Viable but Non-culturable State of Bacterial Cold-water Disease Pathogen *Flavobacterium psychrophilum* at Various Temperatures

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ABSTRACT—Bacterial cold-water disease (BCWD) caused by *Flavobacterium psychrophilum* leads to heavy mortality of ayu *Plecoglossus altivelis* in Japan. Previously, a 28°C warmed water treatment was shown to be an effective treatment for BCWD, since *F. psychrophilum* can not grow and maintain colonies at this temperature. However, it was unclear whether the bacteria might resuscitate after the treatment was over. Therefore, we investigated colony formation, membrane potential and pathogenicity of *F. psychrophilum* at various temperatures (15°C, 23°C, 28°C and 33°C) in sterilized underground water. Within 2 days at 28°C, *F. psychrophilum* completely lost their colony-forming abilities but still maintained their membrane potentials. It seemed that these cells entered into viable but non-culturable (VBNC) state. However, experimental ayu infection revealed that VBNC *F. psychrophilum* cells were unable to cause BCWD, suggesting that the cells were progressing towards death at 28°C.

Key words: viable but non-culturable, *Flavobacterium psychrophilum*, bacterial cold-water disease, *Plecoglossus altivelis*, VBNC, BCWD

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS) (Bernardet *et al.*, 1996). They were originally isolated from coho salmon *Oncorhynchus kisutch* in North America in 1948 (Borg, 1960), but were not found in Japan until 1987, when they were isolated from ayu *Plecoglossus altivelis* (Wakabayashi *et al.*, 1994). BCWD induces heavy mortality of ayu and occurs not only in fish farms but also in natural rivers in Japan (Iida and Mizokami, 1996; Amita *et al.*, 2000). Chemotherapy is effective in curing BCWD of ayu (Ninomiya and Yamamoto, 2001; Sugahara *et al.*, 2010). The excessive use of antibiotics sometimes results in the development of drug resistance among bacteria (Rangdale *et al.*, 1997; Bruun *et al.*, 2000, 2003); residues of bactericidal drugs in fish and the environment are an increasingly important problem (Cabello, 2006), especially with respect to food safety.

An alternative to curing BCWD with chemotherapy is warmed water treatment. The optimum temperature for growth of *F. psychrophilum* is around 15–20°C; growth is not possible at temperatures above 25°C (Pacha, 1968; Bernardet and Kerouault, 1989; Holt *et*

al., 1989; Iida and Mizokami, 1996; Uddin and Wakabayashi, 1997; Lee and Heo, 1998). The warmed water treatment takes advantage of the psychrophilic characteristics of *F. psychrophilum*: When the temperature is raised to 28°C, *F. psychrophilum* in both modified cytophaga (MCY) broth and sterilized underground water completely lose their colony-forming abilities within 2 days (Sugahara *et al.*, 2010). However, the loss of colony-forming ability is not equal to cell death. Bacteria often enter viable but non-culturable (VBNC) state (Colwell *et al.*, 1985) under environmental stresses; in some cases, VBNC cells may be resuscitated and returned to culturable state (Magariños *et al.*, 1994; Wai *et al.*, 1996; Fouz *et al.*, 1998).

In the present study, we investigated VBNC state of *F. psychrophilum* at various temperatures in two environments: sterilized underground water (low-nutrient) and MCY broth (high-nutrient).

Materials and Methods

Bacterial strain and growth conditions

F. psychrophilum PH0424 was used in this study, which was isolated from a diseased ayu kidney at Hiroshima prefecture in 2004. The bacteria were stored at –85°C in culture medium. For the pre-culture, the bacteria were inoculated into 50 mL of MCY broth

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medium (0.2% tryptone [Difco], 0.05% yeast extract [Difco], 0.02% beef extract [Kyokuto Pharmaceutical Industrial Co.], 0.02% sodium acetate, 0.02% calcium chloride, pH 7.2). The inoculated broth was incubated at 15°C for 24 h with shaking (160 rpm). The following experiments were conducted with the bacterial suspensions (10^9 CFU/mL) in the early stationary phase.

