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# Molecular response of *Vibrio parahaemolyticus* to the sanitizer peracetic acid

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ABSTRACT

Peracetic acid (PAA) is a common oxidative sanitizer that is used in the food industry against various microorganisms. Limited information on the response of bacteria to this biocide is available. This study investigates the molecular response of the prevalent seafood-borne pathogenic *Vibrio parahaemolyticus* to PAA using mutants of peroxide scavenging genes. Among *katE1*, *katE2*, *katG1*, *katG2*, *ahpC1* and *ahpC2*, and their regulator *oxyR* gene mutants, *oxyR* and *katE* mutants were highly susceptible to PAA. The growth and lethality of *V. parahaemolyticus* were harmed by 15 ppm of PAA in the △*katE1E2* double mutant, and were significantly ameliorated in the presence of the *katE1* gene in the wild-type strain and the gene-complementary strains that were pre-adapted in 2 ppm of PAA or 100 μM hydrogen peroxide. The application of PAA to these strains induced the accumulation of reactive oxygen species. The reduction of the level of hydrogen peroxide and gene expression during this treatment was influenced by the presence of *katE* genes. This investigation confirmed the major role of *katE1* and a compensatory role of *katE2* in the resistance of *V. parahaemolyticus* to PAA, and demonstrated some minor differences in the responses of this bacterium against PAA and hydrogen peroxide.

## 1. Introduction

Peracetic acid (PAA) is an organic peroxide that is formed by a reaction between hydrogen peroxide ($H_2O_2$) and acetic acid. It is a powerful oxidant and is used primarily as a disinfectant in the food industry (Park et al., 2012; Singh et al., 2018). It is also applied to sanitize medical supplies and wastewater (Stampi et al., 2002) and as a biocide in antifouling process (Peltola et al., 2011). The biocidal activity of PAA against vegetative cells of various bacteria (Jolivet-Gougeon et al., 1996; Sagripanti et al., 1997), bacterial endospores (Leggett et al., 2015), viruses (Zanetti et al., 2007) and fungi (Nakayama et al., 2013) has been evaluated.

As an oxidizing biocide, PAA damages proteins, enzymes and other metabolites, and disrupts cell permeability (Eramo et al., 2017; Finnegan et al., 2010). PAA functions as an organic peroxide by upregulating the expression of an organic hydroperoxide resistance gene (*ohr* homolog of *Streptomyces lividans*) in *S. coelicolor* (Dai et al., 2016) and enhancing the expression of OhrR in *Bacillus cereus* (Ceragioli et al., 2010). Since PAA can dissociate into $H_2O_2$ and acetic acid, it is similar to $H_2O_2$ in activating the catalase and alkylhydroperoxidase reductase genes in bacteria, such as *S. lividans* (Dai et al., 2016) and *Pseudomonas putida* (Anderson and Miller, 2001). Acetic acid, formed after the dissociation from PAA, has been reported to induce the expression of superoxide dismutases and catalases in some bacteria (Bruno-Barcena et al., 2010; Kumar and Rai, 2015; Sanders et al., 1995). The expression of genes in response to $H_2O_2$ or PAA in *B. cereus* differs in 15% of examined genomic genes (Ceragioli et al., 2010). These studies reveal that bacterial responses to PAA or $H_2O_2$ and other peroxides have certain characteristics which may be attributed to the special properties of PAA.

