Inactivation of *Vibrio parahaemolyticus* by Aqueous Ozone^S

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Received: January 29, 2018

Revised: May 21, 2018

Accepted: May 23, 2018

First published online
June 6, 2018

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^SSupplementary data for this
paper are available on-line only at
<http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

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Vibrio parahaemolyticus contamination causes serious foodborne illness and has become a global health problem. As a disinfectant, aqueous ozone can effectively kill a number of bacteria, viruses, parasites, and other microorganisms. In this study, three factors, namely, the aqueous ozone concentration, the exposure time, and the bacterial density, were analyzed by response surface methodology, and the aqueous ozone concentration was the most influential factor in the sterilization ratio. Under low aqueous ozone concentrations (less than 0.125 mg/l), the bacterial cell membranes remained intact, and the ozone was detoxified by intracellular antioxidant enzymes (e.g., superoxide dismutase and catalase). Under high aqueous ozone concentrations (more than 1 mg/l), cell membranes were damaged by the degree of peripheral electronegativity at the cell surface and the concentration of lactate dehydrogenase released into the extracellular space, and the ultrastructures of the cells were confirmed by transmission electron microscopy. Aqueous ozone penetrated the cells through leaking membranes, inactivated the enzymes, inhibited almost all the genes, and degraded the genetic materials of gDNA and total RNA, which eventually led to cell death.

Keywords: *Vibrio parahaemolyticus*, aqueous ozone, response surface methodology, transcriptome, inactivation

Introduction

With the increased consumption of raw seafood in recent years, reports of foodborne illnesses have also gradually increased. Bacterial contamination is the primary hazard, especially pathogenic *Vibrio* species [1]. *Vibrio parahaemolyticus* is widely present in shallow seawater and is attached to the body surfaces of some marine crustaceans, mollusks, and other aquatic organisms, where it grows and reproduces. In most environmental samples, *V. parahaemolyticus* does not contain thermostable direct hemolysin and thermostable direct hemolysin-related hemolysin, which are two virulence factors that are harmful to humans [2]. However, a small number of *V. parahaemolyticus* bacteria that carry these factors are pathogenic and can cause nausea, vomiting, abdominal pain, diarrhea, fever, and other symptoms of acute gastroenteritis. These cases primarily occur when people consume contaminated seafood. In particular, in a patient who already has significant health problems, this

infection may cause dehydration, shock, coma, and even death [3, 4]. Therefore, it is critically important to kill or inactivate *V. parahaemolyticus* in edible raw seafood.

Ozone is unstable in water. It will produce a single atom of oxygen, hydroxyl radicals, and other strong oxidants whose oxidation potential is weaker than fluorine but stronger than chlorine. Aqueous ozone can kill or inactivate bacteria, fungi, protozoa, viruses (including hepatitis A and B viruses, human immunodeficiency virus), and other organisms in surface ocean water and food [5]. The bactericidal effect of aqueous ozone is 600–3,000 times greater than that for the same concentration of chlorine [6]. Highly concentrated aqueous ozone obtained by electrolysis does not contain toxic nitrogen oxides and other byproducts. The US Food and Drug Administration has ruled that ozone can be safely used as a fungicide in food production, and the US Environmental Protection Agency has also agreed to the use of ozone as a disinfectant [7]. Currently, ozone is considered as a good, safe food

preservative and has been widely used in factories to sterilize frozen seafood before packaging [8]. Ozone can also be used to sterilize and disinfect cold storage facilities, processing plant equipment, air, household disinfection cabinet [6], drinking water and wastewater [9, 10], fruits and juice [11, 12], and vegetables [13]. Ozone tolerance varies among microorganisms. Susceptibility to ozone sterilization is related to the type of microorganism [10], its microbial growth period and density [14], the ozone concentration and stress response [15], and other factors [16]. At present, *Escherichia coli* is the primary model organism used to study the mechanism of ozone sterilization [15]. Ozone and its decomposition products increase the permeability of the lipid bilayer in bacterial cell membranes and decrease their integrity [17]. These compounds then react with enzymes, DNA, and RNA, causing the cell contents to leak through the permeable cell membranes [18–20]. This is the primary process underlying *E. coli* sterilization.

