Comparisons of Internal Behavior after Exposure to *Flavobacterium psychrophilum* between Two Ayu (*Plecoglossus altivelis altivelis*) Strains Showing Different Cumulative Mortality to Bacterial Cold Water Disease

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ABSTRACT.   Bacterial cold water disease (BCWD) in ayu (*Plecoglossus altivelis altivelis*) has a serious impact on aquaculture and fisheries. There is known to be a significant difference among ayu strains with regard to mortality caused by BCWD. In this study, the immune response of different ayu strains against *Flavobacterium psychrophilum* infection was observed. One strain was resistant to infection by *F. psychrophilum*, and the other was susceptible to infection by the same bacteria. The number of bacteria in the body was observed in each ayu strain, and the change in bacterial counts was similar. However, there was a significant difference in bacterial count in the spleen between the two strains on days 6, 9, 12 and 15 after exposure. To observe the immune response against *F. psychrophilum*, agglutination assay using serum was performed. An agglutination reaction in the resistant ayu strain was observed in 4 out of 6 ayu on day 6 after exposure, while no reactions in the susceptible ayu strain were observed in any sampled fish until day 12. However, some reactions in the susceptible ayu strain were observed in surviving ayu. These results indicate that there is a correlation between the presence of bacterial multiplication and agglutination reaction against *F. psychrophilum*.

KEY WORDS: agglutination reaction, bacterial count, *Flavobacterium psychrophilum*, *Plecoglossus altivelis altivelis*, strain-dependent.

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Bacterial cold water disease (BCWD) is caused by *Flavobacterium psychrophilum*. This bacterium is considered to be one of most important fish pathogens for ayu, *Plecoglossus altivelis altivelis* [28]. BCWD has been found to occur in both fish farms and natural rivers in Japan [1, 8, 25], and outbreaks of the disease have resulted in significant commercial losses for the aquaculture and fishery industries. It is therefore important to prevent the occurrence of the disease in farm and river environments, and to this end, it is vital to gain a better understanding of host defense mechanisms of ayu against the disease. For several fish, it has been shown that strains differ in their susceptibilities to disease pathogens [3, 4]. Moreover, it has been reported that a difference in spleen size is related to mortality to BCWD among several strains in rainbow trout [6]. A significant difference in cumulative mortality from BCWD has been observed among various ayu stocks. Based on the results of a study using intraperitoneal inoculation, it has been inferred that different mortality among ayu stocks is caused by different immune reactivity within ayu. However, differences among stocks have not been examined with regard to body defense parameters [16]. To introduce an effective immune response against particular pathogens into target fish, the immune mechanisms of the fish must first be studied. Changes in bacterial count in tissues have been investigated in order to compare virulence among bacterial strains [7, 22, 23]. Changes in antibody response have been compared between immunized and control fish in rainbow trout with significant differences identified between them [13]. Thus, strain-dependent differences in cumulative mortality appear to be correlated with pathogen behavior and with immune-reactivity in the host. The spleen is an important immune organ and plays an antigen-trapping role in the mammalian immune response to pathogens. Similarly, in rainbow trout, the spleen is the major organ of resistance to BCWD [5, 17]. The number of *F. psychrophilum* shows similar results in various tissues (spleen, kidney, heart, liver, gills and blood) in ayu with the highest levels seen in the spleen [24]. *F. psychrophilum* in the spleens of infected ayu has been observed at 7 hr after challenge [14]. Thus, bacterial count in the spleen may be useful for assessing bacterial behavior in ayu. Comparison of changes in bacterial counts between susceptible and resistant strains may also be important in evaluating disease control.

Increases in antibody titer caused by BCWD vaccine inoculation have been recognized in ayu [18–20], but speci-

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ficity remains unknown. Moreover, serum antibody titer in the BCWD-resistant strain was higher than that in the susceptible strain after immunization with formalin-killed *F. psychrophilum* [15]. However, the change in infection by *F. psychrophilum* must be compared from the initial phase in order to find differences in immune reactivity between the two strains.

In two genetically different ayu strains (a landlocked strain and an amphidromous strain), there were significant differences in cumulative mortality rate following infection experiments [12]. It was confirmed that there are strain-dependent differences in disease susceptibility. In this study, we compared the changes in *F. psychrophilum* counts in the spleen of both strains after exposure. Moreover, we investigated changes in the agglutination titer against *F. psychrophilum* in the two strains.

