperature. W: sterile distilled water, SyW: Sterile synthetic water. The values followed by the same letter, are not statistically significant within each row.

Strain	Log Reduction		
	US with cold water (W)	US without cold water (W)	Temperature controlled US (SyW)
<i>K-12 wild type</i>	1.67±0.05 ^a	2.50±0.32 ^a	0.81±0.29 ^a
Δ gadA	1.53±0.17 ^a	3.00±0.14 ^b	0.83±0.18 ^a
Δ gadB	1.64±0.06 ^a	2.49±0.40 ^{abc}	1.29±0.29 ^{ab}
Δ gadC	1.77±0.06 ^a	3.33±0.57 ^{abcd}	0.87±0.20 ^a
Δ gadW	0.51±0.08 ^b	0.68±0.07 ^e	1.27±0.03 ^b
Δ gadX	0.29±0.08 ^b	0.68±0.09 ^e	0.85±0.17 ^a
Δ gabT	0.69±0.07 ^c	0.52±0.04 ^{ef}	0.75±0.00 ^a
Δ gabD	0.79±0.07 ^c	0.52±0.02 ^{ef}	1.33±0.32 ^{ab}
Δ rpoS	1.53±0.12 ^a	2.18±0.40 ^a	1.42±0.34 ^{ab}
Δ dnaK	2.11±0.20 ^d	5.42±0.18 ^h	0.98±0.10 ^a
Δ soxS	1.80±0.13 ^{ad}	2.24±0.22 ^{ac}	1.02±0.38 ^{ab}
Δ soxR	1.85±0.18 ^{ad}	3.52±0.27 ^d	1.56±0.53 ^{ab}
Δ oxyR	0.60±0.38 ^{bc}	0.83±0.05 ^e	0.42±0.08 ^c
Δ yneL	1.78±0.12 ^{ad}	2.97±0.15 ^{abcd}	1.22±0.35 ^{ab}

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