In this study, *V. parahaemolyticus* was used to study the effectiveness of aqueous ozone at various concentrations and stress times, and against different bacterial densities. Response surface experiments were used to analyze the effects of the concentration and exposure time of the aqueous ozone solutions and the bacterial density on the survival ratio. Moreover, cell changes were also examined by measuring the enzyme activity, by transmission electron microscopy, and by RNA-Seq technology when exposing the cell to different concentrations of aqueous ozone. These experiments were designed to discern the possible mechanism by which aqueous ozone kills the foodborne pathogen *V. parahaemolyticus* and to provide a theoretical basis for the use of aqueous ozone as a disinfectant in food production facilities and other environments.

Materials and Methods

Production Growth Curve of *V. parahaemolyticus*

V. parahaemolyticus strain ATCC 17802 was obtained from the Chinese General Microbiological Culture Collection Center as previously reported [21, 22]. It was grown in Luria-Bertani broth at 37°C for 18–22 h. To isolate a single *V. parahaemolyticus* cell, a loop of culture containing the bacteria was spread onto the surface of a thiosulfate-citrate-bile salt-sucrose (Qingdao Hope Bio-Technology Co., Ltd., China) agar plate, which was incubated at 37°C for 18–24 h. Large round green translucent colonies were selected from the agar using a loop, and they were then incubated in alkaline peptone water with shaking at 150 rpm at 37°C [23].

To produce growth curves, 1 ml of culture was re-incubated in

200 ml of alkaline peptone water with shaking at 150 rpm and 37°C. Cells were collected at a series of growth stages, and their densities were measured using an Ultrospec 2100 UN/Visible spectrophotometer (Amersham Biosciences, USA) at OD₆₀₀. Moreover, serial decimal dilutions of cells at different growth stages as measured by ultraviolet spectrophotometry were also prepared, and 1 ml of each dilution was pipetted into an empty sterile petri dish. Subsequently, approximately 20 ml of melted plate count agar (Qingdao Hope Bio-Technology Co., Ltd., China), which had been cooled in a water bath at 46°C, was poured into the dish, and it was then mixed thoroughly by gentle tilting and swirling. Twenty minutes later, the dishes were turned up-side-down and incubated at 37°C for 18–24 h. Finally, the total colony forming units were considered as the total viable counts (TVC) [24].

Generation and Measurement of Aqueous Ozone

The aqueous ozone was generated by a high-concentration Ozone Generator (QD-OS-S-300L; Guangzhou Jia Huan Ozone Electric Appliance Co., Ltd., China) at ambient temperatures ranging from 12 to 15°C, because this temperature range has strong inactivation effects against *E. coli* [15]. The concentration of dissolved aqueous ozone was measured by iodometry. We added 0.071 g of KIO₃ and 1.5 g of KI to 50 ml of water in a 250 ml conical flask, and then we added another 50 ml of water. After mixing, 10 ml of acetic acid was added. The resulting iodine was titrated with 0.1 mol/l of sodium thiosulfate until the yellow solution almost disappeared. Sodium thiosulfate was a suitable neutralizer to stop the aqueous ozone reaction without affecting the growth of *V. parahaemolyticus* (Table S1). Furthermore, 1 ml of starch solution (0.5% (w/v)) was added as an indicator, and the 0.1 mol/l sodium thiosulfate titration was continued when the blue solution disappeared at just the right time [25].

Analysis and Optimization of the Sterilization Ratio of Aqueous Ozone by Response Surface Methodology

To analyze the influence of the aqueous ozone concentration on the sterilization ratio, 1 ml of cell cultures with densities of 10⁸ CFU/ml each were mixed with 99 ml of aqueous ozone at concentrations of 0.0625, 0.125, 0.25, 0.5, 1.0, and 2 mg/l. One minute later, 0.1 mol/l of sodium thiosulfate was added to terminate the reaction, and the viable cell number was measured by counting the TVC.

To analyze the influence of the cell density on the sterilization ratio, 1 ml of cell cultures with different densities (10⁶, 10⁸, and 10¹⁰ CFU/ml) were mixed with 99 ml of aqueous ozone at a concentration of 1.0 mg/l. The reaction was also terminated by sodium thiosulfate after 1 min, and the viable cell number was measured by counting the TVC.

The aqueous ozone exposure time is also an important factor that affects the sterilization ratio. One milliliter of cell cultures with 10⁸ CFU/ml was mixed with 99 ml of aqueous ozone at a concentration of 1.0 mg/l. Bacterial samples were exposed for 10,

20, 40, 80, 160, or 320 sec, and their viable cell numbers were measured by counting the TVC. The cells without aqueous ozone treatment were the control. The sterilization ratio was calculated using the following formula:

$$\text{Sterilization ratio (\%)} = \frac{\text{TVC of control} - \text{TVC of treatment}}{\text{TVC of control}} \times 100\%$$

Based on single-factor analysis, the highest and lowest values of aqueous ozone concentrations, bacterial density, and exposure time were determined individually. These three factors (the aqueous ozone concentration, bacterial density, and exposure time) were then set as independent variables, and the sterilization ratio was analyzed using Design-Expert software 7.0.0 [26].

