Acceleration of the Occurrence of Vibriosis in Ayu *Plecoglossus altivelis* by Cyanobacterial Picoplankton *Synechococcus* sp.

Satoru Suzuki,*¹ Toshihide Suenobu,*¹ Hiroto Maeda,*² and Riichi Kusuda*¹

*¹Fish Disease Laboratory, Faculty of Agriculture, Kochi University, Nankoku, Kochi 783, Japan

*²Lake Biwa Research Institute, Otsu, Shiga 520, Japan

(Received June 17, 1994)

Vibriosis in ayu *Plecoglossus altivelis* has occurred in Lake Biwa, Japan, along with the blooming of cyanobacterial picoplankton. To search the factors accelerating the occurrence of vibriosis in ayu, the effect of picoplankton on the mortality of ayu was investigated. Pretreatment of ayu with *Synechococcus* sp. clone P before infection accelerated the occurrence of vibriosis in experimental infections. Moreover, a higher number of *Listonella anguillara* adhered to the skin which was taken from fish reared with clone P. These findings suggest that the acceleration of the occurrence of vibriosis by clone P is caused by facilitating the attachment of *L. anguillara* to the skin.

Key words: vibriosis, ayu, picoplankton, *Synechococcus*

Lake Biwa is the largest freshwater lake in Japan, and one of the world's most ancient lakes which, for a long time, underwent very little ecological change.¹⁾ This lake originally was oligotrophic; however, in recent years the trophic level of the North Basin of this lake changed from oligotrophic to mesotrophic. With the change in the trophic level, bloom formation by phytoplankton has been occurring in the lake.²⁾ The seasonal variation in the numbers of picoplankton is from 10^3 cells/ml to 10^6 cells/ml. In early summer in 1989, the maximum abundance reached to 2×10^6 cells/ml. Three clones of picoplankton were isolated from the bloom, which were named as clones P (pink), G (green), and B (brown) according to their color in culture. All clones were identified as a group of *Synechococcus* based on their biological characteristics and their color substances.²⁾ Occurrence of *Synechococcus* blooming in fresh water of Lake Biwa is the first evidence of this kind in the world.

On the other hand, vibriosis in ayu *Plecoglossus altivelis* frequently occurs accompanied with the blooming.^{3,4)} Since ayu is the most important fish of the fishery industry in Lake Biwa, an outbreak of vibriosis in ayu, concomitant with the bloom causes not only an ecological unbalance in the lake but also much economical loss. Although a certain correlation was observed between the blooming and vibriosis, no attempt to reveal the mechanism of the occurrence of vibriosis caused by the picoplankton blooming has not been performed yet.

The aim of this study was to search the mechanism of vibriosis acceleration in ayu using an experimental infection system. We found the accelerated mortality of ayu when reared with *Synechococcus* sp. clone P before infection. Also the fact is described that the pre-culture of ayu with *Synechococcus* sp. clone P increases the adhesion of the causative bacterium, *Listonella anguillara* (formerly *Vibrio anguillarum*) to ayu skin. The primary effect of *Synechococcus* sp. on vibriosis in ayu is discussed.

Materials and Methods

Fish

Ayu *Plecoglossus altivelis* was used in an experimental infection study, which were kindly provided by Kochi Freshwater Aquaculture Center. Average body weight of fish used in this study was 10 g.

Picoplankton, Bacterial Strains and Their Culture

Cyanobacterial picoplankton, *Synechococcus* sp. clones P and G²⁾ were cultured in a CT medium containing 15 mg $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 10 mg KNO_3 , 5 mg $\beta\text{-Na}_2\text{-glycerophosphate}$, 4 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 μg vitamin B_{12} , 0.01 μg biotin, 1 μg thiamine, 50 mg TAPS and 0.3 ml P IV metal solution in 100 ml and pH was adjusted to 8.2 with HCl. The P IV metal solution contains 19.6 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 3.6 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 100 mg EDTA in 100 ml distilled water. A culture was performed for 4 weeks at 20°C under 1000 lx. For counting cell numbers, the appropriate amount of culture was filtrated through 0.22 μm pore-size Fuji FM-22 filter. The cell numbers on the filter were directly counted under epifluorescent microscope (1000 $\times$) equipped with a 100 W halogen lamp, a 546 nm excitation filter, 580 nm dichroic mirror and a 590 nm barrier filter.