MATERIALS AND METHODS

Fish strains and experimental infection: Two experimental strains, a landlocked strain (La-s) and an amphidromous strain (Am-s), were bred under the same environment by Gifu Prefectural Research for Freshwater Fish and Aquatic Environments. La-s was bred for five generations, originating from landlocked ayu in Lake Biwa in Shiga Prefecture. Am-s was bred for three generations, originating from amphidromous ayu in the Kiso River in Gifu Prefecture. The ancestor populations of the two strains were distinguishable by genetic markers, and the strains have maintained these genetic differences [26]. The average body weights of La-s and Am-s used in this study were 20.0 ± 3.6 and 19.2 ± 3.8 g, respectively. Before exposure, the right (La-s) or left (Am-s) pelvic fin of the experimental fish was cut away as an identifying marker. Cultured fish in two strains were used for attempted isolation of *F. psychrophilum* from gills and kidneys 10 days before the beginning of this trial. Bacteria were not detected in sampled fish (data not shown). These cultured strains did not show distinctive symptoms of BCWD and developed BCWD in both immature and mature stages. Experimental infection was performed based on reported procedures with some modifications [16]. Briefly, 24 fish (La-s) were housed in an upper tank (100-*l* tank) with five fish killed by BCWD. Discharged water from the upper tank was introduced into two 500-*l* tanks for experimental infection. The two strains of fish were mixed and cultured in the two tanks (for La-s and Am-s, n=75 and 76, respectively, in tank 1; and n=78 and 67, respectively, in tank 2). Water temperature ranged from 16.5 to 17.4°C. Dead fish were removed from the experimental tanks daily and were counted. The dead, surviving and sampled fish from the experimental tanks were identified by cuts on the pelvic fin.

Number of F. psychrophilum in spleen: Prior to the experiment and on days 3, 6, 9, 12, 15, 18, 21, 24 and 27 after exposure, three La-s fish and three Am-s fish were scheduled to be sampled from the surviving fish in both 500-*l* tanks. However, three La-s fish were sampled on days 15, 18, 21 and 24, and two fish were sampled on day 27, due to the number of dead fish in the tanks. The spleen was removed

from the sampled fish, followed by homogenization with a pellet mixer on ice, and this slurry was suspended in a centuple volume of spleen weight with modified Cytophaga broth [27]. Bacterial count (CFU/g) in the spleen was determined by plating 10-fold serial dilutions on modified Cytophaga agar plates [27]. Plates were incubated for at least 5 days at 15°C. Subsequently, four yellow colonies were randomly selected and confirmed as *F. psychrophilum* by PCR with the following primers, PSY-G1 [10] and *fpPPIC1* [30].

Agglutination assay: In order to observe the body defense response to *F. psychrophilum*, we used agglutination assay. Before exposure and on days 6, 12, 18 and 24 after exposure, peripheral blood was collected with a syringe from the caudal vessels of sampled fish. Blood was left for 60 min on ice. Subsequently, serum supernatant was collected by centrifugation at $20,000 \times g$ for 5 min at 4°C, and serum was stored at −80°C until use. *F. psychrophilum* ATCC49418 was cultured with Cytophaga agar [2] for 3 days at 18°C, inoculated into 100 m*l* of Cytophaga broth and cultured for 3 days at 18°C. Cultured cells were washed twice with 0.85% physiological saline using centrifugation at $10,000 \times g$ for 5 min at 4°C. Harvested cells were suspended in 10 m*l* of 0.85% saline with 0.2% (v/v) formalin to kill the cells and were placed for 24 hr at 37°C. Formalin-killed cells (FKCs) were used as antigen to determine agglutination activity. Agglutination titer in the sera from the two strains was measured using the micro-titer method. After serum was treated at 56°C for 30 min to inactivate complement activity, a two-fold dilution series was generated using 1% PBS. The volume used for each serum dilution was added to an equal volume of FKCs. The mixture was then incubated at 4°C overnight. Agglutination titer was assessed by visual observation. Agglutination titer value was maximal attenuation magnification to minimal agglutinate.