Measurement of the Zeta Potential of the Cell Surface

Cell cultures (10^8 CFU/ml; 1 ml) were mixed with 99 ml of aqueous ozone at concentrations of 0.0625, 0.125, 0.25, 0.5, 1.0, and 1.2 mg/l for 1 min each. After the reactions were terminated by sodium thiosulfate, the samples were centrifuged and resuspended in stroke-physiological saline solution. They were then washed and resuspended in 1 M KNO₃ (pH 6.2) twice. The zeta potential of the suspended cells was measured using a Zetasizer Nano device (Malvern, UK) [27].

Measurement of Enzymatic Activity

The lactate dehydrogenase (LDH) content can be used to determine the permeability of cell membranes. As the method used to measure the zeta potential, the cell pellets were exposed to aqueous ozone and were subsequently disrupted ultrasonically. The LDH content was then measured using an LDH activity assay kit (Nanjing Jiancheng Bioengineering Institute, China). Cells that were neither treated with aqueous ozone nor ultrasonically processed were used as the spontaneous group. Cells that were lysed directly by ultrasound were set as the maximum LDH released group. The damage ratio of the cell membrane was expressed by the following equation:

$$\text{Damage ratio (\%)} = \frac{\text{Treatment LDH} - \text{Spontaneous LDH}}{\text{Max LDH} - \text{Spontaneous LDH}} \times 100\%$$

A total superoxide dismutase (T-SOD) assay kit (Nanjing Jiancheng Bioengineering Institute, China) and a catalase (CAT) assay kit (Nanjing Jiancheng Bioengineering Institute, China) were used to measure the intracellular SOD and CAT activities, respectively. The measuring methods and equations were the same as those used for LDH in accordance with the manual.

Examination of the Cell Membranes by Transmission Electron Microscopy (TEM)

Cell cultures (10^8 CFU/ml; 1 ml) were mixed with 99 ml of aqueous ozone at a concentration of 1.0 mg/l for 1 min. In the control sample, stroke-physiological saline solution was used instead of aqueous ozone. Cells were first fixed with 2.5% glutaraldehyde solution in phosphate buffer (pH 7.0) for more

than 4 h, and then washed with phosphate buffer three times. The samples were subsequently fixed with 1% OsO₄ in phosphate buffer (pH 7.0) for 1–2 h and then washed three times in the phosphate buffer. The cells were then dehydrated with a graded ethanol series (50%, 70%, 80%, 90%, and 95%) for approximately 15–20 min at each step, and transferred into a mixture of ethanol and isoamyl acetate (1:1 (v/v)) for 30 min, followed by the purification of the isoamyl acetate for 1–2 h. Furthermore, cells were dehydrated in a Hitachi Model HCP-2 critical point dryer with liquid CO₂. At the final step, the dehydrated samples were coated with gold-palladium and observed by TEM (300 kV TEM H-9500; Hitachi, Japan).

Detection of the Integrity of Genetic Material by Electrophoresis

As with the method for measuring the zeta potential, the same number of cell pellets were exposed to different concentrations of aqueous ozone. The samples were then centrifuged and their genomic DNA was extracted using a MiniBEST Bacterial Genomic DNA Extraction Kit (TaKaRa, China), and the total RNA was extracted by TRIzol reagent (Invitrogen, USA). The nucleotide concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The integrity of the nuclei acid was verified using 1% (w/v) agarose gel electrophoresis by loading the same volume of gDNA or total RNA.