Causative bacterium of vibriosis, *Listonella anguillara* strain PT87041 was used in this study, which was isolated from ayu having typical vibriosis in 1987 in Tokushima, Japan. *L. anguillara* was cultured for 24 h at 25°C in the medium containing 5 g polypepton, 1 g yeast extract, 1 g proteose peptone, 1 g bonito extract and 20 g NaCl in 1 l and pH was adjusted to 7.8 with NaOH. While preparing a solid medium, 1.5% bactoagar was added. The LD₅₀ of this strain against ayu was evaluated to 2.5×10^3 cells/ml, which was measured by a water-born challenge according to the method of Kawano *et al.*⁵⁾ and the calculation method of Reed–Muench.⁶⁾

Direct Toxicity of Picoplankton to Ayu

Twenty five fish in each group were reared at 18°C in 50 l aquaria with recycling water under no feeding. *Synechococcus* sp. clones P and G were added to the aquaria with final concentrations of 4.2×10^6 and 2.4×10^6 cells/ml, respectively. In the control group, CT medium was employed. The fish mortality in each group was recorded. Attachment of *Synechococcus* sp. to fish was not surveyed in this study.

Experimental Infection of Ayu with *L. anguillara*

As described above, 25 ayu in each group were used for infection study. An experimental infection was performed according to the method of water-borne route of Kawano *et al.*⁵⁾ with some modifications. In brief, fish were immersed in 5% NaCl solution for 5 sec followed by exposure to water containing *L. anguillara* for 5 min. The fish mortality was surveyed

for 4 to 5 days after infection. Reisolation of the bacterium was performed in all dead fish. Two experimental groups were set up as follows.

i) Rearing of fish with picoplankton after infection

After infecting with 6.4×10^4 cells/ml of *L. anguillara*, ayu were reared with *Synechococcus* sp. clone P or G. Number of cells of clones P and G were 4.2×10^6 and 2.4×10^6 cells/ml, respectively. A CT medium was added to the control group.

ii) Rearing of fish with picoplankton before infection

Ayu were reared with 3.4×10^6 cells/ml of *Synechococcus* sp. clone P for 24 h before infection. The pre-treated ayu with picoplankton were challenged with 1.0×10^4 cells/ml of *L. anguillara*, followed by rearing without picoplankton. The fish pretreated with CT medium were used as the control group.

Adhesion of *L. anguillara* to Ayu Skin

Cell numbers of *L. anguillara* attached on the skin surface of ayu were counted by using semi-*in vitro* system. Skin was removed from ayu, which was placed on the inner side of the cap of a glass vial and was tightly held between a vial edge and the cap. The surface area of the fixed skin was 0.8 cm^2 . To examine the effect of coexistence of picoplankton and *L. anguillara*, mixture of 1.0×10^6 cells/ml of *Synechococcus* sp. clone P or G and 1.0×10^7 cells/ml of *L. anguillara* was accommodated in the vial. The vial was incubated for 1 h at 25°C to expose the skin to the mixture. The skin was removed and washed thoroughly in phosphate buffered saline (PBS). The number of adhering bacteria was counted by the plate count method and expressed as a number per cm^2 area of the skin. Adhesion of *L. anguillara* to the skin of ayu pre-reared with picoplankton was also examined. Fish employed for this experiment were reared for 24 h in water containing 4.8×10^6 cells/ml of clone P or 2.1×10^6 cells/ml of clone G before removal of the skin. The control fish were reared in water added with CT medium. The skin was exposed to 3.2×10^7 cells/ml of *L. anguillara*. Cell numbers attached to the skin were measured as described above. The total viable bacterial cells on the skin surface of the fish used in this experiment were also surveyed. One cm^2 surface area of the fish skin was wiped with sterile cotton pin, which was then well suspended in PBS. Cell numbers were counted by the plate count method.