PAA and other oxidizing biocides are variably effective against microorganisms, depending on their interactions with microbial macromolecules and the microbial defense mechanisms (Finnegan et al., 2010). Limited information is available regarding the mechanism of action and molecular response of bacteria to PAA. In this investigation, the association of different peroxide scavenging genes in response to PAA was examined in *Vibrio parahaemolyticus*, which is a prevalent seafood-borne pathogenic bacterium in many Asian countries and has become a global problem since the spread of its pandemic strain (Wong et al., 2000). The results of this study demonstrated that *katE1* is the chief resistant gene among peroxide scavenging genes in protecting this pathogen against PAA.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*V. parahaemolyticus* strain KX-V231 (Kanagawa phenomenon

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**Table 1**
*Vibrio parahaemolyticus* strains and plasmid used in this study.

| Strain | Description | Source |
|---|---|---|
| *V. parahaemolyticus* | | |
| KX-V231 | Wild-type, serotype O3:K6, $KP^+$, $tdh^+$, clinical isolate | (Chen et al., 2016) |
| Δ*oxyR* | KX-V231Δ*oxyR* | (Chung et al., 2015) |
| Δ*katE1* | KX-V231Δ*katE1* | (Chen et al., 2016) |
| Δ*katE1/C* | KX-V231Δ*katE1* containing pSCB01-katE1 | (Chen et al., 2016) |
| Δ*katE2* | KX-V231Δ*katE2* | (Chen et al., 2016) |
| Δ*katE1E2* | KX-V231Δ*katE1*Δ*katE2* | (Chen et al., 2016) |
| Δ*katG1G2* | KX-V231Δ*katG1*Δ*katG2* | (Yu et al., 2016) |
| Δ*ahpC1C2* | KX-V231Δ*ahpC1*Δ*ahpC2* | (Chung et al., 2014) |
| KX-V231V | KX-V231 containing pSCB01 | (Chen et al., 2016) |
| Plasmid | | |
| pSCB01 | Derived from pBR328 and pDS132, *mobRP4*, $Ap^r$, $Cm^r$, $Tc^r$ | (Wang et al., 2013) |
| pSCB01-katE1 | pSCB01 with *katE1* | (Chen et al., 2016) |

positive, serotype O3:K6) and other mutant strains were used in this study (Table 1). These strains were cultured at 37 °C on Tryptic Soy Agar (Becton-Dickinson Diagnostic Systems, Sparks, MD, USA) that was supplemented with 3% sodium chloride (TSA-3% NaCl), or in Tryptic soy broth-3% NaCl (TSB-3% NaCl), which was shaken at 160 rpm. The culture in TSB-3% NaCl was incubated at 37 °C with shaking for 2.5 h, to enter the exponential phase and was used as inoculum in the following experiments. Chloramphenicol (final concentration of 5 μg/ml) was added to the media as required for the cultivation of the *V. parahaemolyticus* strains containing the cloning vector or complementary gene.

### 2.2. Inhibition of bacterial growth on agar medium

The inhibition of bacterial growth by PAA was evaluated by disk diffusion assay. *V. parahaemolyticus* cultures in the exponential phase were spread on a Luria-Bertani (LB, Becton-Dickinson)-3% NaCl agar plate. Paper discs (6 mm, Creative Media Products, Taiwan) that had absorbed 10 μl of 1000 or 10,000 ppm of peracetic acid (PanReac AppliChem, Barcelona, Spain) were then placed on the bacterial lawn of the agar plate. After incubation at 37 °C for 17 h, the sizes of the inhibition zones were measured.

### 2.3. Inhibition of bacterial growth in broth medium

The chemical stress-adapted and non-adapted bacterial cultures were challenged by PAA or $H_2O_2$. To prepare the stress-adapted cells, bacterial strains were cultured in LB-3% NaCl in the presence of 2 ppm of PAA (26.30 μM) or 100 μM $H_2O_2$ statically at 37 °C for 2.5 h, harvested by centrifugation at 12,000 × *g* for 2 min, and resuspended in fresh medium.

To quantify the inhibition of growth, the *V. parahaemolyticus* cultures were diluted to an initial absorbance at 600 nm ($OD_{600}$) of 0.015 using a Smart Spec 3000 (Bio-Rad, Hercules, CA, USA). Aliquots (200 μl) of these bacterial suspensions were dispensed into the wells of a microtiter plate, to which 15 ppm of PAA (197.24 μM) or 130 μM $H_2O_2$ (Santoku Chemical Industries, Tokyo, Japan) were added and incubated statically at 37 °C for 8 h. Bacterial growth was quantified by measuring $OD_{590}$, using a Biotek EPOCH2 microplate reader (Winooski, VT, USA).

