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Susceptibility of *Vibrio parahaemolyticus* to disinfectants after prior exposure to sublethal stress

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ABSTRACT

In the present study, *Vibrio parahaemolyticus* 690 in phosphate buffered-saline containing 3% NaCl was subjected to sublethal stresses: heat shock at 42 °C for 15 min, acid adaptation at pH 5.0 for 30 min, or cold shock at 20 °C for 4 h. The effect of sublethal stress on the susceptibility of *V. parahaemolyticus* to a chlorine-containing disinfectant (Clidox-S) and a quaternary ammonium compound (Quatricide) at 25 and 40 °C was investigated. It was found that the sublethal stresses examined enhanced the resistance of *V. parahaemolyticus* 690 to both disinfectants. Depending on the kinds of sublethal stress, *V. parahaemolyticus* 690 showed various degrees of enhanced resistance to disinfectants. Furthermore, the phenomenon of enhanced resistance to the disinfectants was more marked at 40 than at 25 °C.
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1. Introduction

Vibrio parahaemolyticus is a gram-negative, moderately halophilic, foodborne pathogen. Food poisoning outbreaks associated with *V. parahaemolyticus* have been reported throughout the world (Beuchat, 1982). In China, Japan, Taiwan and other coastal Asian nations where people often consume raw or minimally processed seafood in their daily diet, *V. parahaemolyticus* is considered one of the leading bacterial pathogens causing gastroenteritis (Anonymous, 2011; Bhuiyan et al., 2002; Hara-Kudo et al., 2003; Honda and Lida, 1993). Therefore, the importance of taking adequate measures to prevent the contamination and proliferation of *V. parahaemolyticus* in food has received considerable attention from regulatory agencies, food industry and consumers.

Chemical disinfectants such as chlorine-containing compounds and quaternary ammonium compounds (QACs) are frequently applied to food contact and non-food contact surfaces in food industry and food service kitchens. Furthermore, chlorine dioxide, one of the chlorine-containing disinfectants, has been used to treat drinking water and to eliminate foodborne bacteria in various kinds of food with satisfactory results (Allende et al., 2006; Kang et al., 2008; Kim et al., 1999; Shin et al., 2004; Wang et al., 2010).

During food preparation, processing, and service, microorganisms found in contaminated food may be subjected to various kinds of sublethal stresses such as high or low temperature, or acidity, which may alter them in ways that have a bearing on food safety. Indeed, various investigators (Browne and Dowds, 2001, 2002; Chiang et al., 2006; Lee et al., 2007; Periago et al., 2002a,b; Wong et al., 2002) have reported that stress responses occur in cells of microorganisms that have previously been exposed to sublethal adaptation treatments. Sublethal stress might alter the properties of the microorganism and enable it to develop resistance to subsequent lethal stresses. Lin et al. (2002) observed that heat shock and cold shock reduced the susceptibility of *Listeria monocytogenes* to disinfectants. On the other hand, cold shock reduced while heat shock increased the susceptibility of *Salmonella* Typhimurium to disinfectants. Moreover, Lin et al. (2011) and Moorman et al. (2005) reported that acid adaptation enhanced the resistance of *L. monocytogenes* and *Listeria innocua*, respectively, to the disinfectant tested, while acid adaptation reduced resistance in the susceptibility of both *Salmonella* Typhimurium and *Escherichia coli* O157:H7 to disinfectants as observed by Leyer and Johnson (1997) and Stopforth et al. (2003), respectively. These reports have demonstrated the complexity of microbial stress response and suggest that microbial susceptibility to disinfectants might not only vary

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with microorganism but also be affected by the kind of sublethal stress to which the microorganism has been subjected. In the present study, the susceptibility of *V. parahaemolyticus* to a chlorine and a QACs disinfectants after prior exposure to various sublethal stresses was investigated to help better develop adequate control measures against this dangerous microorganism.

2. Materials and methods

2.1. Test organism and growth medium

In the present study, *V. parahaemolyticus* 690, a clinical strain capable of producing thermostable direct hemolysin was used as the test organism. It was obtained from Professor H. C. Wong (Dept. of Microbiology, Soochow University, Taipei, Taiwan). The test organism was first activated by two successive transfers in Tryptic soy broth (TSB) (Difco, Detroit, MI, USA) supplemented with 3% NaCl (Merck, Darmstadt, Germany) (TSB-3% NaCl) at 37 °C overnight. The activated culture was further grown in TSB-3% NaCl for 4 h and served as the inoculum to prepare the sublethal-stressed and control cells.

2.2. The sublethal-stress treatment of V. parahaemolyticus

Before exposure to sublethal stress, the prepared culture of *V. parahaemolyticus* was first centrifuged (3000 × *g*) for 10 min at 37 °C. The pellets were washed twice with phosphate buffer solution (PBS, 0.1 M, pH 7.0) supplemented with 3% NaCl (PBS-3% NaCl). For the preparation of heat-shocked and cold-shocked cells, the washed cells were suspended in pretempered PBS-3% NaCl and then submerged in a temperature controlled circulating water bath (BT-150, Yin-Der, Taipei, Taiwan) at 42 °C for 45 min and at 20 °C for 4 h, respectively, according to the method described by Chang et al. (2004) and Lin et al. (2004). The procedures described by Wong et al. (1998) were followed to prepare the acid-adapted cells of *V. parahaemolyticus*. Essentially, the washed cells of *V. parahaemolyticus* were suspended in PBS-3% NaCl that had its pH adjusted to 5.0 with 6 N HCl and incubated at 37 °C for 30 min. The washed cells in PBS-3% NaCl without exposure to sublethal stress served as the non-stressed (control) cells.