Statistical analysis: *F. psychrophilum* counts in the spleen were compared between La-s and Am-s by one-way ANOVA for each sampling day. In this study, the detection limits of the bacterial counts in the spleen and agglutination titer were 1,000 CFU/g and 3 ($\log_2$), respectively. If the bacterial counts or agglutination titer was less than the detection limits, we assumed the counts to be 999 CFU/g or 2 ($\log_2$), respectively.

RESULTS

Bacterial number in fish spleen: During exposure to bacteria, the number of dead fish differed between La-s and Am-s, and the trend in cumulative number of dead fish was similar for each strain in both tanks. Cumulative number of dead fish in the two tanks on each day is shown in Fig. 1. Dead fish were observed on day 3 after exposure in La-s. The number of dead fish in La-s increased rapidly after day 7, and all fish were lost through sampling or death from the two tanks after day 24. In total, 118 of La-s fish (n=153) died, and 35 fish were used for measurements up to day 24 after exposure. Am-s fish began dying after day 10, but the cumulative number of dead fish of Am-s was lower than that of La-s. Am-s stopped dying at day 22 after the exposure.
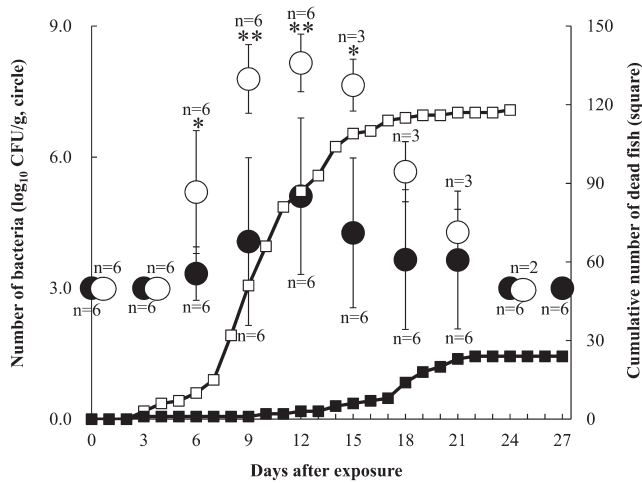
Fig. 1. The changes of *F. psychrophilum* counts (circle, mean and standard deviations) in the spleens and cumulative number of dead fish (square) by BCWD for landlocked strain (white) and amphidromous strain (black). Significant differences of the bacterial count between the two strains were indicated (*: $P<0.05$, **: $P<0.01$).
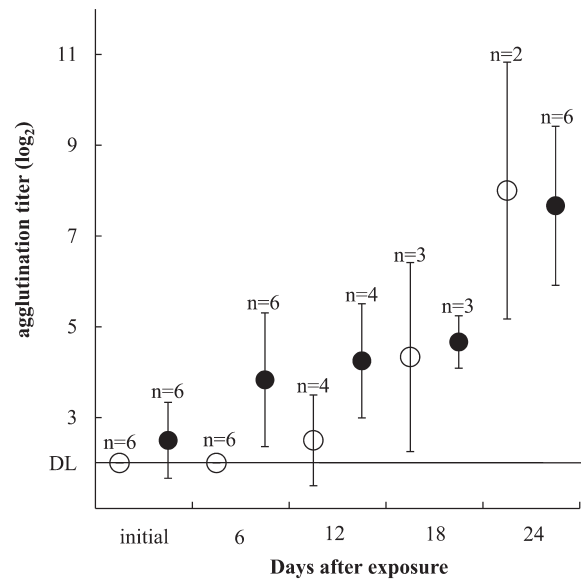

Fig. 2. The changes of agglutination titers in landlocked (white circle) and amphidromous (black circle) strains. The values represent the mean and standard deviations. DL: Detection limit.