Survival/growth of F. psychrophilum at various temperatures in sterilized underground water or MCY broth

We investigated the survival/growth of *F. psychrophilum* at various temperatures in sterilized underground water or MCY broth. The underground water was filtered through a membrane filter (pore size: 0.22 μ m) and sterilized by autoclaving at 121°C for 15 min. Basically, the experiments were conducted according to the method described by Sugahara *et al.* (2010). The bacterial density at the start of the experiment was adjusted to 10^8 CFU/mL. In the survival experiment with sterilized underground water, duplicate bacterial suspensions were kept at 15°C, 23°C, 28°C and 33°C. In the growth experiment with MCY broth, they were kept at 28°C only. One mL of experimental samples were collected from the bacterial suspensions on Hours 0, 6, 12 and Days 1, 2, 3, 5, 10 for use in the following experiments.

Cell counting

Total cell numbers were estimated by a direct count (Porter and Feig, 1980). The samples were fixed by 1% glutaraldehyde and stained with 4',6-diamino-2-phenylindole (DAPI, Wako) at 5°C for 30 min. The treated samples (100 μ L) were diluted 10-fold serially with sterilized phosphate-buffered saline (PBS) and 100–500 μ L of diluted samples were then filtered through black polycarbonate filters (pore size: 0.22 μ m, diameter: 25mm, Millipore). The filters were observed under a fluorescence microscope (Eclipse 80i with UV-1A filter, Nikon).

Numbers of culturable *F. psychrophilum* were estimated by a drop-plate method (Hoben and Somasegaran, 1982) with MCY agar (Sugahara *et al.*, 2010).

In the survival experiment with sterilized underground water, numbers of culturable *F. psychrophilum* were also estimated by the most probable number (MPN) method (Rowe *et al.*, 1977) with MCY broth in 96-well microtiter plates. Each of five wells received 20 μ L of bacteria and 180 μ L of MCY broth. The samples were 10-fold serially diluted with MCY broth and incubated at 15°C for 7 days. Positive wells (those with turbidity) were scored visually and MPN were calculated using a standardized table.

Bacterial membrane potential

Membrane potential was estimated with

LIVE/DEAD BacLight Bacterial Viability Kits (Molecular Probes). The experiments were conducted according to the manufacture's protocol with slight modifications. SYTO 9 and propidium iodide were mixed (1:1), and 30 μ L of the mixture was added to 970 μ L of sterilized PBS. Ninety microliters of the bacterial suspension was added to 10 μ L of the dye mixture and incubated in the dark at 15°C for 1 h. The stained sample was placed onto a microscope slide and covered with a cover slip. A fluorescence microscope (Eclipse 80i with B-2A filter, Nikon) was used to count at least 300 cells; the total cell count was estimated by direct counts of DAPI-stained samples described above.

Pathogenicity of F. psychrophilum to ayu

In order to investigate the pathogenicity of *F. psychrophilum* to ayu, fish were experimentally infected with bacterial cells that had been kept in the sterilized underground water at 15°C, 23°C, 28°C and 33°C for 1, 2 and 3 days. The Ayu had been captured in Lake Biwa in December 2008 and reared to an average weight of 4.3 g in a concrete tank at Shiga Prefectural Fisheries Experimental Station, Japan in running underground water at 18°C. Prior to the infection experiments, BCWD had never occurred in the rearing tank. After fish were anesthetized with FA 100 (Tanabe Pharmaceutical Co.), 20 μ L of the bacterial suspension was injected into the peritoneal cavity. The fish were then placed into duplicate 60-L tanks. Each tank contained 25 individuals. The fish were reared without food in running underground water at 18°C for 21 days. Dead fish were immediately removed from the tank and checked for BCWD symptoms.

Results

Survival of F. psychrophilum at various temperatures in sterilized underground water

There were no increases in total cells or colony counts in any temperature throughout the experiment (Figs. 1a–1d). In the 15°C and 23°C treatments, colony counts were 10^5 and 10^4 CFU/mL, respectively, at the end of the experiment (Figs. 1a and 1b). The colony-forming ability completely disappeared within 2 days in the 28°C and 33°C treatments (Figs. 1c and 1d). MPN accurately reflected colony numbers in all temperature treatments (Figs. 1a–1d).

