UNRAVELLING THE ANTIMICROBIAL MECHANISM OF ULTRASOUND WASTEWATER TREATMENT

SPITERI D.[1], CHOT-PLASSOT C.[1], KARATZAS K.A.[2], SCERRI C.[3] and VALDRAMIDIS V.[1]

[1] Department of Food Studies and Environmental Health, University of Malta, [2] Department of Food and Nutritional Science, University of Reading, [3] Department of Physiology and Biochemistry, University of Malta
E-mail: vasilis.valdramidis@um.edu.mt

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 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 gene deletions related with genes affecting specific functional properties to *E. coli*. These were: dnak *soxR, soxS, oxyR, rpoS, gadA/gadB, gadC* and *yneL*. *E. coli* K-12 Δ*oxyR* , appears to be more resistant to the treatment, whereas the mutant K-12 Δ*dnaK* was more sensitive with approximately 2.5 log reduction in comparison with the wild type strain of *E. coli* K-12. This is due to the fact that *dnaK* gene participates in the response to general stress especially more to hyperosmotic stress, which could be due to the result of an increased concentration of free radicals forming around the cell. These free radicals could facilitate cellular membrane disruption, inactivating the normal process of the cell. The other *E. coli* deleted genes tested (*soxR*, *soxS*, *rpoS, gadA, gadB, gadC, yneL*) didn't seem to be involved in protection against ultrasound.

Keywords: ultrasound, *E.coli* K12, Δ*oxyR*, Δ*dnaK*, Δ*soxR*, Δ*soxS*, Δ*rpoS,* Δ*gadA,* Δ*gadB,* Δ*gadC,* Δ*yneL*

1. Introduction

Europe has extensive water resources compared to other regions of the world, and water has long been considered as an inexhaustible public commodity. Governments have long pondered on the idea of recycling and reusing wastewater to provide an additional dependable water resource, whilst also reducing the environmental impacts.

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 current EU Urban Waste Water directive (91/271/EEC). Thus European countries have a long history in water management, particularly in the treatment of wastewater. A study published in 2006 by Bixio *et al.*, summarises the European water reuse practices and sets out the map of the water reclamation technologies and reuse applications. The study mentions that almost 70% of the population are facing water stress.

Most European countries use chlorination as the main method of disinfection, as it destroys target organisms by oxidising cellular material (EPA, 1999). Chlorine can easily be produced and is relatively cheap, while also is highly effective in killing pathogens (Lenntech, 2014). However the Netherlands is the only EU country that limits the use of chlorine for water treatment (Lenntech, 2014). The United States uses another similar alternative, chloramine, which like chlorine also provides a suitable residual protection in drinking water.

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). Ultrasound generates elastic vibrations and waves whose frequency is over 15-20 kHz. Whilst ultrasound can stimulate the activity and growth of microorganisms at low intensities and small influence durations, at greater intensities it depresses and destroys microorganisms. Long term water treatment by ultrasound of high power leads therefore to disinfection (Vasilyak, 2010).

The disinfection capacity of sonication in water is due to the phenomenon of acoustic cavitation, which is the formation and collapse of micro-bubbles occurring in milliseconds and producing extreme temperature and pressure gradients (Drakopoulou *et al*., 2009). Indeed, the collapse of these micro-bubbles leads to extremely high local temperatures and pressures. These conditions have shown to result in the generation of highly reactive radicals. Ultrasound is therefore able to inactivate bacteria and de-agglomerate bacterial clusters through a number of physical, mechanical, and chemical effects caused by acoustic cavitation (Antoniadis *et al*., 2007).

The objectives of this study are two-fold:

- To unravel the antimicrobial mechanisms of ultrasound by studying the responses of *E.coli* mutant strains;
- To assess the inactivation of the bacteria mutants using different liquid media, i.e., sterile distilled water and synthetic wastewater.

2. Material and Methods

2.1. Bacterial strains and cultural conditions

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.