To evaluate bactericidal activity, the *V. parahaemolyticus* cultures in LB-3% NaCl were adjusted to an $OD_{600}$ of 0.3, to which PAA was added and incubated at 37 °C for one to three hours. Survivors were counted by standard dilution plate counting on LB-3% NaCl agar (Chen et al., 2016).

### 2.4. Analysis of reactive oxygen species

Bacterial cells in aliquot (1 ml) of the treated culture were harvested by centrifugation, rinsed and resuspended in 1 ml of phosphate-buffer saline, reacted with 1 μl of 2′-7′-dichlorofluorescein diacetate (DCFDA, Life Technologies, Carlsbad, CA, USA) for 30 min in darkness, and 100 μl of the reactant was used to determine the cellular reactive oxygen species (ROS). Fluorescence at excitation/emission (495/520 nm) was measured using a GloMax®-Multi Microplate Multimode Reader (Promega, Fitchburg, WI, USA). The ROS were quantified as fluorescence per cfu/ml (Muras et al., 2016).

### 2.5. Change of hydrogen peroxide

A 10 ml volume of *V. parahaemolyticus* culture in LB-3% NaCl in the exponential phase was adjusted to an $OD_{600}$ of 0.3 and challenged with 15 ppm of PAA for 5, 10, 15 or 20 min, and the residue of $H_2O_2$ in the culture was quantified by FOX assay (Jiang et al., 1992). Briefly, 1 ml of the culture was sampled and centrifuged, and 100 μl of the supernatant was mixed with 400 μl of 25 mM $H_2SO_4$, and reacted with 500 μl of iron-xylenol solution, which consisted of 25 mM $H_2SO_4$, 3.6 mM butylated hydroxytoluene, 2.5 mM ferrous sulfate heptahydrate and 100 μM xylenol orange in a water/methanol mixture (1:9 v/v) at 37 °C. The reaction proceeded for 1 h and $OD_{560}$ was determined. The percentage $H_2O_2$ residue of the sample was calculated as the $OD_{560}$ at 1 h divided by that at time zero.

### 2.6. Expression of antioxidative genes

The expression of antioxidative genes, such as *katE1*, *katE2*, *katG1*, *katG2* and *ahpC1*, in different *V. parahaemolyticus* strains under the stress of 0, 5 or 7.5 ppm of PAA for 1 h was determined by the reverse transcription-polymerase chain reaction (RT-PCR), which was described in our previous publications (Chen et al., 2016; Lai et al., 2009) and the primers used were presented in Table 2. The expression of 16S rRNA was used as the internal control.

**Table 2**
Primers used in RT-PCR.

| Target | Designation | Sequence (5′ → 3′) | Amplicon, bp |
|---|---|---|---|
| 16SrDNA | q16SrRNA-F | TCCCTAGCTGGTCTGAGA | 222 |
| | q16SrRNA-R | GGTGCTTCTTCTGTCGCT | |
| *katE1* | VPA1418-F | TACGACCGTTGCTGGTGA | 235 |
| | VPA1418-R | TTCTGGCAGCGATGTCCA | |
| *katE2* | VPA0305-F | AGAGTTGTGCACGCTCGT | 228 |
| | VPA0305-R | CCCTACCAGATCCCAGTT | |
| *katG1* | VPA0768-F | GTGGTCATACCGTGGGTA | 237 |
| | VPA0768-R | GGCTCTTCTTCAGTTCCC | |
| *katG2* | VPA0453-F | TGCATGGCTCCATGACCA | 257 |
| | VPA0453-R | CGCATGCCATGACATACG | |
| *ahpC1* | VPA1683-F | CTACCCAGCAGACTTCAC | 227 |
| | VPA1683-R | CTTCACGCATCACACCGA | |