Twenty-four Am-s fish (n=143) died, and 54 fish were used for measurement. Sixty-five fish survived until the end of the experimental infection period. There were no significant differences between bacterial counts in La-s and in Am-s, between the two tanks (P=0.578 and 0.525, respectively); therefore, we decided that the bacterial counts in the spleen between the two strains could be compared. *F. psychrophilum* was detected in the spleens of both strains from days 6 to 21 after exposure, although the bacterial counts differed. Bacteria in La-s were detected in most of the sampled fish from days 6 to 21. Bacteria in Am-s were detected in two fish on days 6 and 9, in most of the fish on days 12 and 15 and in only one fish on days 18 and 21. There were significant differences in splenic bacterial counts between La-s and Am-s on days 6, 9, 12 and 15 ($P<0.05$ or $P<0.01$) (Fig. 1). The average bacterial counts ($\log_{10}$) on day 6 in the spleens of La-s and Am-s were 5.2 and 3.3 (CFU/g), respectively. Bacterial counts ($\log_{10}$) on days 9, 12 and 15 in La-s were 7.8, 8.2 and 7.6 (CFU/g), respectively, and those in Am-s were 4.1, 5.1 and 4.3 (CFU/g), respectively. The average of bacterial counts in both fish strains increased from days 6 to 12 and decreased thereafter.

Agglutination assay: Agglutination assay was performed using the micro-titer method. Agglutination titers are shown in Fig. 2. Titers were below the detection limit in La-s fish tested before exposure and on day 6 after exposure. An increase in agglutination titers was observed in one fish on day 12 and in most fish measured on days 18 and 24. In contrast, agglutination reaction was confirmed in two tested Am-s fish before exposure and in 4 of 6 fish on day 6 after exposure. Moreover, an increase in agglutination titers was observed for all tested fish from day 12 onwards.

DISCUSSION

There was a strong association between the mortality caused by BCWD and increases in *F. psychrophilum* count in ayu in this study. Growth of *F. psychrophilum* was observed in all La-s fish sampled at the peak death rate (days 9, 12 and 15), and many fish died from BCWD. In contrast, although *F. psychrophilum* was detected in most sampled Am-s on days 12 and 15, numerous Am-s fish survived until the end of the experiment. Moreover, the average bacterial count for Am-s was significantly lower than that for La-s. These results suggest that bacterial multiplication in ayu was inhibited by host-defense mechanisms in Am-s. In other words, the results show that the mortality caused by BCWD is a consequence of bacterial multiplication in ayu. It has been reported that *F. psychrophilum* is present in various organs in ayu within a short period after immersion exposure [14].

It has been suggested that the ability to control bacterial number in ayu is the most important factor for resistance to BCWD. Agglutination titer against *F. psychrophilum* in La-s increased with bacterial infection. A comparable result was reported using different ayu strains [11]. In contrast, some of the sampled fish in Am-s showed an agglutination reaction against *F. psychrophilum* in their sera before exposure. Moreover, titers were observed for 4 of 6 tested fish on day 6 in the early phase of infection. The presence of agglutination reaction may be related to inhibition of bacterial multiplication in ayu. There are significant differences in cumulative mortality caused by BCWD between every generation of La-s and Am-s [12]. The difference in cumulative mortality of BCWD among the stocks used in experiments is reported to be genetically inherited [15]. Therefore, the difference in

agglutination activity between the two strains may also be a genetic characteristic to affect cumulative number of dead fish.

In the non-specific immune response, natural antibodies [9] or lectin [29] is thought to be involved in an agglutination reaction. In this study, it was inferred that there are natural antibodies present in Am-s serum, as Am-s serum showed an agglutination reaction against *F. psychrophilum* before exposure. This result was not considered to be due to a specific antibody resulting from *F. psychrophilum* infection before the experiment, as *F. psychrophilum* was never isolated from this strain prior to experimental infection. Moreover, the strain has never developed BCWD since the first generation, and both the Am-s and La-s used in this study were cultured in a facility blocked from the outside environment using a single ground water source. These results suggest that *F. psychrophilum* had no chance of contacting either strain before the experiment.

It has been demonstrated that there is a correlation between disease resistance and inhibition of bacterial multiplication during early infection. As there were no effects on infection due to different bacteria, it appears that the agglutination activity was due to an antibody. Some Am-s fish showed agglutination activity against *F. psychrophilum*, and Am-s may have a higher opsonic activity than La-s fish against the pathogen. Opsonization by fish antibody and complement has been shown to support the phagocytic response against pathogens in salmon [21]. Phagocytes in the head, kidney and spleen of rainbow trout have also been observed to play a role in BCWD protection [5, 17]. Therefore, it is necessary to research the opsonic effects in ayu serum.

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