Transcriptome Sequencing and Analysis

The cell cultures (10^8 CFU/ml; 10 ml) were mixed with 990 ml of aqueous ozone at concentrations of 0.1 and 1.0 mg/l for 1 min. When the reactions were terminated, the cells were subsequently spun down at 3,000 g for 1 min by a swing rotor centrifuge (Eppendorf, Germany). The above steps of termination and centrifuge lasted less than 5 min. The TRIzol reagent was immediately added to the centrifuge tube to inhibit RNase activity. The cells were homogenized with TRIzol reagent and then with chloroform. Finally, total RNA was precipitated in the aqueous layer with isopropanol by centrifuging and submitted to Zhejiang Tianke Co., Ltd. (Hangzhou, China). Contaminating DNA in the RNA samples was removed by TURBO DNase (Ambion), and then the ribosomal RNA was removed using a MICROBExpress Bacterial mRNA Enrichment Kit (Ambion, USA) according to the manual. The integrity and concentration of the purified and enriched mRNA were assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The rRNA-depleted samples were subsequently fragmented and then synthesized into cDNA using SuperScript III Rev Transcriptase (Invitrogen, USA) combined with random primers (Invitrogen, USA). Paired-end library construction was performed using 2 µg of total RNA, and its quality was verified again by the Bioanalyzer. RNA-Seq was performed on the Illumina HiSeq 2000 platform [28].

Adapters and poor quality base pairs were filtered out by Trimmomatic software [29], and repeated reads were removed by FastUniq software [30]. Those clean reads were then mapped to

the *V. parahaemolyticus* reference genome (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Vibrio_parahaemolyticus_RIMD_2210633_uid57969) using TopHat 2.0.11, and differential expression analysis was performed using Cuffdiff 2.0.2 according to the fragments per kilobase of the exon model per million mapped reads [31, 32]. The criterion for a significant up-regulation or down-regulation was a p -value < 0.05.

Quantitative PCR

The total RNA extraction, the removal of DNA contamination, and the cDNA synthesis were performed as described for the transcriptome sequencing. A Power SYBR Green Master Mix (Applied Biosystems, USA) was used for quantitative PCR (qPCR), which was conducted using a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, USA). The 16S rRNA gene was selected as an endogenous control [33], and the target gene IDs and primer sequences are listed in Table S2. The qPCRs and the calculation of the relative expression levels of genes were performed as previously described [34, 35].

Statistics

All the analyses were performed in triplicate. Mean values were reported with standard deviations for each case. One-way analysis of variance (ANOVA) was performed for all data, and mean separations were conducted by Tukey's multiple range test (SPSS 13.0). Differences were considered significant at p -values < 0.05.

Omics Data

All Illumina RNA sequencing data were submitted to the NCBI Short Read Archive (<http://www.ncbi.nlm.nih.gov/sra>), and their accession numbers are SRR6488512–SRR6488514.

Results and Discussion

Ozone Degradation Tendency in Water

Ozone is much more stable as a gaseous phase than an aqueous phase. Gaseous ozone has been used to sterilize and disinfect air and household disinfection cabinets, whereas aqueous ozone was used to sterilize drinking water,

Fig. 1. Aqueous ozone degradation and its sterilization effects on *Vibrio parahaemolyticus*.

(A) Tendency of aqueous ozone to degrade in water at room temperature. Significant change ($p \leq 0.05$) of the aqueous ozone concentration is indicated by lowercase letters. (B) Sterilization ratio under different aqueous ozone concentrations while the bacterial density and exposure time were fixed. (C) Sterilization ratio under different bacterial densities while the aqueous ozone concentration and exposure time were fixed. (D) Sterilization ratio under different exposure times while the aqueous ozone concentration and bacterial density were fixed.

Table 1. Ranges and levels of variables in the Box-Behnken statistical experimental design.

Independent variables	Symbol	Coded variable level		
		Minimum	Medium	Maximum
Aqueous ozone concentration (mg/l)	A	0.063	0.631	1.2
Bacterial density (log ₁₀ CFU/ml)	B	4	6	8
Time exposed to aqueous ozone (sec)	C	10	165	320

wastewater, fruits, and juice [6, 9, 10]. *V. parahaemolyticus* is widely present in shallow seawater and attached to the body surfaces of marine food product. Therefore, aqueous ozone was selected to sterilize *V. parahaemolyticus*. However, the stability of aqueous ozone is affected by its concentration, temperature, pH, presence of organic matter and metal ions, and UV exposure [36]. In order to determine the ozone degradation tendency in water, the aqueous ozone concentration was detected by iodometry when produced. The aqueous ozone (3.3 mg/l) started to break down dramatically in the first 20 min at room temperature, and it remained stable for 45–75 min; moreover, the change in absolute value of aqueous ozone concentration was relatively smooth from 60 to 75 min (Fig. 1A). Therefore, aqueous ozone was chosen as the experimental material at 60 min.