Results

Toxicity of Picoplankton to Ayu

When ayu were reared in the presence of picoplankton, one and five fish died in the clone P treated-group and clone G treated-group, respectively (Fig. 1). However, since four fish died in the control group and no symptoms were observed in any of the dead fish, the death of fish in all groups is thought to be due to physiological stress. Bacteria were not isolated from the kidneys of any of the dead fish.

Mortality of Ayu Rearing with *L. anguillara* after Infection

Mortality of ayu reared with picoplankton after infection with *L. anguillara* was surveyed. As shown in Fig. 2, 19 to

Fig. 1. Direct toxicity of picoplankton.

Ayu were reared with picoplankton clone P (□) or G (△). Control group (○) was reared with only a CT medium.

24 fish died within 4 days after the challenge in all groups. All dead fish showed typical symptoms of vibriosis, such as pop-eye and hemorrhage in the base of fins and on the body surface. Reisolation of *L. anguillara* from the kidneys of all dead fish indicates that all fish were killed by *L. anguillara* infection.

Mortality of Ayu Treated with Picoplankton before Infection

To evaluate the effect of pretreatment with picoplankton, ayu were reared in water containing picoplankton for 24 h before infection. As shown in Fig. 3, twenty three fish died within 96 h after infection. However, all fish succumbed until 60 h in the group pre-treated with *Synechococcus* sp. clone P. Moreover, it was observed in the picoplankton treated-group that mucus was secreted on the body surface before the challenge. Symptoms were developed and the reisolation of *L. anguillara* was successfully done in all fish as described above.

Adhesion of *L. anguillara* to Ayu Skin

Semi-*in vitro* experiments to count the cell numbers of *L. anguillara* adhering to ayu skin were performed by using skin taken from healthy and picoplankton pre-treated fish. In this assay system, since picoplankton cannot grow on

Fig. 2. Effect of picoplankton on mortality of *L. anguillara*-infected ayu.

Ayu were reared with picoplankton clone P (□) or G (△) after challenging with *L. anguillara*. To control group (○), a CT medium was added.

Fig. 3. Pretreatment effect of picoplankton clone P on the mortality of ayu.

Fish were reared for 24 h with picoplankton clone P before infection with *L. anguillara*, and reared in picoplankton free water after challenged (□). Control group (○) was not pretreated.

Fig. 4. Numbers of bacteria adhering to ayu skin. Ayu skin was exposed to the mixture of *L. anguillara* and picoplankton.

Fig. 5. Numbers of bacteria adhering to ayu skin which was taken from the fish reared with picoplankton.

the medium, most of the colony forming bacteria were identified as *L. anguillara* by an agglutination test using antiserum against serotype J-O-1. As shown in Fig. 4, when the skin from healthy fish was exposed to a mixture of picoplankton and *L. anguillara*, no significant differences were observed in the numbers of bacteria adhering among the three groups tested. There was a tendency in the groups with picoplankton that the viable count was slightly lower than the control group without picoplankton. Few viable cells could be counted on the surface of the healthy skin exposed to PBS. Therefore, most of the bacteria found in the skin exposed to *L. anguillara* suspension are thought to be *L. anguillara*.

Adhesion of *L. anguillara* to the skin of fish reared in water containing picoplankton was examined. The results are shown in Fig. 5. Cell numbers adhering to clone P-treated fish skin was significantly higher than other two groups. There was no difference between the control group and clone G-treated group. Total viable counts on the fish

Fig. 6. Viable bacterial counts on the skin surface of normal ayu and picoplankton pretreated-ayu.

skin were also measured in this experiment. As shown in Fig. 6, no significant difference was observed among the three groups, although the numbers in clone P-treated group were higher than others. This indicates that the most of bacteria attached on the skin of clone P pre-treated fish were mainly *L. anguillara*.

