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Survival of Bacteria at a Subfreezing Temperature (–1°C)

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Preservation of foodstuffs at temperatures around –1°C has attracted special interest recently. We investigated whether bacteria contaminating foodstuffs, especially contaminating fish, were killed or survived at –1°C compared with 37°C. Survival rates of *Escherichia coli* K12 and *Staphylococcus aureus* IFO12732 in nutrient broth at –1°C for 7 days were 52% and 31%, respectively. However, the survival rate of *Vibrio parahaemolyticus* in nutrient broth containing 3% NaCl at –1°C for 7days was only 0.03%. When the bacteria were kept in a soy sauce solution containing alcohol and some seasonings (the soy sauce solution) at –1°C, survival rates of *E. coli* K12 and *S. aureus* IFO12732 after 2 days were 56% and 54%, respectively, but *V. parahaemolyticus* was completely killed after 24 h at –1°C in the soy sauce solution. When *E. coli* K12 and O157 and *V. parahaemolyticus* were incubated at –1°C in the soy sauce solution containing some pieces of raw fish (the improved soy sauce solution), 3 strains of the bacteria were not killed. These results indicate that bacteria contaminating fish are not killed at –1°C and that storage of fish at –1°C is not always effective in diminishing food poisoning.

Key words: *Escherichia coli*; *Staphylococcus aureus*; subfreezing point; survival rate; *Vibrio haemolyticus*

During distribution and storage, many food commodities need to be kept without loss of food quality. Food spoilage may be caused by bacterial growth and bacterial metabolites, which lead to pH-changes and formation of toxic compounds, off-odors and gas, and by oxidation of lipids and pigments, which lead to formation of compounds with undesirable flavor and discoloration (Barnes, 1994; Huis in't Veld, 1996).

Many methods have been developed for maintaining the quality of foods, such as dehydration of foods, smoking of meats or fish, preservation with salt and cold storage. There are 2 main methods for cold storage of foods: storage in the refrigerator and in the freezer. In recent years, great interest has been taken in the storage of foods and aging of processed foodstuffs at a subfreezing temperature (–1°C) (Yamane, 1986, 1987, 1996). For example, a flatfish which was kept for several hours at –1°C began to swim in warm sea water (Yamane, 1996). In order to keep foods fresh for longer times, foodstuffs such as viable fish, meats or vegetables, are increasingly stored at –1°C. However, it is not clear whether microorganisms contaminating the foods are viable at –1°C or not. In this study we investigated bacterial survivals at –1°C, and the contaminating bacteria attached to the surface of the fish after soaking them in the soy sauce, which is a Japanese original cooking addition.

Materials and Methods

Bacterial strains and preparation of bacterial suspension

Escherichia coli K12, *Staphylococcus aureus* IFO12732 and a clinically isolated strain of *Vibrio parahaemolyticus*, all stored in our labo-

Abbreviation: CFU/mL, colony forming units per milliliter

ratory at –85°C, were used in these experiments. *Escherichia coli* O157, which was an isolate from a patient with hemolytic colitis in Tottori Prefecture in 1996.

E. coli and *S. aureus* were separately transferred from a stored culture to a nutrient agar slant, which consisted of 0.5% (w/v) beef extract (Bacto beef extract, Difco Lab., Detroit, MI), 1% polypeptone (Nihon Seiyaku Co., Ltd., Tokyo, Japan), 0.5% NaCl and 1.5% agar. After incubation overnight at 37°C, a loopful of bacteria was inoculated into fresh nutrient broth, which consisted of 0.5% beef extract, 1% polypeptone and 0.5% NaCl. The cultures were incubated at 37°C overnight with shaking. Bacteria in the stationary phase were collected by centrifugation at 3,000 revolutions/min for 10 min, resuspended in fresh nutrient broth and prepared at a concentration of approximately 10^8 organisms/mL. Bacterial suspensions in test tubes were put in ice-water before use.

When *V. parahaemolyticus* was used for the experiment, the medium was nutrient broth containing 3% NaCl or nutrient agar containing 3% NaCl.

Treatment of bacteria at –1°C and 37°C

Five microlitters of bacterial suspensions were placed into tubes with 5 mL of fresh nutrient broth medium (final concentration of approximately 10^5 organisms/mL). The tubes were placed in an incubator with a refrigerating machine (Hitachi Incubator CR-14C, Tokyo), and the bacteria were cultured at –1°C or 37°C with shaking (reciprocating motion at 120 times/min) for up to 7 days. Pure water begins to freeze at 0°C, but bacterial suspension in nutrient broth did not freeze at –1°C. In order to certify bacterial survival after incubation at –1°C for various intervals, 0.2 mL of bacterial culture kept at –1°C was transferred to a test tube containing fresh nutrient broth and incubated at 37°C with shaking.