Sterilization Effect of Aqueous Ozone on *V. parahaemolyticus*

Initial preliminary experiments were conducted following the single-factor experimental method to determine the most influential parameters that affected the bacterial sterilization ratio. Three parameters, the aqueous ozone concentration, aqueous ozone exposure time, and bacterial density, exerted dominant effects during *E. coli* inactivation [16], and they were selected as single factors in this study. The three experimental parameters were optimized on basis of the sterilization ratio obtained from the single-factor study. Fig. 1B shows the effects of different concentrations of aqueous ozone on *V. parahaemolyticus* when the bacterial density and the exposure time were fixed. The sterilization was gradually enhanced with the increasing aqueous ozone concentration, and the relationship was linear. However, when both the concentration and the aqueous ozone exposure time were fixed, the sterilization efficacy declined with the increasing bacterial density (Fig. 1C). With fixed concentrations of aqueous ozone and bacterial density, the sterilization effect was enhanced when the exposure time was increased (Fig. 1D). The sterilization trends of aqueous ozone on *V. parahaemolyticus* were consistent with those reported in *E. coli* [15, 16], and

thus the next step was to determine the dominant factor in the sterilization effect.

Key Factor Analysis for the Sterilization Ratio of Aqueous Ozone

The above three selected experimental parameters were optimized using response surface methodology by considering them as independent variables. A Box-Behnken design was adopted to evaluate the combined effect of the three independent variables using 17 sets of experiments. Both the ranges and levels of independent variables as well as details of designed experiments are shown in Tables 1 and 2, respectively.

Combined with the results of Tables 1 and 2, the formation of second-order quadratic polynomial models

Table 2. Box-Behnken experiments.

Serial No.	Aqueous ozone concentration (mg/l)	Bacterial density (log ₁₀ CFU/ml)	Aqueous ozone treatment time (sec)	Sterilization ratio (%)
1	0.06	4	165	10.48
2	1.20	4	165	100.00
3	0.06	8	165	4.76
4	1.20	8	165	100.00
5	0.06	6	10	5.91
6	1.20	6	10	100.00
7	0.06	6	320	7.64
8	1.20	6	320	100.00
9	0.63	4	10	26.81
10	0.63	8	10	26.00
11	0.63	4	320	53.32
12	0.63	8	320	45.14
13	0.63	6	165	25.54
14	0.63	6	165	35.16
15	0.63	6	165	27.31
16	0.63	6	165	33.36
17	0.63	6	165	30.48

Table 3. ANOVA results of the response surface quadratic model for the sterilization ratio.

Source of variance	Sum of squares	Degrees of freedom	Mean square	F-value	p Value
Model	19,358.26	9	2,419.78	62.81	<0.0001
A	17,224.57	1	17,224.57	447.12	<0.0001
B	27.07	1	27.07	0.70	0.4262
C	280.61	1	280.61	7.28	0.0271
AB	8.19	1	8.19	0.21	0.6571
BC	13.56	1	13.56	0.35	0.5694
AC	10.43	1	10.41	0.28	0.5945
A ²	1,601.91	1	1,601.91	41.58	0.0002
B ²	65.33	1	65.33	1.70	0.2291
C ²	51.99	1	51.99	1.35	0.2789
Residual	308.18	7	38.52		
Lack of fit	243.59	3	60.90	3.77	0.1134
Pure error	64.60	4	16.15		
Total	19,666.44	16			
	R ² = 0.9843	Adj R ² = 0.9687			

was developed using Design-Expert 7.0.0 software to establish the sterilization ratio of *V. parahaemolyticus* when treated with aqueous ozone as follows:

$$\text{Sterilization ratio (\%)} = 40.4247 - 2.0881 \times A - 12.5508 \times B + 0.0256 \times C + 1.2576 \times AB - 0.0059 \times BC + 0.153 \times AC + 60.2987 \times A^2 + 0.9848 \times B^2 + 0.0001 \times C^2$$

The experimental data for the sterilization ratio were statistically analyzed by variance (Table 3). The *F*-value of the model was 62.81, and the corresponding *p*-value was <0.0001, which indicated the model was highly significant. The lack of *F*-value fit at 3.77 was not significant, because the corresponding *p*-value was >0.05. An insignificant *p*-value can be used to predicate the lack of fit [26]. The predicted *R*² value of 0.9843 was consistent with the corresponding adjusted *R*² value of 0.9687. In a model, better *R*² values should be close to 1 to fit the experimental data [26]. The independent variables of the quadratic polynomial models *A* and *C*, and the quadratic term *A*² were highly significant because the *p*-value for them was <0.05, whereas the remaining model, interaction, or quadratic terms had high *p*-values of more than 0.05. Therefore, model *A* of the aqueous ozone concentrations was found to be the most influential factor in the sterilization ratio, but all the other factors were also important.

