Mathematical Analysis for Growth Depression of *Vibrio parahaemolyticus* in Shrimp under a High Carbon Dioxide Atmosphere

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Growth depression of *Vibrio parahaemolyticus* in pink shrimp under a high CO₂ atmosphere was studied. A strain was isolated from prawn and identified as *V. parahaemolyticus*. The identified strain was used to inoculate pink shrimp and stored at 20°C under air, N₂, and CO₂. The plate count under CO₂ 16 h after inoculation was significantly lower than those under air and N₂. The plate count under a steady state of CO₂ was lower by log 3.6 to log 3.7 than those under air and N₂. The growth of *V. parahaemolyticus* in/on the shrimp was fitted and simulated using the Gompertz equation and a new logistic model, to determine its growth rate parameters. CO₂ reduced the value of the parameter equivalent to maximum plate count.

Keywords: *Vibrio parahaemolyticus*, shrimp, carbon dioxide, Gompertz model, logistic model

Introduction

Pink shrimp (*Pandalus borealis*; Krøyer, 1838) is an important seafood resource in the world (Simard et al., 1992). As pathogens such as *Vibrio parahaemolyticus* (Fujino et al. 1951) Sakazaki et al. 1963 emend. West et al. 1986), a Gram-negative, halophilic asporogenous rod, can contaminate shrimp, hygienically distribution of shrimp in the market is a major concern (Levin, 2006). It is well known that CO₂ inhibits the growth of aerobes (Dixon and Kell, 1989), and the appearance of the shrimp degrades very rapidly after harvest due to enzymatic activity of polyphenol oxidase (EC 1.14.18.1), which appears as black spots on the surface (Gonçalves et al., 2003). Chen et al. (1992) reported that high CO₂ pressure inhibited the activity of polyphenol oxidase, and Gonçalves et al. (2003) depressed the appearance of black spots on pink shrimp using a modified atmosphere with a high CO₂ concentration.

Their findings suggest that CO₂ is effective for maintenance of both appearance and hygiene of shrimp. However, the effect of CO₂ on the growth depression of *V. parahaemolyticus* as a facultative anaerobe is still unclear.

In the present study, a strain of *V. parahaemolyticus* was isolated, and growth depression of the bacterium by CO₂ was investigated using the Gompertz equation, a kinetic model widely used for analysis of microbial growth (Buchanan, 1993; Ross and McMeekin, 1994), and a new logistic model proposed by Fujikawa et al. (2003).

Materials and Methods

Isolation and identification of a strain for experiments

Isolation of a new strain of *V. parahaemolyticus* for the present experiments was attempted. A strain of *V. parahaemolyticus* could be obtained from various cultures, like ATCC. However, the properties of these cultures may differ their original culture, due to repeated subculturing needed for maintenance (Makino and Fujisawa, 2001). Therefore, the isolation and identification of fresh microorganisms was performed in the present study.

*V. parahaemolyticus* was isolated from 30 g prawn (*M. supranus japonicus*; Bate, 1888) purchased from a local market (harvested in Vietnam) using alkaline peptone water (Oxoid, Ltd., Basingstoke, UK), a CHROMagar Vibrio plate (CHROMagar Microbiology, Paris, France), triple sugar iron
(TSI) (Eiken Chemical Co., Ltd., Tokyo, Japan) slant and 0 to 10% sodium chloride solutions.

The isolated strain was tested to confirm the identity of the strain to be *V. parahaemolyticus* by TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). The base sequence of 16S rDNA (Gürtler and Stanisich, 1996), the mol% guanine + cytosine (G + C) of the DNA (Katayama et al., 1984) and DNA-DNA hybridization (Ezaki et al., 1989) with NBRC 12711 (Biological Resource Center, National Institute of Technology and Evaluation, Tokyo, Japan) as the type strain of *V. parahaemolyticus* were performed for the identification.