Bacterial membrane potential and shapes of F. psychrophilum by LIVE/DEAD kit

Percentage of live cells of *F. psychrophilum* at various temperatures in sterilized underground water or MCY broth estimated by LIVE/DEAD kit was shown Table 1. Even though there was no ability of colony formation in the 28°C and 33°C in the sterilized underground water on Day 2 and 1, respectively, the staining

Fig. 1. Number of total cells, colony counts, numbers of live and dead cells, and MPN of *F. psychrophilum* in sterilized underground water, incubated at (a) 15°C, (b) 23°C, (c) 28°C and (d) 33°C. Each point shows mean (n=2). Dotted lines show the detection limit (50 CFU/mL) of colony count. Under the detection limit of colony count, number of bacteria in the water was treated as 0.

Table 1. Percentage of live cells of *F. psychrophilum* at various temperatures in sterilized underground water or MCY broth estimated by LIVE/DEAD kit

		Incubation time							
		0	Hour 6	Hour 12	Day 1	Day 2	Day 3	Day 5	Day 10
Sterilized underground water	15°C	98.7*	98.5	97.8	99.5	97.4	97.7	97.1	24.4
	23°C	98.7	97.0	96.4	99.0	87.4	52.2	15.8	8.8
	28°C	98.7	88.8	62.4	44.8	13.3	7.6	2.3	0.0
	33°C	98.7	30.6	12.7	12.9	2.1	0.2	0.8	0.0
MCY broth		28°C	97.0	71.4	63.6	55.0	38.1	8.2	0.7

*, mean (n=2)

for membrane potential indicated that about 13% of the total cells were still alive.

At the beginning of the experiments, only the typical shapes of *F. psychrophilum* were observed (Fig. 2). These shapes were maintained at all temperatures in the sterilized underground water (Fig. 2). However, in the 28°C MCY broth treatment, *F. psychrophilum* changed their shapes at Hour 6 and maintained the new, long-cell phenotype (twice as long as normal) until the end of experiment (Fig. 2).

Pathogenicity of *F. psychrophilum* to ayu

High numbers of live cells (10^7 – 10^8 cells/mL) and colony counts (10^6 – 10^8 CFU/mL) were observed in the 15°C and 23°C sterilized underground water treatments during all 3 days (Figs. 3a and 3b). Correspondingly, 40–66% of the fish in these treatments were killed from *F. psychrophilum* infections. On the other hand, *F. psychrophilum* cells, which lost the colony-forming ability at 28°C and 33°C in the sterilized underground water, had no pathogenicity to fish (Figs. 3c and 3d).

Fig. 2. *F. psychrophilum* in sterilized underground water or MCY broth stained by LIVE/DEAD kit. Green fluorescence indicates bacterial cells with intact membranes; red fluorescence indicates bacterial cells with damaged membranes. The bars indicate 5 μ m.

Fig. 3. Changes in the pathogenicity of *F. psychrophilum* after incubation at various temperatures (15°C, 23°C, 28°C and 33°C) in sterilized underground water. The bacterial suspension (20 μ L/fish) was injected into the peritoneal cavity of ayu. Mortality rates were calculated at 21 days after the bacterial challenge. Each point shows mean (n=2). Dotted lines show the detection limit (50 CFU/mL) of colony count. Under the detection limit of colony count, number of bacteria in the water was treated as 0.

Discussion

F. psychrophilum grows optimally at 15–20°C (Pacha, 1968; Bernardet and Kerouault, 1989; Holt *et al.*, 1989; Iida and Mizokami, 1996; Uddin and Wakabayashi, 1997; Lee and Heo, 1998), and is not able to grow at 25°C or more (Sugahara *et al.*, 2010). Among its optimum temperature range, *F. psychrophilum* could survive longer and maintain pathogenicity under starvation conditions such as sterilized stream and lake water (Madetoja *et al.*, 2003; Vatsos *et al.*, 2003). Therefore, it is not surprising to obtain the results in this study that *F. psychrophilum* maintained its colony-forming ability for at least 10 days in the sterilized underground water at 15°C and 23°C, and is unable to maintain the ability at 28°C.