2.3. Disinfectant solutions

In the present study, disinfectants included: Quatricide, a QAC-base disinfectant with *n*-alkyl dimethyl ethylbenzyl ammonium chloride and *n*-alkyl dimethyl benzyl ammonium chloride as the active ingredients (Pharmacal Research Lab Inc., 2010), and Clidox-S, a chlorine dioxide disinfectant (Pharmacal Research Lab Inc.). The parent solution of these disinfectants was first prepared according to the manufacturer's instructions. The Clidox-S solution was prepared by mixing Clidox-S, distilled water, and Clidox-S activator at a ratio of 1:18:1 (v:v:v). The Quatricide solution was prepared using ca 59.1 mL (two ounces) of Quatricide mixed with 3785.4 mL (one gallon) of distilled water (w/v). Based on results obtained from preliminary experiments, the parent solution of the Clidox-S and Quatricide was further diluted with sterile RO water by 10- and 50-fold, respectively, to prepare the test disinfectant solution which contained chlorine dioxide, 6.40 ppm or QACs, 3.461 ppm.

2.4. The viability of sublethal stressed and control V. parahaemolyticus exposed to disinfectants

To examine the susceptibility of the test organism to disinfectants, 1.0 mL of either heat-shocked, cold-shocked, acid-adapted, or control cell suspension was inoculated into 50.0 mL of the diluted Quatricide or Clidox-S at a final population of ca 10^{6-7} cfu/mL at 25 or 40 °C. Exposure time for the Quatricide treated sample was 10 min while exposure to Clidox-S was 50 min. At specific exposure intervals, 1.0 mL of sample was withdrawn and combined with 1.0 mL of DeyEngley neutralizing broth (Sigma, Saint Louis, MO, USA) to neutralize components that might be lethal to the test organism (Sutton et al., 1991).

2.5. Enumeration of viable V. parahaemolyticus

To determine the viable population of *V. parahaemolyticus*, samples were first serially diluted in PBS-3% NaCl. The viable counts were then made by pour-plating on Tryptic soy agar (Difco) supplemented with 3% NaCl after 24 h of incubation at 37 °C.

2.6. Statistical analysis

The mean value and standard deviation were calculated from the data obtained from the three separate experiments. In each experiment, two samples were taken at the specific time intervals for the determination of the viability of the test organism. Data were compared using Ducan's multiple range test method in SAS, version 8 (SAS Institute, Cary, NC).

3. Results and discussion

3.1. The viability of sublethal-stressed and control V. parahaemolyticus exposed to Quatricide

In the present study, *V. parahaemolyticus* was first exposed to various sublethal stresses. These sublethal stresses have been reported to alter the susceptibility of *V. parahaemolyticus* to subsequent lethal stresses (Chang et al., 2004; Lin et al., 2004). Additionally, alterations in protein expression were also noted in the cells of *V. parahaemolyticus* after acid and heat shock treatments (Chiang et al., 2008; Wong et al., 1998).

Quatricide, being a QACs, is capable of interacting with the cytoplasmic membrane, causing cell leakage and membrane damage of microorganisms (Ioannou et al., 2007). In the presence of Quatricide at 25 °C, the survival of *V. parahaemolyticus*, regardless of sublethal stress, decreased as the exposure period was extended (Fig. 1A). Although the survival of the control and the sublethal-stressed cells determined after 2 min of exposure showed no significant difference ($P > 0.05$), it was generally observed that the survival rate of control *V. parahaemolyticus* was lower than the sublethal-stressed cells as the exposure period was further extended. After 10 min of exposure to Quatricide at 25 °C, the control *V. parahaemolyticus* showed a population reduction of 4.36 log cfu/mL (Table 1). In contrast, the sublethal-stressed *V. parahaemolyticus* exhibited a population reduction ranging from 3.28 to 3.81 log cfu/mL, which varied with the sublethal stress, yet was significantly less ($P < 0.05$) than that of the control *V. parahaemolyticus*. This clearly demonstrated that heat shock, cold shock, and acid shock treatments conducted in the present study enabled *V. parahaemolyticus* to be more resistant to Quatricide. Furthermore, the extent of enhanced resistance of *V. parahaemolyticus* to Quatricide varied with the sublethal stress treatments. Among the various sublethal stresses examined, the level of enhanced resistance to Quatricide induced by cold shock at 20 °C for 4 h was the least.

When exposure to Quatricide at 40 °C, the viable population of the control and sublethal-stressed *V. parahaemolyticus* also reduced with the extension of the exposure period (Fig. 1B). However, the viable population of *V. parahaemolyticus*, regardless

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