Discussion

In early summer in 1989 and 1990, vibriosis in ayu occurred in the North Basin of Lake Biwa, Japan, accompanied with the blooming of cyanobacterial picoplankton.^{3,4)} Such an incidence has not been reported so far anywhere else around the world. It is still unclear whether the blooming of the picoplankton in Lake Biwa is caused by augmentation of eutrophic level of the lake. Also, the cause of a synchronous increase in mortality of ayu with blooming is not known.

In this study, the effect of picoplankton on the occurrence of vibriosis in ayu was investigated. Direct toxicity of picoplankton to ayu was not observed. Coexistence of picoplankton with *L. anguillara* infected-ayu also did not affect the mortality. However, a noteworthy finding is that the pretreatment of ayu with picoplankton clone P before infection accelerated the occurrence of vibriosis. Moreover, secretion of skin mucus was observed in the fish when pretreated with clone P. It was reported that the primary step of *L. anguillara* infection in ayu occurs on the skin surface,^{7,8)} whereas the skin mucus has preventive effect on the infection.⁹⁾ From these findings, it is suggested that the picoplankton excessively stimulates the production and secretion of mucus on the skin surface; this result leads to the attachment of *L. anguillara* on the skin.

These effects of picoplankton on ayu found *in vivo* study were further examined *in vitro*. When skin obtained from healthy ayu was exposed to the mixture of picoplankton and *L. anguillara*, bacterial numbers attached to the skin were not significantly different among experimental groups. However, bacterial numbers adhering to the skin which was taken from fish reared with picoplankton clone P was much more than other groups. This indicates that the acceleration of the occurrence of vibriosis by clone P is caused by facilitating the attachment of *L. anguillara* to the skin. This

effect was not observed by clone G, suggesting that the two clones have different effects on the occurrence of vibriosis in ayu. At present, three clones of Synechococcal picoplankton have been isolated from Lake Biwa. If the two clones belong to different species, it is possible that the difference in their effects depends on their species specificities. Also, whether various clones of picoplankton show a synergistic effect is an interesting point to be clarified. Moreover, naturally attached bacterial numbers on the skin of fish pretreated with picoplankton were not significantly different from the control. This suggests that picoplankton acts against not only ayu but also *L. anguillara* in the process of occurrence of vibriosis. The effect of picoplankton on *L. anguillara* will be reported in the near future.

Acknowledgments We wish to thank Dr. Y. Yoshida for his advice and arrangement of this study. This study was supported in part by a Grant-in-Aid for Scientific Research from the Fisheries Agency, Ministry of Agriculture, Forestry, and Fisheries of Japan.

References

- 1) S. Horie: Paleolimnological problems of Lake Biwa-ko, *Mem. Coll. Sci. Univ. Kyoto, Ser. B (Biology)*, **28**, 53–71 (1961).
- 2) H. Maeda, A. Kawai, and M. M. Tilzer: The water bloom of Cyanobacterial picoplankton in Lake Biwa, Japan. *Hydrobiologia*, **248**, 93–103 (1992).
- 3) Biwako Kenkyusho News (Oumia), No. 29, 7 (1989).
- 4) Biwako Kenkyusho News (Oumia), No. 33, 7 (1990).
- 5) K. Kawano, T. Aoki, and T. Kitao: Immersion vaccination and water-borne challenge of ayu (*Plecoglossus altivelis*) against vibriosis. *Fish Pathol.*, **18**, 143–149 (1983).
- 6) L. J. Reed and H. Muench: A simple method of estimating fifty per cent endpoint. *Am. J. Hyg.*, **27**, 493–497 (1938).
- 7) N. Funahashi, T. Miyasaki, K. Koderu, and S. Kubota: Histopathological studies on vibriosis of ayu (*Plecoglossus altivelis*) *Fish Pathol.*, **8**, 136–143 (1974) (in Japanese).
- 8) T. Kanno, T. Nakai, and K. Muroga: Mode of transmission of vibriosis among ayu *Plecoglossus altivelis*. *J. Aquat. Anim. Health*, **1**, 2–6 (1989).
- 9) K. Kawai and R. Kusuda: Mechanisms of protection in ayu orally vaccinated for vibriosis—I. Protection activity in body surface mucus. *Fish Pathol.*, **16**, 1–8 (1981).