In another experiment, 5 µL of bacterial suspensions were transferred into 5 mL of a soy sauce containing alcohol (*mirin*) and some seasonings (the soy sauce solution), and incubated at –1°C or 37°C with shaking.

The soy sauce solution itself was further improved giving it a milder taste by incubating it at –1°C for 24 h with small pieces of raw fish meat before adding the bacterial suspension. A block of fish at a size of approximately 1.5 cm × 1.5 cm × 2 cm was put into the 17 × 130 mm test tube and 5 mL soy sauce solution was added (the improved soy sauce solution), and the tube was kept at –1°C for 24 h. To this suspension 5 µL of bacterial suspension was inoculated and incubated at –1°C or 37°C with shaking.

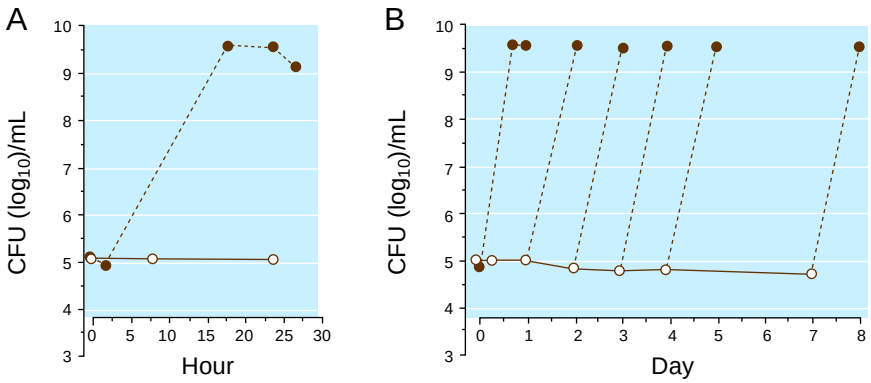

Fig. 1. Survival rate of *Escherichia coli* K12 in nutrient broth at –1°C. Approximately 1×10^5 *E. coli* K12 in 5 mL of nutrient broth were incubated at –1°C or 37°C with shaking. At various intervals, 0.2 mL of bacterial culture was removed and viable cells were counted by colony counting methods using 10-fold serial dilutions. **A:** Survival curve of *E. coli* K12 at –1°C (○) and 37°C (●). **B:** Survival rate of *E. coli* K12 after incubation at –1°C (○) for up to 7 days. On days 0, 1, 2, 3, 4 and 7, 0.2-mL aliquots of bacterial culture were put into 5 mL of fresh nutrient broth and incubated at 37°C (●).

Colony forming units and calculation of survival rate

At various intervals after incubation at –1°C or 37°C, 0.2-mL aliquots of the bacterial suspensions were removed, diluted serially with saline and plated onto nutrient agar plates with or without 3% NaCl for bacterial enumeration by colony counting. Survival rate was calculated by division of colony forming units per milliliter (CFU/mL) on fixed day by CFU/mL on day 0.

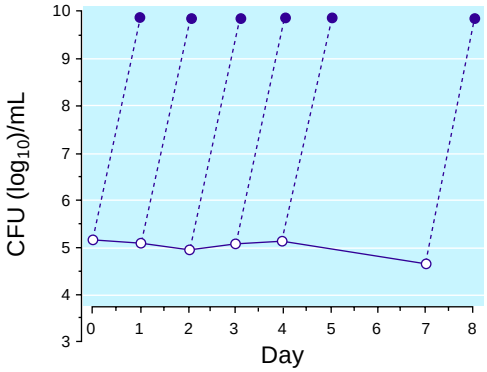

Fig. 2. Survival rate of *Staphylococcus aureus* IFO12732 after incubation in nutrient broth at –1°C (○) for 7 days. On days 0, 1, 2, 3, 4 and 7, 0.2 mL of bacterial culture was put into the fresh nutrient broth, and grown at 37°C (●).

Results

Survival rates in nutrient broth

Bacterial broth medium in test tubes did not freeze at –1°C for at least 7 days even in static cultures. When *E. coli* K12 was incubated at –1°C for 24 h, viable cell count was 100%, and the number of organisms did not increase (Fig. 1A). The survival rate of *E. coli* K12 after incubation at –1°C for 7 days was 52%. When 0.5 mL of *E. coli* K12 kept at –1°C for 7 days was put into fresh nutrient broth medium and incubated at 37°C, the bacteria grew to saturation within 24 h (Fig. 1B).

