Low-concentration Sorbic Acid Promotes the Induction of *Escherichia coli* into a Viable but Nonculturable State

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The effect of food preservatives and sanitizers at low concentrations on the induction of *Escherichia coli* into a viable but nonculturable (VBNC) state was investigated. When *E. coli* was incubated in physiological saline at 37°C, the viable cell count measured by plate counting was approximately 3-logs lower than that measured by flow cytometry after 30 days. This difference, and morphological changes in cells, confirmed the transition of *E. coli* into a VBNC state. Adding 10 µg/l of sorbic acid significantly promoted the induction of *E. coli* into a VBNC state. This effect was not seen with benzoic acid or sodium hypochlorite at the same concentration. Resuscitation of *E. coli* VBNC cells was successful when they were grown in nutrient broth containing sodium pyruvate. These results suggest that the presence of low concentrations of food additives in a food manufacturing environment may act as potential triggers for bacterial VBNC induction.

Key words : Viable but nonculturable / Sorbic acid / Benzoic acid / Sodium hypochlorite / Resuscitation.

Recent studies have gradually made it clear that a wide variety of pharmaceutical ingredients are present in the water environment (Azuma et al., 2016; Tiedeken et al., 2017; Vasquez et al., 2014). The concentration level of these pharmaceutical ingredients in the water environment ranges from ng/L to µg/L, which is lower than the concentration level (mg/L) that exerts pharmacological action in the human body. However, because pharmaceutical ingredients are designed to exhibit specific physiological effects at the targeted site in the body at low concentrations (Ankley et al., 2007; Boxall et al., 2012), concerns about toxic effects on ecosystems inhabiting the water environment, health effects on human beings through drinking water and promotion of emergence of drug-resistant bacteria have also been reported by researchers (Azuma, 2018; Kümmerer, 2009; Sharma et al., 2016). Many chemical substances and food additives are also used in food factories, and some are discharged into the environment. Although these food additives are also diluted to much lower concentration than concentrations that exert an effect such as food preservation in foods, they may have some effect on environmental microorganisms as well as for the pharmaceutical ingredients.

In aquatic environments, it has been demonstrated that many bacteria, including human pathogenic bacteria, can enter the viable but nonculturable (VBNC) state when exposed to adverse environmental conditions (Nilsson et al., 1991; Oliver, 2016; Xu et al., 1982). Today, VBNC are broadly defined as cells that enter a non-culturable state in response to stress, while maintaining a detectable but reduced metabolism (e.g., decreased respiration, nutrient transportation, and synthesis of macromolecules), relatively high ATP levels, and aspects of cellular integrity such as an intact chromosome content and cell membrane (Kassem et al., 2013; Xavier et al., 2014). From a food hygiene perspective, contamination from VBNC bacteria may be underestimated during standard routine laboratory procedures, resulting in an improper assessment of contaminated pathogens in the food and environment (Dreux et al., 2007; Panutdaporn et al., 2006; Zhang et al., 2015). Recently, it has been reported that weak acids used as food preservatives induced the transition of bacteria (Besnard et al., 2002; Cunningham et al., 2009) and yeast (Liao et al., 2010) into a VBNC state.

In this study, we investigated whether the weak acids and sanitizers that are used as food additives in Japan influence the induction of *E. coli* cells into a VBNC state.
at low concentrations. Utilization criteria for sorbic acid, which is the preservative most commonly consumed by Japanese people per day (Matsumoto, 2014), in Japan’s Food Sanitation Act (Japan External Trade Organization, 2011) are determined as follows. For example, not more than 3 g/kg, not more than 2 g/kg, not more than 0.2 g/kg, and not more than 0.05 g/kg, for cheese, fish paste products, wine, and lactic acid bacteria beverages, respectively. We found that sorbic acid induced an E. coli VBNC state at much lower concentrations than the concentrations used for food preservation.

Strain E. coli NBRC 3301 stored in 10% glycerol solution at −80°C was thawed and preincubated in nutrient broth (NB, Eiken Chemicals Co. Ltd., Tokyo, Japan) at 37°C for 24 h. The preincubated E. coli were washed five times (3000 × g, 5 min) and resuspended in 1 ml of sterile physiological saline (0.85%) to a concentration of 10^10 CFU/ml.