The Cell Membrane Was Damaged by Aqueous Ozone Treatment

Regarding unicellular organisms, the interfacial surface physiology that is regarded as maintaining optimal cell function is particularly important. Ionized substituents and phosphoryl groups on the outer cell envelopes cause bacterial surfaces to possess a net negative electrostatic charge. Thus, the degree of peripheral electronegativity of a cell surface can be assessed by using the zeta potential [27, 37]. Lower zeta potentials could be observed in dead bacterial cells [38]. In this study, the zeta potential would stabilize if the aqueous ozone concentration was lower than 0.2 mg/l. When the concentration was higher than 0.4 mg/l, the zeta potential decreased gradually with the increasing aqueous ozone concentration. When the concentration was 1.2 mg/l, the zeta potential was reduced by 30.5% compared with the control sample (Fig. 2A). The result suggested that aqueous ozone influenced the overall polarity of the bacterial surface that might underlie the integrity of the cell membrane.

LDH is present in almost all living cells (animals, plants, and prokaryotes), catalyzing the conversion of lactate to pyruvate and back to lactate. As a soluble cytoplasmic enzyme, it is released into the extracellular space when the plasma membrane is damaged [39]. With the increasing aqueous ozone concentration, the amount of enzyme was also increased (Fig. 2B), showing a dose-response relationship, and suggesting the cell envelope was damaged during ozonation.

Furthermore, the ultrastructure of the cell was observed by TEM to determine the morphological changes in the internal and external cell structures after applying aqueous ozone. After aqueous ozone treatment, the irregular shape and rupture of the cell wall were observed, and the majority of the cells appeared to be hollow, which may have been caused by the loss of cell contents (Fig. 2C), such as LDH. Control cells without aqueous ozone treatments had intact cell walls and membranes, and a uniform cell cytoplasm (Fig. 2D). The effect was similar to that observed in *E. coli* in which the surface structure was damaged after ozonation as shown by both TEM and scanning electron microscope analyses [17, 40]. Therefore, the cell membrane integrity can be damaged by aqueous ozone, which results in loss of the cellular contents (*e.g.*, enzyme, genetic materials) and finally leads to cell death.

Intracellular Antioxidant Enzymes Detoxified a Certain Concentration of Aqueous Ozone

Reactive oxygen species can induce damage in the

Fig. 2. Cell membrane damage after aqueous ozone treatment.

(A) The degree of peripheral electronegativity on the cell surface was assessed by using the zeta potential. (B) Cell membrane permeability was assessed by observing the lactate dehydrogenase content. Significant change ($p \leq 0.05$) of the aqueous ozone concentration is indicated by lowercase letters. (C) TEM examination of cells under aqueous ozone treatment of 1 mg/l for 1 min. (D) TEM examination of cells without aqueous ozone treatment.

genetic material and then lead to genetic lesions (e.g., mutagenic activity and tumorigenicity). Both superoxide dismutase and catalase are the most important and common enzymes involved in the decomposition of reactive oxygen species, and their levels are related to the concentration of reactive oxygen species within the cell [41]. The SOD activity showed a progressive increase after the cells were treated with low concentrations of aqueous ozone (0–0.125 mg/l). When the concentration of aqueous ozone was 0.125 mg/l, the SOD activity was 89.61 ± 4.85 U/mg protein, which was 116% of the control sample (Fig. 3A). This

finding illustrated that a low concentration of aqueous ozone might diffuse into the cell and then stimulate the activity of the SOD antioxidant. However, as the aqueous ozone concentration increased, the SOD activity decreased. The activity was 39.68 ± 4.32 U/mg protein when treated with 1.2 mg/l aqueous ozone, which was 52% of the control sample. A high concentration of aqueous ozone exceeded the antioxidant capacity of the intracellular SOD, which broke the antioxidant balance. The effects on CAT were similar to those of SOD when treating with aqueous ozone (Fig. 3B). The CAT activity also showed a progressive

Fig. 3. Intracellular antioxidant enzyme activity of cells after aqueous ozone treatment of 0–1.2 mg/l for 1 min. (A) SOD activity. (B) CAT activity. Significant change ($p \leq 0.05$) of the aqueous ozone concentration is indicated by lowercase letters.