Table 1: Information on the *E. coli* (strain K12) genes deleted for the mutants studied (adapted from 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.

The pure cultures of strains were stored in vials at -80°C in a freezer. 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) 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.

2.2. Ultrasound treatments

The ultrasonic equipment used in this study is the device UP200St from Hielscher. It is composed of an ultrasonic generator UP200St-G (200 W, frequency 26 kHz), and a transducer UP200St-T that can be integrated in a sound protection box. A temperature probe is linked to the transducer and measures the temperature of the solution treated throughout the ultrasonic treatment, this temperature profile is recorded on an integrated SD/USB ComboCard. Two modes of treatment exist: the continuous mode and the pulsed mode, i.e., regular alternation between on and off mode.

The working solution to be treated was prepared by diluting 2 mL of the working culture in 298 mL of sterile water or synthetic wastewater in a 500 mL sterile beaker. The synthetic wastewater was prepared as described in 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). The solution was later transferred to a jacketed beaker, which was used to pass cold water, to avoid temperature increase during ultrasound. It was prepared in the same way for all the mutants of the study. In each case, a 14 mm diameter sonotrode was used, and placed 2 cm deep in the solution to be treated. The sonotrode was carefully cleaned between each experiment. Decimal dilutions in Ringer's solution and plating were determined before and after each treatment in order to calculate the microbial levels (in CFU/mL) and thus establishing the microbial reduction.

The first series of treatments consisted in applying an ultrasound treatment to the working solutions of bacteria during 3 minutes in continuous mode, for all the mutants of *E. coli* mentioned previously using three conditions: 1) Controlled temperature I: Beaker is surrounded by a cold water bath to keep the temperature lower than 45°C; 2) Without controlled temperature: Beaker was not placed in cold water bath in order to study the effect of heat generated by ultrasound; 3) Controlled temperature II: Synthestic waste water was placed in a Jacketed beaker, which was used to control the temperature and thus not allow it to increase more than 37°C.

3. Results

Figure 1 shows the log microbial levels of three different *E. coli* strains under similar conditions but different medium. In this experiment the medium effect on free radical formation during ultrasound treatments has been studied. Results indicate that the only significant difference was observed in the *dnaK* mutant. It must be emphasized that in this case the *dnaK* mutant is mostly affected by temperature. This is further shown in Figure 2, which illustrates the behaviour of all the mutant strains. The mutants are compared to the wild type strain of *E. coli* K-12. It appears clearly that the mutant K-12 Δ*oxyR* seems more resistant to the treatment (reduction of 0.60 log) whereas the mutant K-12 Δ*dnaK* looks nearly as sensitive as the wild type after 3 minutes of

continuous treatment, even though temperature was controlled. For all the other mutants a reduction similar to the reduction obtained for *E. coli* K-12 wild type is observed. On average most of the mutants, similarly to the wild type, showed a 1 log reduction. This easily concludes that the a general reduction due to ultrasound treatment may occur, due to cavitation.