**Storage of the isolated strain under CO<sub>2</sub> condition** Pink shrimp (*Pandalus eous* Makarov, 1935) (17.7 ± 1.1 g per shrimp) harvested on January 21, 2009, at Tajima fishery harbor (Japan), without any chemical treatment for depressing black spots, were sterilized in an autoclave for 15 min at 121°C to eliminate the influences on the growth of the tested strain by other microorganisms. A suspension (0.1 mL) containing 10<sup>4</sup> CFU/mL of the isolated *V. parahaemolyticus* strain was inoculated in a sterilized shrimp.

Two contaminated shrimp were put in a sterilized disposable petri dish and eight such prepared petri dishes were enclosed in a 7-L inner volume acrylic chamber (As One Co., Osaka, Japan); three such chambers were prepared. One chamber including air was closed under ambient temperature. Atmospheres in the other two chambers were replaced by flowing pure CO<sub>2</sub> and N<sub>2</sub> gases from gas cylinders for 5 min at a rate of 10 L/min. The concentration of CO<sub>2</sub> or N<sub>2</sub> in each chamber was over 99%. The atmospheric pressure in all the chambers was 1.01325 × 10<sup>5</sup> Pa. Atmospheres including air or high CO<sub>2</sub> concentration were prepared as control or test plots. Atmospheres including a high N<sub>2</sub> concentration (equivalent to air without O<sub>2</sub>) were prepared in order to confirm the effect of O<sub>2</sub> on the growth of the strain because the absence of O<sub>2</sub> from the atmosphere containing high CO<sub>2</sub> concentration may affect growth. It would be difficult to judge whether CO<sub>2</sub> depressed the growth simply by comparison between air and CO<sub>2</sub>, even if the growth is depressed in CO<sub>2</sub>. The chambers were stored at 20°C and one petri dish per chamber was removed every 4 h until 28 h from the start of storage for estimation of plate count. The storage temperature was selected as a lower one than that used in a previous study (Kimura et al., 1997) to investigate the growth depression of *V. parahaemolyticus* by CO<sub>2</sub> as Dixon and Kell (1989) reported that growth depression is stronger at a reduced temperature. The atmospheres in the chambers were replaced by the same gases as the start of storage just after each sampling. The petri dishes were also removed from the chamber 48 h after the start of storage. The contaminated shrimp before storage was used for the colony count at 0 h.

A sampled shrimp was put in a sterilized plastic bag with 90 mL saline and homogenized for 30 s using a stomacher. A 1.0-mL filtrate of homogenate was sampled and subjected to serial 10-fold dilutions. A 0.1-mL diluted suspension was uniformly spread on thiosulfate citrate bile sucrose agar plates. The plates were incubated for 18 h at 37°C, after which the number of colonies appearing on the plates were counted and the plates containing between 30 and 300 colonies were used.

Plate count data over time were plotted on a scatter graph. Several sigmoidal functions are effective for approximating the growth curves (Zwietering et al., 1990). Growth curves were generated from experimental data obtained in this study using the Gompertz equation (Eq. 1), which has been employed most often (Buchanan, 1993; Ross and McMeekin, 1994) in conjunction with SigmaPlot ver. 9.01 (SPSS, Inc., Chicago, IL, USA), a nonlinear least square fitting program that employs the Marquardt-Levenberg algorithm:

\[
N_t = N_0 + Ce^{-(e^t - e^M/t)}
\]

where \( N \) is the plate count (CFU/g), \( C \) is the difference between the initial and maximum plate count (CFU/g), \( e \) is the Naperian base, \( t \) is the storage time (h), \( M \) is the time at which the absolute growth rate is maximum (h), \( B \) is the maximum relative growth rate (relative growth rate at \( M \)) (h), and 0 is the start of storage.

Lag phase duration \( L \) (h) was estimated using Eq. 2 from the Gompertz equation (Zwietering et al., 1990).

\[
L = M - \frac{1}{B}
\]

Experimental data for plate counts were analyzed by the Tukey-Kramer honestly significant difference test (\( P < 0.05 \)) using JMP ver. 8.0 software (SAS Institute Inc., Cary, NC, USA).