A solution of 10-fold dilution series from 10 µg/l to 0.01 µg/l of sorbic acid, benzoic acid, or sodium hypochlorite (Wako Pure Chemicals Ind., Ltd., Osaka, Japan) was prepared with sterile saline (0.85%). The test bacterial suspension (100 µl) was added to the test tube containing the food additive solution (10 ml). Because the bactericidal effects of the food additives used in this study are lower when the temperature is lower (Matsuda, 1998), the experimental temperature was set at 37°C as the temperature where the food additives can begin to exhibit the bactericidal effect. The tube was then sealed and incubated at 37°C for 30 days with shaking at 110 strokes/min. These test substances had no antimicrobial activity against E. coli at 10 µg/l and 0.01 µg/l for 24 h (data not shown).

A portion (100 µl) of the incubated food additive solution was sampled every 5 days, and the viable cell count was measured by both plate counting and flow cytometry. In the plate count method, the samples were diluted with sterile saline and cultured on nutrient agar plates (Eiken Chemicals) at 37°C. After 24 h, the number of colony forming units was counted to calculate the viable cell count.

To measure viable E. coli by flow cytometry, a LIVE/DEAD® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used as a fluorescent stain. The BacLight™ Bacterial Viability Kit contains a mixture of SYTO® 9 and propidium iodide fluorescent dyes. SYTO® 9 permeates all cells, binding to DNA, causing a green fluorescent signal. Propidium iodide only enters cells that have significant membrane damage, which is an indication of nonviability and binds to nucleic acids with higher affinity than SYTO® 9. The combination of these two dyes provides a rapid and reliable method for discriminating between live and dead bacteria in VBNC studies (Da Silva and Martinis, 2013; Hasan et al., 2013; Su et al., 2009). Staining was performed according to the manufacturer’s instructions, and fluorescence was measured using a flow cytometer (Cell Lab Quanta SC MPL, Beckman Coulter, Inc., Brea, Calif., USA) to estimate the viable cell count. All experiments were performed in triplicate. Data are presented as the mean ± standard error of the mean.

Figure 1A shows the viable cell counts of E. coli incubated in sterile saline measured by the agar plating method and flow cytometry. The decrease in the viable cell count, measured by flow cytometry, was less than one order of magnitude during the 30 days. However, using the agar plate method, bacterial counts were determined to be reduced by approximately 3-logs. Furthermore, Gram staining confirmed that the morphology of the rod-shaped E. coli became small cocci with a diameter of 0.8 to 1.0 µm (data not shown).

Many researchers have reported similar morphological changes in E. coli that transformed into a VBNC state (Aurass et al., 2011; Munna et al., 2013). In physiological saline at 37°C, the E. coli cells used in this study did not wholly transfer to the VBNC state even after 30 days of incubation, and only a few cells were observed to be in the VBNC state. However, interestingly, the addition of 10 µg/l of sorbic acid did not decrease the number of viable cells as measured by flow cytometry compared with the control but the agar plate method showed that sorbic acid dramatically decreased culturable bacteria below the limit of detection (<100 CFU/ml) by day 15 (Fig. 1B). Therefore, the addition of sorbic acid significantly promoted the induction of E. coli into the VBNC state compared with the results in physiological saline.

Benzoic acid (10 µg/l), a preservative similar to sorbic acid, did not result in further induction of E. coli into a VBNC state compared with the control (Fig. 1C). Sodium hypochlorite (NaClO) is a sanitizer used in food processing. Studies have shown that NaClO treatment (effective chlorine concentration = 1 mg/l) induced a VBNC state in E. coli and Salmonella (Oliver et al., 2005). E. coli went into a VBNC state with NaClO treatment (10 µg/l) but not to the extent that it did with sorbic acid (Fig. 1D). In addition, treatment with any of the compounds at 0.01 µg/l or less had no effect on E. coli (data not shown).