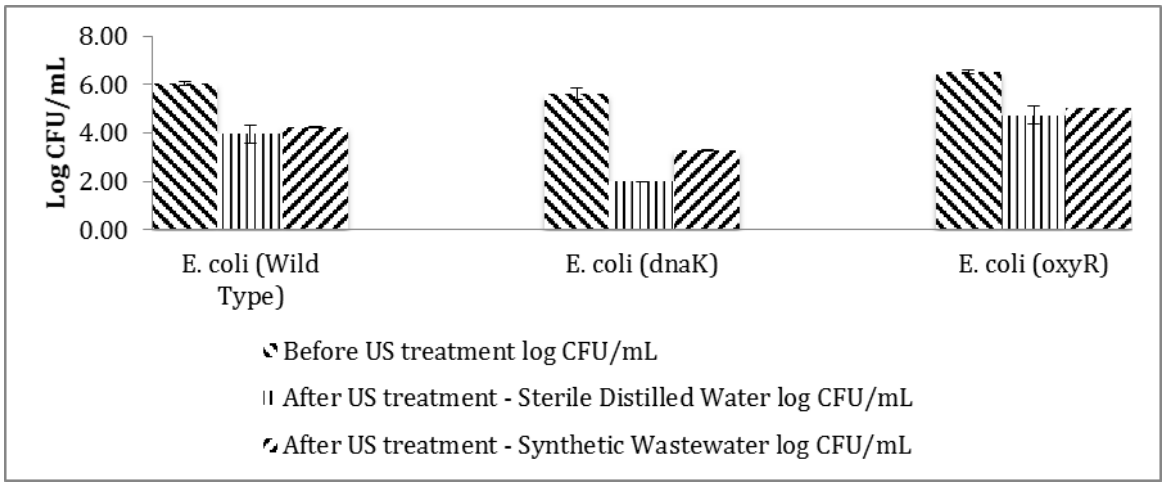

Figure 1: Graph showing difference between synthetic wastewater and sterile distilled water

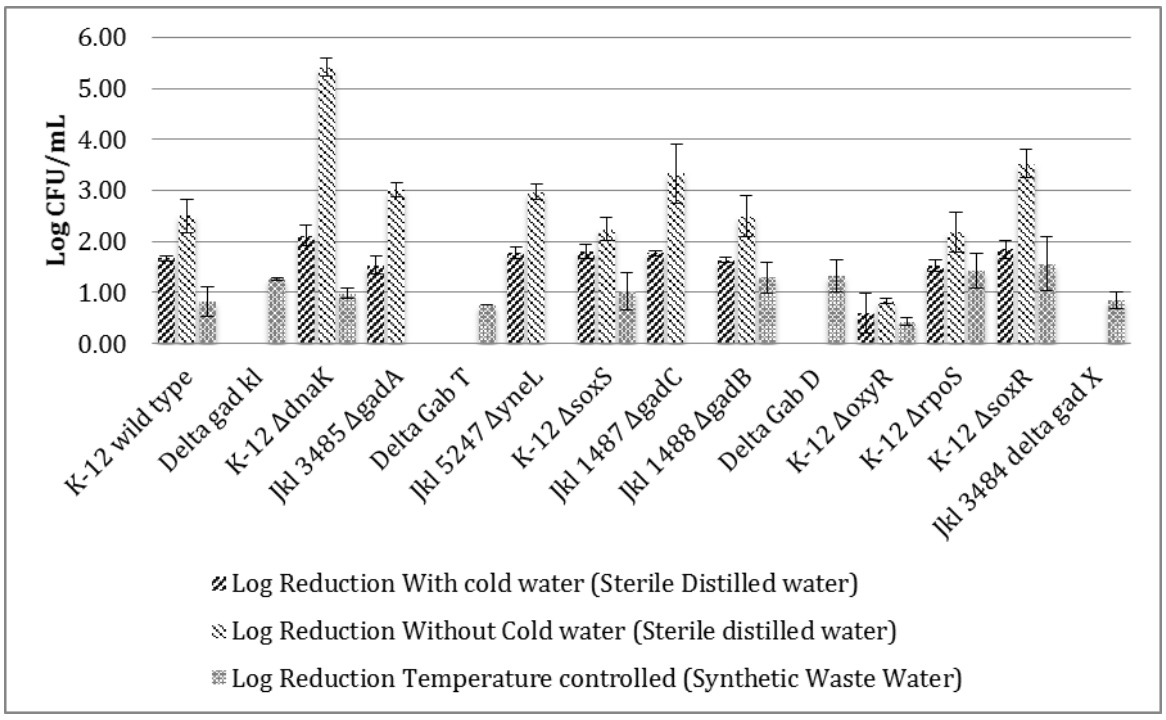

Figure 2: Graph showing log reduction of different mutants under both controlled and freely increasing temperature