increase with low concentrations of aqueous ozone (0–0.0625 mg/l). The activity of CAT was 0.29 ± 0.07 U/mg protein when treated with 1.2 mg/l aqueous ozone, which was 37.47% of the control sample. This phenomenon conformed to the characteristics of the hormesis, a dose-response relationship characterizing a biphasic dose response to stressors with favorable response to a low dose but detrimental effect at a higher dose. Almost all of the organisms have this characteristic. The hormetic response, a low-dose ozone or oxidative preconditioning promoting the synthesis of enzymes (e.g., SOD, CAT, GPx, and GR) to defend oxidants, has been demonstrated in experimental animals and patients [42]. In this study, both SOD and CAT in *V. parahaemolyticus* conferred protection to the cells at a certain low concentration but lost activity at high concentrations of aqueous ozone treatment, suggesting

Fig. 4. Genetic materials in the cells after aqueous ozone treatment of 0–1.2 mg/l for 1 min. (A) Degradation of gDNA. (B) Degradation of total RNA.

hormesis existed in *V. parahaemolyticus*, and aqueous ozone was its stressor.

Aqueous Ozone Degraded Genetic Materials

The genetic materials DNA and RNA are repositories of information in every living cell, and thus their integrity and stability are crucial to life [43]. Previous studies showed that ozone treatment led to *E. coli* DNA lesions with mutations, in which the massive breakdown of DNA would be lethal if not repaired by DNA polymerase [20]. Therefore, some investigators introduced ozone to degrade antibiotic resistance genes that were escaping to the environment after cell membranes were damaged in *E. coli*

and other bacteria from wastewater [19, 44]. In this study, both genomic DNA and total RNA were visualized by ethidium bromide staining after *V. parahaemolyticus* cells were treated with a series of aqueous ozone concentrations (Figs. 4A and 4B). A low concentration of aqueous ozone (less than 0.25 mg/l) had little effect on the integrity of the genomic DNA and total RNA. When the concentration was approximately 0.5 mg/l, the bands of genomic DNA and total RNA began to fade. If the concentration was greater than 1 mg/l, the sterilization ratio was more than 56.53% (Fig. 1B), and the damage to the genomic DNA and total RNA very obviously resulted in faint bands, suggesting the massive breakdown of both DNA and RNA was lethal for cells. Moreover, the read numbers from the transcriptome sequencing showed that the high-concentration treatment of 1 mg/l (3,737,032) produced much fewer numbers than either the control (5,695,016) or the low concentration of 0.1 mg/l (5,632,096) (Table S3), indicating the gene expression profile only corresponded to surviving bacteria. Therefore, the genetic materials of *V. parahaemolyticus* were barely damaged by a low concentration (≤ 0.1 mg/l), whereas they were severely degraded at high concentrations (≥ 1 mg/l). This phenomenon might be one of the most important reasons to respond to the linear relationship between the sterilization ratio and the aqueous ozone concentration (Fig. 1B).

Transcriptome Sequencing of Whole-Genome Expression Profiling for Cells under Aqueous Ozone Stress

To obtain a deep understanding of the molecular

mechanisms of *V. parahaemolyticus* in response to aqueous ozone stress, large-scale transcriptome sequencing of cells was performed under aqueous ozone stress using the Illumina sequencing technology. After removing the adaptor and the low-quality and repeated sequences, as well as possible viruses, the reads were obtained and could be mapped to the *V. parahaemolyticus* reference genome (RIMD_2210633) (Table S3), and then they were assembled de novo to generate 8,440 unique transcripts (Fig. S1). A total of 4,832 genes mapped exactly to all the genes within the *V. parahaemolyticus* RIMD_2210633 strain [32]. To validate the RNA-Seq expression profile data, 21 genes were selected randomly for qPCR confirmation (Tables S2 and S4). A high correlation was observed between the two methods ($R^2 = 0.9299$) identified by a simple linear regression equation as follows: $y = 1.1944x - 0.1005$ (Fig. 5A).

The above results (Figs. 2–4) showed that high concentrations of aqueous ozone significantly affect the phenotype of cells (e.g., cell membrane, enzyme, and genetic materials). A genome-level screening using RNA-Seq would be more suitable for uncovering the molecular mechanism of aqueous ozone sterilization. There were 75 and 47 genes that were up- or down-regulated by more than 2-fold in response to exposure to 0.1 and 1 mg/l aqueous ozone, respectively ($P < 0.05$) (Table S5). The overlapping genes (VPA0061, VPA0084, VPA0171, VPA1017, VP2010, VP2421, and VP2422) were summarized in a Venn diagram (Fig. 5B), and their relative expression levels under different concentrations of aqueous ozone treatment were provided (Fig. 5C).

