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

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SIGNIFICANCE AND IMPACT OF THE STUDY

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

EXTENDED ABSTRACT

Ultrasound creates cavitation phenomena, resulting in the formation of several free radicals, namely OH• and H•, due to the breakdown of the H_2O molecule. These

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

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

INTRODUCTION

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

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

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

As of recently, the application of ultrasonic technology has received wide attention in water and wastewater treatment and environmental remediation areas, including the application for disinfection purposes (Chen, 2012; Han *et al.*, 2013; Cesaro and Belgiorno, 2016). Ultrasound generates elastic vibrations and waves whose frequency 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/cm^2 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.

RESULTS AND DISCUSSION

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

The temperature profiles obtained show that from the three different treatments, all showed a significant difference on the heating rate between the three different set-ups. The controlled temperature treatment resulted in 0.1029°C/s and a final temperature 39.5°C, non-temperature controlled treatment with a heating rate of 0.2008°C/s and a final temperature of 58.3°C and with just cold water 0.1209°C/s with a maximum temperature of 44.5°C. Thus, it is evident that in some of the mutants, the log reduction observed, is related to ultrasound activity rather than the temperature as shown in table 2. In fact, according to Patil et al. (2011), the *soxR*, *soxS*, *oxyR*, *rpoS* and *dnaK* genes have been reported to play an important role in the protection against reactive oxygen radicals. As explained previously, one of the phenomena induced by cavitation is the formation of radicals $H^{\bullet}$ and $OH^{\bullet}$ and of H_2O_2 (Joyce *et al*. 2003), which are known to provoke oxidative stress in bacteria. The experimental results show that not all mutants were affected in the same way by the ultrasonic treatment.

Two of the most affected mutants were found to be Δ*oxyR* and Δ*dnaK* (temperature) mutants. The OxyR subunit of RNA polymerase is the master regulator of hydrogen

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

Under the conditions tested, the mutant K-12 Δ*oxyR* appeared to be more resistant to the treatment whereas the K-12 Δ*dnaK* was more sensitive in comparison with the wild type strain (Table 2). The *dnaK* would therefore play a role in the protection against ultrasound treatment of the bacteria, and the corresponding mutant also shows a great sensitivity to the heat generated during the ultrasonic treatment. An interesting observation that needs to be noted is that involving Δ*oxyR*. The *oxyR* controls the expression of a set of genes that constitute the *oxyR* regulon. The OxyR protein is produced constitutively and is oxidized by H_2O_2. The oxidized form of OxyR binds to promoter regions of target genes and activates transcription by protein–protein contact with RNA polymerase. The OxyR-activated genes have direct and indirect antioxidant functions in the defence of the cell, such as removal of H_2O_2 by catalase and the protection of DNA from oxidative attack by the Dps protein (Pomposiello and Demple

2001). The current results show that this mutant was more resistant to ultrasound indicating that the produced H_2O_2 during ultrasound treatments is not stable.

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

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

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

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

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

Materials and Methods

Bacterial strains and preparation of inoculum

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

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

Experiments were performed in two types of liquid systems: (i) sterile water (SW) and (ii) synthetic wastewater (SyW). The working solution to be treated was prepared by diluting 2 mL of the working culture in 298 mL in SW or SyW in a 500 mL sterile beaker. The SyW was prepared as described by Antionadis et al. (2007) and Ayyildiz et al. (2011), i.e., peptone 64.0g/L; Meat Extract 44.0g/L; Urea 12.0g/L; K_2HPO_4 11.2g/L; NaCl 2.8g/L; $CaCl_2.2H_2O$ 1.6g/L; $MgSO_4.7H_2O$ 0.8g/L).

Ultrasound treatments

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

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

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

Statistical analysis

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

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Conflict of interest

No conflict of interest declared.

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

Gene	Protein encoded	Protein functions
dnaK	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.
soxR	Redox-sensitive transcriptional activator SoxR	Activates the transcription of the *soxS* 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 *in*

		vivo to modulate the transcriptional activity of SoxR in response to specific types of oxidative stress.
soxS	Regulatory protein SoxS	Transcriptional activator of the superoxide response regulon of *E.coli* that includes at least 10 genes such as *sodA*, *nfo*, *zwf* and *micF*; facilitates the subsequent binding of RNA polymerase to the *micF* and the *nfo* promoters.
oxyR	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
rpoS	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
gadA *gadB*	Glutamate decarboxylase alpha Glutamate decarboxylase beta	Convert glutamate to gamma-aminobutyrate (GABA); the gad system helps to maintain a near-neutral intracellular pH when cells are exposed to extremely acidic conditions.
gadC	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.

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

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)
K-12 wild type	1.67±0.05^{a}	2.50±0.32^{a}	0.81±0.29^{a}
ΔgadA	1.53±0.17^{a}	3.00±0.14^{b}	0.83±0.18^{a}
ΔgadB	1.64±0.06^{a}	2.49±0.40^{abc}	1.29±0.29^{ab}
ΔgadC	1.77±0.06^{a}	3.33±0.57^{abcd}	0.87±0.20^{a}
ΔgadW	0.51±0.08^{b}	0.68±0.07^{e}	1.27±0.03^{b}
ΔgadX	0.29±0.08^{b}	0.68±0.09^{e}	0.85±0.17^{a}
ΔgabT	0.69±0.07^{c}	0.52±0.04^{ef}	0.75±0.00^{a}
ΔgabD	0.79±0.07^{c}	0.52±0.02^{ef}	1.33±0.32^{ab}
ΔrpoS	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}
ΔsoxR	1.85±0.18^{ad}	3.52±0.27^{d}	1.56±0.53^{ab}
ΔoxyR	0.60±0.38^{bc}	0.83±0.05^{e}	0.42±0.08^{c}
ΔyneL	1.78±0.12^{ad}	2.97±0.15^{abcd}	1.22±0.35^{ab}

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