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 on bacteria. The experimental results show that all mutants were not affected in the same way by the ultrasonic treatment. Under the conditions tested, the mutant K-12 Δ*oxyR* appeared to be more resistant to the treatment whereas the mutant K-12 Δ*dnaK* was more sensitive in comparison with the wild type strain of *E. coli* K-12. The *dnaK* gene would therefore play a role in the protection against

ultrasound treatment of the bacteria, and the mutant with a deletion in this gene also show a great sensitivity to the heat generated during the ultrasonic treatment. An interesting observation that needs to be noted is that involving the mutant with a deletion in the *oxyR* gene. The *oxyR* gene 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 defense 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 & 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.

Two of the most affected strains were found to be Δ*rpoS* (general stress) and Δ*dnaK* (temperature) mutants. The RpoS subunit of RNA polymerase is the master regulator of the general stress response in *E. coli* as it positively regulates approximately 500 genes, i.e. about 10% of the genes of the *E. coli* genome. It is essential for survival in stationary phase as well as under a variety of stress conditions (Hengge, 2009). The DnaK protein is, among other, essential for growth of the cells 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 & Walker, 1989). The deletion of the *dnaK* gene in the K-12 Δ*dnaK* mutant can explain the fact that this 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 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. Without the *dnaK* gene, the bacteria were more sensitive 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 and cavitation effects.

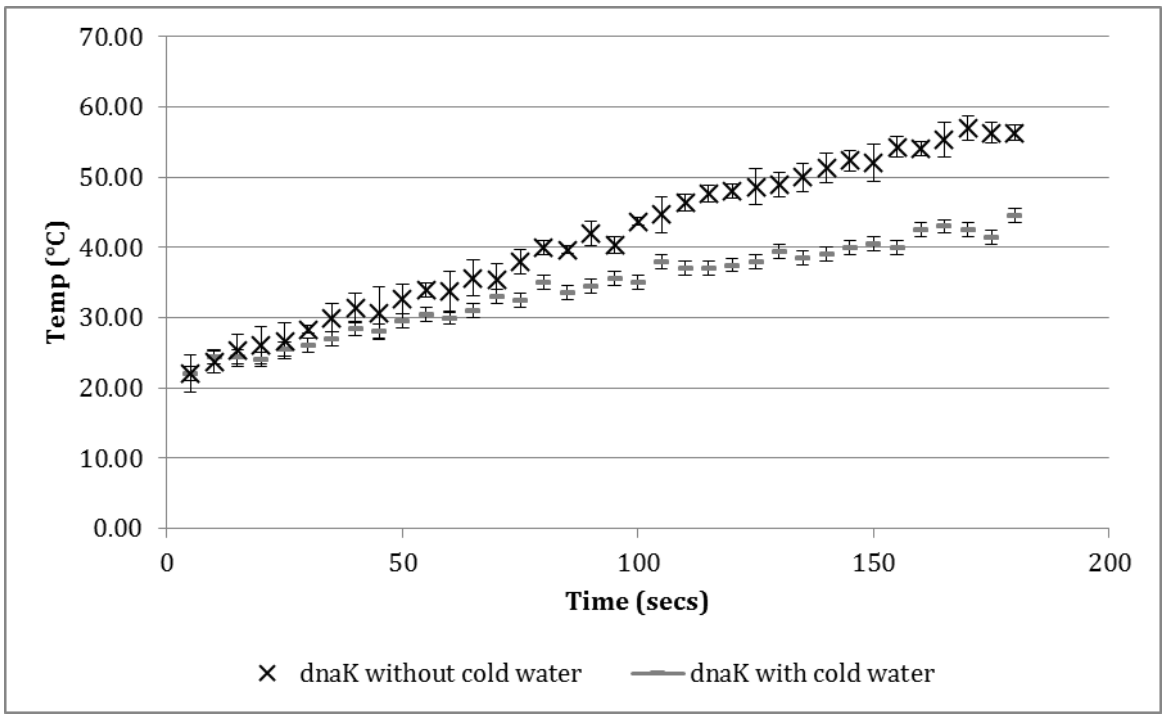

Figure 3: Temperature profiles for four mutants with and without a cold water bath.