The condition, where bacteria are capable of physiological upkeep but are no longer able to form colonies, is known as the viable but non-culturable (VBNC) state. This state can be caused by environmental stress, such as nutrient starvation (Colwell *et al.*, 1985). The phenomenon of VBNC has been reported among several fish pathogens, such as *Vibrio anguillarum* (Eguchi *et al.*, 2003), *Aeromonas salmonicida* (Morgan *et al.*, 1991), *Photobacterium damsela* subsp. *piscicida* (= *Pasteurella piscicida*) (Magariños *et al.*, 1994), and *P. Photobacterium damsela* subsp. *damsela* (Fouz *et al.*, 1998). Previously, *F. psychrophilum* that were cultured for 2 weeks in MCY broth at 14°C did not exhibit any colony formation, even though live cells could be detected with LIVE/DEAD kit for at least 10 weeks (Vatsos *et al.*, 2003). Our results describe another instance of VBNC of the bacteria; moreover, the present research indicates that *F. psychrophilum* will enter the VBNC state in sterilized underground water at 28°C.

During the VBNC state, cells often become smaller and form dwarf cells, which is thought to be a response that facilitates survival during long periods of starvation (Morgan *et al.*, 1991; Morita, 1993; Fouz *et al.*, 1998; Eguchi *et al.*, 2003; Vatsos *et al.*, 2003). Previous work has shown that *F. psychrophilum* cells became round when kept in starvation conditions for 4 weeks in sterilized stream water at 14°C (Vatsos *et al.*, 2003). We did not detect this pattern here. However, we observed morphological changes: *F. psychrophilum* in MCY broth at 28°C elongated to about twice the length of typical cells (Fig. 3). This is a response that has been seen in bacteria exposed to antibiotics or higher temperatures (Kogure *et al.*, 1979; Addinall *et al.*, 1996), and is caused by errors in DNA synthesis and cell division. We also observed this response in *F. psychrophilum* cells incubated in MCY broth containing nalidixic acid at 15°C (data not shown). Here, the 28°C treatment may have damaged some part of the cell division or DNA.

There have been attempts to resuscitate VBNC

cells using heat shock (Wai *et al.*, 1996), a shift from cold to room temperature (Fouz *et al.*, 1998), inoculation to a liquid medium (Vatsos *et al.*, 2003), and passage through a host (Magariños *et al.*, 1994). In the present study, we tried to resuscitate VBNC *F. psychrophilum* by inoculating them into MCY broth and infecting them into host fish. We found that the former technique was not successful at resuscitating the bacteria (Fig. 1). Because of maintaining its colony-forming ability, the bacteria in sterilized underground water at 15°C and 23°C were lethal when injected into ayu. However, VBNC bacteria that had been incubated at 28°C and 33°C were not pathogenic. This result indicates that even when *F. psychrophilum* maintained their membrane potentials, they no longer possessed pathogenicity against ayu after they lost their abilities to form colonies. *F. psychrophilum* at 28°C in sterilized underground water on Day 1 had kept a small amount of culturable cells (10^3 CFU/mL), but they did not kill ayu. Lethal dose 50% (LD50) of the strain used in this study against ayu was 1.8×10^7 CFU/mL (data not shown). Therefore, *F. psychrophilum* cells less than 10^3 CFU/mL were clearly insufficient to kill ayu. It is still unknown whether *F. psychrophilum* populations were completely eradicated, or had merely declined to undetectable levels. However, since the VBNC cells did not revive under any of the treatment conditions, and the VBNC cells of *F. psychrophilum* at 28°C had no pathogenicity to ayu, it appears likely that the bacteria were progressing towards death. Additionally, no *F. psychrophilum* cells were detected in the organs of experimentally-infected fish after three days of the 28°C treatment (Sugahara *et al.*, 2010). Thus, the current work confirmed the effectiveness of the 28°C warm water treatment against *F. psychrophilum* and indicated that its effects were long-lasting.

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