Viability of *S. aureus* was also good (31%) after 7 days in nutrient broth at –1°C (Fig. 2). By contrast, *V. parahaemolyticus* was not resistant to the low temperature (–1°C). When *V. parahaemolyticus* was kept in nutrient broth containing 3% NaCl at –1°C, viable cell count decreased gradually, with 15% survival on day 1, 6.2% on day 2, 0.6% on day 4, and 0.03% on day 7. At each time, transfer of a 0.2-mL aliquot of *V. parahaemolyticus* culture to 5 mL of fresh nutrient broth containing 3% NaCl revealed that the bacteria could grow to saturation within 14 h (Fig. 3).

Survival of bacteria in the soy sauce solution

When *E. coli* K12 was kept in the soy sauce solution, the survival rates at –1°C and 37°C after 2 days, were 56% and 0%, respectively

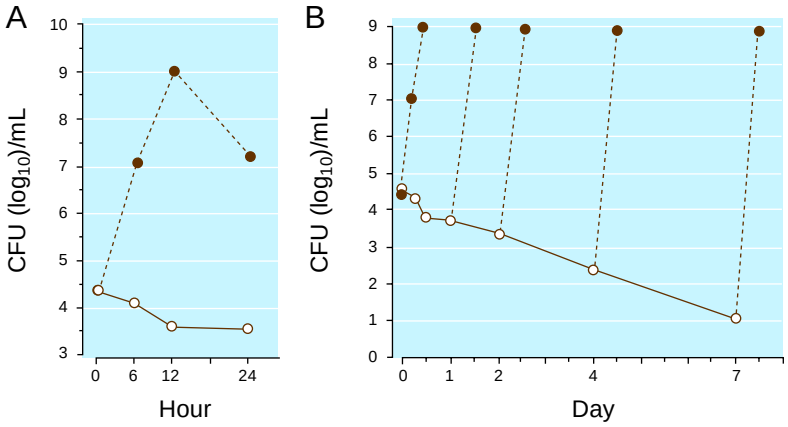

Fig. 3. Survival rate of *Vibrio parahaemolyticus* in nutrient broth with 3% NaCl at –1°C. Symbols are the same as shown in Fig. 1.

Table 1. Survival of *E. coli*, *S. aureus* and *V. parahaemolyticus* after incubation at –1°C and 37°C in the soy sauce solution

Bacteria	Temperature of incubation	CFU (log_{10})/mL		
		on day 0	on day 1	on day 2
E. coli K12	–1°C	6.57	6.52	6.32
	37°C	6.57	0**	0**
S. aureus IFO12732	–1°C	5.75	5.60	5.48
	37°C	5.75	0**	0**
V. parahaemolyticus	–1°C	4.12	0**	0**
	37°C	4.12	0**	0**

Data are typical of 2 or 3 experiments.
** $P < 0.01$ relative to the value on day 0.

(Table 1, from the expression: $10^{6.32}/10^{6.57} \times 100$).

In contrast to growth in nutrient broth, *E. coli* K12 in the soy sauce solution could not grow at 37°C, and was instead killed at 37°C. *S. aureus* also survived for 2 days at –1°C (54%), but was killed at 37°C. *V. parahaemolyticus* was killed in the soy sauce solution both at –1°C and 37°C.

Survival of bacteria in the improved soy sauce solution

E. coli K12 was not killed in the improved soy sauce solution at –1°C, and also retained viability after a 1-day incubation in the improved soy sauce solution at 37 °C (Table 2). *E. coli* O157 in the improved soy sauce solution was gradually killed by incubation at 37°C, but was not killed completely on day 2. Survival rates of *V. parahaemolyticus* in the improved soy sauce solution at –1°C and 37°C on day 1 were 1.1% and 0.08%, respectively, and those on day 2 further decreased. *E. coli* K12 and O157 in the soy sauce solution and the improved soy sauce solution, and *V. parahaemolyticus* in the improved soy sauce solution were more easily killed at 37°C than at –1°C, but when the raw fish suspended in the soy sauce solution was incubated at 37° for 2 days it was partially degraded by enzymes of the fish itself.

Table 2. Survival of *E. coli* K12 and *E. coli* O157 after incubation at –1°C and 37°C in the soy sauce solution containing some pieces of raw fish (the improved soy sauce solution)

Bacteria	Temperature of incubation	CFU (log_{10})/mL		
		on day 0	on day 1	on day 2
E. coli K12	–1°C	6.74	6.56	6.79
	37°C	6.74	3.40	0**
E. coli O157†	–1°C	6.96	7.06	6.90
	37°C	6.96	5.58	2.00**
V. parahaemolyticus	–1°C	5.25	3.30	2.17
	37°C	5.25	1.86**	0**

† Clinical isolate from a patient with hemorrhagic colitis in Tottori Prefecture in 1996.
** $P < 0.01$ relative to the value on day 0.