A new logistic model proposed by Fujikawa et al. (2003) was also used to simulate growth of *V. parahaemolyticus*.

\[
\frac{dN_t}{dt} = B \cdot N_t \left(1 - \frac{N_t}{N_s - C} \right) \left(1 - \frac{N_s}{N_0} \right)^n
\]

where \( n \) is an adjustment factor.

Equation 3 was solved numerically with the 4-order Runge-Kutta method using EQUATRUN-G for Windows ver. 3.0.5 (Omega Simulation, Tokyo, Japan). As the initial value of \( N_s, N_0(1 - 10^{-5}) \) was input to avoid calculation error.

**Results and Discussion**

**Isolation and identification of a strain** The results of isolation and identification are presented in Table 1. The properties were consistent with those of *V. parahaemolyticus* (Baumann and Schubert, 1984).
The mol% G + C of the DNA was 45.7%, with the range of 46 to 47 being equivalent to *V. parahaemolyticus*, according to Baumann and Schubert (1984). Homology of 16S rDNA of the isolated strain with the type strain of *V. parahaemolyticus* (ATCC 17802) was 98.9% (Aporon DB-BA ver. 3.0) or 99.3% (GenBank/DDBJ/EMBL). However, DNA-DNA hybridization with the type strain is needed for identification of a species, according to Wayne *et al.* (1987) that stated that the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness. Therefore, a homology of 81% would be high enough to identify the isolated strain as *V. parahaemolyticus*.

**Growth analysis of isolated *V. parahaemolyticus* in shrimp under high CO₂ condition** Growth of the test strain in shrimp over time using three kinds of atmospheres with approximation and simulation curves generated using the Gompertz equation and the new logistic model is shown in Fig. 1. The measured data were well fitted to both models. Kinetic parameters calculated from Eq. 1 are presented in Table 1.

### Table 1. Characteristics of the strain isolated from prawn.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Results</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHROMagar Vibrio Plate</td>
<td>Lilac colony</td>
<td></td>
</tr>
<tr>
<td>Triple Sugar Iron Slant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slant</td>
<td>Red</td>
<td></td>
</tr>
<tr>
<td>Butt</td>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride</td>
<td></td>
<td>Isolation</td>
</tr>
<tr>
<td>0%</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>3%</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8%</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>16S rDNA</td>
<td>99.3, 98.9%</td>
<td>Identification</td>
</tr>
<tr>
<td>mol% guanine + cytosine of DNA</td>
<td>45.70%</td>
<td>Identification</td>
</tr>
<tr>
<td>DNA-DNA hybridization with type strain*</td>
<td>81%</td>
<td></td>
</tr>
</tbody>
</table>

*NBRC 12711

**Fig. 1.** Growth kinetics of *Vibrio parahaemolyticus* in pink shrimp under air (○), N₂ (□), and CO₂ (△) at 20°C. The symbols represent the mean ± SE of data from four experiments. Three solid lines denote nonlinear least square curves approximated using the Gompertz equation. Three dashed lines denote simulated curves generated by a new logistic model (Fujikawa *et al.*, 2003). Values of adjustment factor *n* were 0.42 (air), 0.26 (N₂) and 0.4 (CO₂), respectively.
Table 2. Kinetic parameters in the Gompertz model $N_t = N_0 + Ce^{-e^{-t/M}}$ for the growth of *Vibrio parahaemolyticus* in pink shrimp under various environmental conditions at 20°C.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Atmosphere</th>
<th>Kinetic parameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shrimp</td>
<td>Air</td>
<td>$N_0 = 3.4$</td>
<td>Present study</td>
</tr>
<tr>
<td>Shrimp</td>
<td>$N_2$</td>
<td>$C = 6.2$</td>
<td>Present study</td>
</tr>
<tr>
<td>Shrimp</td>
<td>CO$_2$</td>
<td>$B = 0.17$</td>
<td>Present study</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>Air</td>
<td>$M = 13$</td>
<td>Yoon et al.</td>
</tr>
</tbody>
</table>