Fig. 5. Gene expression profiling of cells after aqueous ozone treatment.

(A) Gene expression levels with a correlation between RNA-Seq (x-axis) and qPCR (y-axis). Each Log_2 ratio of fold changes was compared between RNA-Seq and qPCR. (B) A Venn diagram of up- or down-regulated genes after exposure to aqueous ozone treatments. (C) Gene expression levels determined by qPCR. Error bars represent the standard deviation.

Fig. 5. Continued.

Fig. 6. Gene Ontology enrichment grouping in *V. parahaemolyticus* under aqueous ozone treatment. (A) Aqueous ozone 0.1 mg/l treatment; and (B) 1.0 mg/l treatment.

The *VPA0061* gene is annotated as a MATE family efflux transporter that is involved in exporting metabolites across the cell membrane and responding to multidrug resistance in many bacteria and eukaryotic cells [45]. Under low-concentration aqueous ozone treatment, its expression level was much higher than that of the control, which suggested that it acted as a pump to transport the ozone out of the cell to reduce oxidant harm. When the aqueous ozone concentration was higher than 0.25 mg/l, the expression level of the *VPA0061* gene was inhibited, indicating that the DNA and RNA began to enter into an unstable status and could not complete their transcription progress. The *VPA0084*, *VPA0171*, *VPA1017*, and *VP2421* genes are annotated as encoding a transporter, hydrolase, transcriptional regulator, and AAA family ATPase, respectively. Their expression levels were inhibited when cells were exposed to aqueous ozone. The *VP2010* and *VP2422* genes are annotated as encoding sensory histidine kinase and prepilin peptidase. With an increasing aqueous ozone concentration (0–1 mg/l), the expression levels of *VP2010* and *VP2422* were gradually increased; however, both of them were decreased at the highest concentration (1.2 mg/l). Therefore, the destruction of the genetic materials and the inhibition of gene expression are important methods for killing bacteria.

In *E. coli*, the oxidative stress-related genes *soxRS* and *oxyR* were confirmed to play an important role in protecting against ozone stress through DNA repair and the removal of radicals [17, 46], and the regulon RpoS also conferred protection against radicals [17]. The *VPA0335* gene, which was annotated as encoding a SoxR protein in *V. parahaemolyticus*, was screened out through transcriptome sequencing (Table S5) and was inhibited between the control and the aqueous ozone exposure concentrations (0.0625–1.2 mg/l) by qPCR (Fig. 5C). However, neither the *soxS* nor the *rpoS* gene was identified by transcriptome sequencing (Table S5). Under oxidative stress, SoxR induces the expression of SoxS that further promotes the SoxRS regulon in *E. coli* to confer protection against stress [46]. Thus, *V. parahaemolyticus* might have other regulon(s) to confer a certain increased degree of SOD and CAT activity at low concentrations of aqueous ozone (Figs. 3A and 3B) with the exception of SoxRS.

Enrichment analysis is a useful method for interpreting RNA-Seq data through the automatic functional categorization of gene lists [47]. Differently expressed genes at the 2-fold up- or down-regulation levels under each aqueous ozone-treated set were searched using the local Blast2go database [48]. The outputs include the three independent domains of

biological processes, molecular function, and cellular components. Under low aqueous ozone concentration treatment, the majority of the most abundant genes were involved in many biological processes, including the regulation of biological processes, biological regulation, single organism signaling, cell communication, regulation of cellular processes, single transduction, signal transducer activity, and responding to stimuli (Fig. 6A). These findings indicated that *V. parahaemolyticus* received a stimulation from ozone and then transmitted the signal to the intracellular space, which induced the action of the membrane pump system to eliminate the ozone from the cell. The remnants of ozone were detoxified by intracellular antioxidant enzymes (e.g., SOD and CAT). Under high aqueous ozone concentrations, the expression levels of almost all genes were inhibited (Fig. 6B), indicating that the ozone molecules entered the cell from the damaged membrane, and then inhibited the enzymes and destroyed the genetic materials, which could represent a way of killing bacteria with high concentrations of aqueous ozone.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31301566 and 31271954), Specialized Research Fund for the Doctoral Program of Higher Education (No. 20133326120008), and Food Science and Engineering—the Most Important Discipline of Zhejiang Province (No. JYTSP20142071).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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