Discussion

The relationship between bacterial growth and temperature has been investigated in detail (Ratkowsky, 1982; Heitzer, 1991; Zwietering, 1991). Theoretical models of bacterial growth were developed for temperatures between 5 and 50°C. However, it is well known that bacterial growth is inhibited at temperatures below 4°C (Davey, 1994). As an exception, psychrophilic bacteria, yeasts and moulds can grow at temperatures below 4°C (Huis in't Veld, 1996; Berry, 1997). Preservation of foods by low temperature is one of the best strategies for keeping foods fresh (Yamane, 1986).

There are many studies on the bacterial growth in food (Majeed, 1990; Nerbrink, 1993; Baranyi, 1994; Barnes, 1994). Deterioration of food quality is partially due to spoilage by bacteria. Furthermore, up to this time in experimental laboratories, bacteria in stab media have been stored in the refrigerator (4°C) for short terms, and bacteria in an appropriate suspending media have been preserved in the freezer (–30°C or –85°C) or in liquid nitrogen (–196°C) for longer terms. So, it is important to know whether food-contaminating bacteria can survive at low temperatures, especially at –1°C, or not.

In our study, when *E. coli*, *S. aureus* and *V. parahaemolyticus* were suspended in nutrient broth with or without 3% NaCl and incubated at –1°C or at 37°C, the 3 species of bacteria used grew at 37°C, but did not grow at –1°C. However, *E. coli* and *S. aureus* in the soy sauce solution were killed at 37°C and only partially killed at –1°C. Because the soy sauce solution contains high concentrations of NaCl and alcohol, *E. coli* and *S. aureus* with higher metabolic activity (i.e., at 37°C) might have been more easily killed than resting bacteria (i.e., at –1°C). However, *V. parahaemolyticus* did not survive well at –1°C. Species of *Vibrio* vary with respect to the temperatures at which growth occurs. *V. parahaemolyticus* grows at temperatures above 20°C, but viability decreases gradually at temperatures below 15°C (Baumann, 1984). *V. parahaemolyticus* was completely killed in the soy sauce solution at –1°C and at 37°C during a 24-h period (Table 1). Killing of *V. parahaemolyticus* at –1°C might be due to the low temperature and inhibitory components in the soy sauce solution, such as NaCl and alcohol, and killing at 37°C might be due to metabolic activities of bacteria and the inhibitory components in the soy sauce solution.

In Japan and other countries in South-East Asia, a soy sauce solution containing alcohol and seasoning (the soy sauce solution) is often used for marinating raw fish before cooking. The soy sauce attaches and sinks into the fish, which makes it taste good. It is important to know whether raw fish containing *E. coli* in the soy sauce solution might be killed or not. We intended to test bacterial survival in the soy sauce solution following the cooking procedure as closely as possible. Another reason for testing the survival rate of *E. coli* in the soy sauce solution is an outbreak of food poisoning by *E. coli* O157 all over Japan in 1996 (Infectious Disease Surveillance Center, 1997). As people in Japan often eat raw fish, they were afraid that fish were contaminated with pathogenic microorganisms such as *E. coli* O157, *Salmonella*, *V. parahaemolyticus*, etc.

In contrast to our expectation that survival rates of *E. coli* K12 and O157 and *V. parahaemolyticus* in the improved soy sauce solution were the same as those in the soy sauce solution, 3 strains of the bacteria suspended in the improved soy sauce solution survived at –1°C for 2 days and also at 37°C for at least 1 day (Table 2). The improved soy sauce solution may contain some components of relatively low molecular weight, which oozed from the fish and might have neutralized the toxic substances in the soy sauce solution against bacteria. Another possibility is that the fish meat may absorb the unsuitable substances for bacteria surviving in the soy sauce solution, such as NaCl, alcohol and other ingredients. We used codfish in our experiment, and as we get codfish commercially, the condition of the fish was different with the change of season. Sometimes the fish was contaminated, and gram-positive rods and fungi were multiplied after 1- or 2-day culture. Thus, the details of the

experimental methods exhibit difficulties, such as the components of soy sauce and alcohol (*mirin*), the kinds and conditions of fish (frozen and thawed, or fresh raw); in these cases, we could only show general tendencies.

From these data, we conclude that when foods are preserved at –1°C, most contaminating bacteria can survive for a long time, just as the bacteria on the food can survive (or grow) at 4°C. Heating of the fish stored at –1°C may be necessary for complete killing of bacteria. Although the quality of foodstuffs might be maintained at –1°C for a relatively long term, bacteria on the foods might also survive at this temperature. Consequently, the storage of foodstuffs, especially fish at –1°C, can not always prevent food poisoning.

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