$N$ is the plate count (CFU/g), $C$ is the difference between the initial and maximum plate count (CFU/g), $e$ is the Naperian base, $t$ is the storage time (h), $M$ is the time at which the absolute growth rate is maximum (h), $B$ is the maximum relative growth rate (relative growth rate at $M$) (/h), $0$ is the start of storage, and $L$ is the lag phase duration (h): $L = M - 1 / B$

Table 2. Furthermore, kinetic parameters calculated from the data published in a previous paper (Yoon et al., 2008) are presented in Table 2. Except for Yoon et al. (2008), no study has reported time-course changes in the plate count of *V. parahaemolyticus* at 20°C. The parameter values among experiments under air and $N_2$ in the present study, as well as those in the previous study (Yoon et al., 2008) were similar, although the strain and media were different. The Gompertz parameters in Table 2 were substituted into Eq. 3 to simulate the growth of *V. parahaemolyticus*. According to the results in Fig. 1, the parameters were effective for predicting the growth of the strain by plural equations.

Plate counts of the strain 16 h after inoculation were clearly depressed by CO$_2$, which was supported by statistical analysis (significance at the 95% level). This result agrees with the description of Dixon and Kell (1989) that CO$_2$ depresses the growth of many kinds of microorganisms. Parameter $C$, the difference between the initial and maximum plate counts in CFU/g is equivalent to the plate count at steady state 28 h after inoculation. Therefore, CO$_2$ may significantly reduce the value of $C$. However, Kimura et al. (1997) reported that atmospheres containing 20-100% CO$_2$ did not significantly depress the growth of *V. parahaemolyticus*. Therefore, this is the first report to note growth depression of *V. parahaemolyticus* by CO$_2$. Three differences were observed in the experimental methods between the present study and that of Kimura et al. (1997). The strain isolated here was used to inoculate shrimp and incubated at 20°C, while in the previous study (Kimura et al., 1997), strain IFO 12711 (equivalent to NBRC 12711) was used to inoculate the surface of plate count agar slant (Nissui Pharmaceutical Co., Ltd.) and incubated at 30°C. The kind of strain and media may affect the result of the experiments. It is known that the effect of CO$_2$ for growth depression increases with decreasing temperature (Dixon and Kell, 1989). Solubility of gases into liquids increases with decreasing temperature (Gevantman, 1995). CO$_2$ amounts dissolved in a cell at 20°C may be larger than that at 30°C and the depression effect of the gas on the growth of the strain in the present study appeared easier than that in the previous study (Kimura et al., 1997). Joseph et al. (1982) reported that the minimum growth temperature of *V. parahaemolyticus* is 9 to 10°C. Therefore, the depression effect of CO$_2$ at temperatures lower than 20°C has to be evaluated. On the other hand, Dixon and Kell (1989) reported that a decrease in pH caused by an increase in CO$_2$ dissolved in culture media may not affect the growth of microorganisms.

There were no significant differences between the plate counts in air and $N_2$, as expected, since *V. parahaemolyticus* is a facultative anaerobe that grows under O$_2$-independent atmospheres (Baumann and Schubert, 1984). Taken together, our results strongly support that growth was depressed not by the anoxia condition, but by CO$_2$.

The lag phase duration was not particularly longer under CO$_2$ than those under the other atmospheres. This may be due to the duration from 5.0 to 7.6 h being too short for observing the effect of gases on the duration time.

The results presented in this paper are novel, and we showed that CO$_2$ can cause significant growth depression of *V. parahaemolyticus* inoculated in shrimp. Our findings may be applied to gas displacement packaging of shrimp. The application methods of the depression effect by CO$_2$ should be investigated further in the future. The mechanism of growth depression of microorganisms by CO$_2$ still remains unclear (Dixon and Kell, 1989), and more investigations to clarify this mechanism are desired.

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References