Cunningham et al., (2009) reported that potassium sorbate at 50 mM induced L. monocytogenes into the VBNC state within 24 h at 37°C and a pH = 4. Because most weak acids exist in the molecular state (un-ionized form) on the acidic side of pKa, the antimicrobial activity of a weak acid, such as sorbic acid, becomes higher. A weak acid in the molecular state can easily pass through the cell membrane (Ekland, 1983).
The pKa of sorbic acid is 4.76 (Eklund, 1983), and induction of VBNC in L. monocytogenes was due to damage by sorbic acid, which permeated through the cell membrane at a low pH (Cunningham et al., 2009). However, the sorbic acid concentration in their study was approximately 700,000 times more than that in the current study and is therefore not comparable. The pH of the sorbic acid solution at 10 µg/l in this study was almost 5.6 (> pKa = 4.76), which was almost the same as that of physiological saline alone (pH = 5.9). Therefore, the promotion of the VBNC state may have occurred due to other factors besides the environmental pH. In the aqueous solution of pH 5.6, only 13% of the added sorbic acid is present in a molecular state, which easily permeates the cell membrane when calculated based on the value of pKa. It is thought that such an extremely low concentration of sorbic acid molecules invade and act inside the cell. However, although the pH of the benzoic acid solution at 10 µg/l was also 5.6, no promotion effect was observed.

The pKa of benzoic acid is 4.19 (Hollingsworth et al., 2002), which is lower than that of sorbic acid. When calculated based on the value of pKa, about 4% of the benzoic acid is present in the molecular state in aqueous solution at pH 5.6 and is about 1/3 the proportion of sorbic acid molecules at the same pH. This difference might be the reason that no promoting effect on the benzoic acid state was observed with benzoic acid. Possibly, the sorbic acid molecules at extremely low concentration permeate the cell membrane and act on E. coli, but it is not clear at the present stage if this is true.

We also investigated recovery from the VBNC state to a culturable state. E. coli cells induced into the VBNC state by sorbic acid (10 µg/l) on day 15 were used. The solution containing the VBNC E. coli bacteria was diluted with sterile saline and added to 10 ml of NB or NB supplemented with 1% of pyruvic acid to a concentration of 10^4 viable cells/ml. This concentration was estimated based on the viable cell count measured by flow cytometry. A portion of the NB medium (250 µl) was aliquoted into a 96-well plate and incubated at 37°C. Absorbance was measured at 620 nm with a microplate reader (TECAN Infinite® 200 PRO, Switzerland) for up to 24 h.

When the normal, nontreated E. coli (10^4 viable cells/
ml) were incubated in NB, the absorbance at 620 nm began to increase after 5 h (Fig. 2). In the case of adding 1% pyruvic acid to this state, a slightly faster threshold was observed than that without pyruvic acid, but the difference was not significant. However, VBNB E. coli treated with 10 μg/l sorbic acid did not grow in NB alone within 24 h. When the bacteria in the VBNB state are migrated from a low nutrient condition to a high nutritional condition, as with NB, disturbances in the physiological mechanisms occur, which produce reactive oxygen species that damage the cells. Pyruvic acid neutralizes active oxygen and promotes resuscitation from the VBNB state (Calabrese and Bissonnette, 1990; Czechowicz et al., 1996; Kimura et al., 2017; Morishige et al., 2013). When 1% sodium pyruvate was added to NB, VBNB began to grow exponentially after 17 h, resulting in successful resuscitation.

Addition of sorbic acid, even at an extremely low concentration, promoted induction to the VBNB state. The VBNB cells could return to the culturable state in the presence of pyruvic acid. The concentration of sorbic acid used in this study was significantly lower than that used in food preservation. In terms of future research, it is necessary to investigate what kind of stress the sorbic acid at this concentration causes physiologically and genetically to E. coli. The food industry uses large amounts of preservatives such as sorbic acid. The results obtained in this study suggest that even if food additives contained in food manufac-

turing environments and wastewater from the food industry are in extremely low concentration, they may act as a potential trigger for induction into a bacterial VBNB state.

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