4. Conclusions

In conclusion it can be summarized, that ultrasound plays an important role on *E. coli* inactivation albeit a small log difference was observed. Nonetheless an increase in the treatment of ultrasound may increase the inactivation effect resulting in higher inactivation of most of the mutants together with the wild type bacteria. Most of the mutants show a 1 log reduction when compared with the wild type strain. This will easily conclude that the a general reduction due to ultrasound treatment may occur, due to cavitation. However further investigation using the K-12 Δ*oxyR* and the K-12 Δ*dnaK* will allow us to understand the role of certain genes In ultrasound treatment.

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

REFERENCES

1. Antoniadis, A., Poulios, I., Nikolakaki, E. and Mantzavinos, D. (2007). Sonochemical disinfection of municipal wastewater. Journal of Hazardous Materials, **146**, 492-495
2. Ayyildiz, O., Sanik, S. and Ileri, B., 2011. Effect of ultrasonic pretreatment on chlorine dioxode disinfection efficiency. Ultrasonics Sonochemistry 18 (2011) 683–688
3. Bukau, B. and Walker, G.C. (1989). Cellular defects caused by deletion of the Escherichia coli dnaK gene indicate roles for heat shock protein in normal metabolism. Journal of Bacteriology, **171**, 2337-2346
4. Chen, D. (2012). Applications of ultrasound in water and wastewater treatment. In: Handbook on Applications of Ultrasound - Sonochemistry for Sustainability, Boca Raton: CRC Press, 373-405
5. De Koning, J., Bixio, D., Karabelas, A., Salgot, M., and Schafer, A. (2008). Characterisation and assessment of water treatment technologies for reuse. Desalination, **218**, 92-104
6. Drakopoulou, S., Terzakis, S., Fountoulakis, M.S, Mantzavinos, D. and Manios, T. (2009). Ultrasound-induced inactivation of gram-negative and gram-positive bacteria in secondary treated municipal wastewater. Ultrasonics Sonochemistry, **16**, 629-634
7. Gogate, P.R. (2007). Application of cavitational reactors for water disinfection: current status and path forward. Journal of Environmental Management, 85, 801-815
8. Hengge, R. (2009). Proteolysis of σ^S (RpoS) and the general stress response in Escherichia coli. Research in Microbiology, **160**, 667-676
9. Joyce, E., Phull, S.S., Lorimer, J.P. and Mason, T.J. (2003). The development and evaluation of ultrasound for the treatment of bacterial suspensions - A study of frequency, power and sonication time on cultured Bacillus species. Ultrasonics Sonochemistry, **10**, 315-318
10. Lazarova, V., Savoye, P., Janex, M.L., Blatchley III, E.R. and Pommepuy, M. (1999). Advanced wastewater disinfection technologies: state of the art and perspectives. Water Science and Technology, **40**, 203-213
11. Neis, U. and Blume, T. (2004). Improved wastewater disinfection by ultrasonic pre-treatment. Ultrasonics Sonochemistry, **11**, 333-336
12. Patil, S., Valdramidis, V.P., Karatzas, K.A.G., Cullen, P.J. and Bourke, P. (2011). Assessing the microbial oxidative stress mechanism of ozone treatment through the responses of Escherichia coli mutants. Journal of Applied Microbiology, **111**, 136-144
13. Pomposiello, P.J. and Demple, B. (2001). Redox-operated genetic switches: the SoxR and OxyR transcription factors. Trends in Biotechnology, **19**, 109-114
14. Vasilyak, L.M. (2010). Ultrasound application in systems for the disinfection of water. Surface Engineering and Applied Electrochemistry, **46**, 489-493.