Toxicity evaluation of a strain isolated from pollack digestive tract using *Paramecium caudatum*

Song-guk Rim[1], Chol Hong[1], Yong-jin Ri[1], Kyong-ryong Nam[1] and Hui-won Kim[2]*
1. Department of Genetic Engineering, Institute of Microbiology, The State Academy of Sciences, Unjong, Pyongyang City, DPR KOREA
2. Department of Daily Food, Institute of Microbiology, The State Academy of Sciences, Unjong, Pyongyang City, DPR KOREA
*khw1980@star-co.net.kp

Abstract
A strain was isolated from the digestive organs of Walleye pollack residing in the East Sea of the DPR Korea, which not only produces cryophilic protease, organic acid but also has intestinal adhesion property and bacteriostatic activity against pathogens. The strain was classified and identified as Pseudomonas fragi by genetic system classification methods and registered as KCCC 10110 at the Korean Centre for Culture Collection (KCCC) of the State Academy of Sciences, DPR KOREA. In vivo and in vitro toxicity of Pseudomonas fragi KCCC 10110 was evaluated using Paramecium caudatum.

In the treatments with P. fragi KCCC 10110, Bacillus subtilis KCCC 11214 and Lactobacillus lactis KCCC 13208, all of these being well known as non-toxic and probiotic for animals and fishes, higher microbial counts and normal movement of P.caudatum were observed while significant lower cell numbers and abnormal movement of P.caudatum were shown in the treatments with pathogenic strains Clostridium tetani 473, Salmonella typhosa 901 and Aeromonas punctata 823. These results suggest that probiotic and pathogenic bacteria have different influence on Paramecium caudatum and that the strain isolated from Walleye pollack digestive tract is non-toxic.

Keywords: *Paramecium caudatum*, toxicity, probiotics, *Pseudomonas fragi.*

Introduction

Paramecium caudatum is widely used in toxicity, water quality and contamination test of food, medicine, livestock and fish industry due to its cultivation easiness and high sensitivity to toxic substances[2]. The lethal time, kinetic and morphological changes of *P. caudatum* are good indicators for the toxicity evaluation of many kinds of toxic substances. The toxicity assessment of bioactive substances based on the physiological response of paramecium gives a suggestion to the possibility for the toxicity evaluation of the candidate probiotic bacteria isolated from fish.[3,12]

In general, the microbial pathogenicity is defined by invasive and pathogenic microbial toxicity. Invasiveness is the ability of pathogenic microorganisms to invade into and proliferate in the host tissues. The toxicity of pathogens to organisms is caused by the toxins of microorganisms and the toxins are divided into exotoxins and endotoxins. The presence of microbial exotoxin was evaluated with supernatant obtained by centrifugation after the liquid-culture and the presence of endotoxin was evaluated with the cell itself by *P. caudatum*.[7]

From the data on the toxicity assessment of many organic and inorganic substances and the ability of the paramecium to assess *in vivo* and *in vitro* toxicity, we designed to use paramecium to examine the toxicity of the isolates from Walleye pollack gut.[1,7,10,13]

In general, pathogenicity evaluation of the probiotic candidate strains isolated from fish requires time-consuming efforts demanding labor. Our best knowledge, no toxicity test data of probiotic. In this study, we examined and summarized the effects of various isolates on the kinetic status and survival of Paramecium.[2,6,11,15]

Material and Methods

Culture and cell counting of *Paramecium caudatum*: *P. caudatum* was isolated from freshwater samples and identified as *P.caudatum* by their kinetic state, flocculation, shape and size under the optical microscope. It was incubated at room temperature (20 ± 2 °C) using rice straw exudate.

Then, cells were collected at the exponential growth stage and the testing strain cultures were suspended in the supernatant obtained by centrifugation. The time of lethality and the change in kinetic morphology were observed. The parameciums were fixed with fixing solution (ethanol: acetic acid = 3:1) on a cytometer and the cell numbers were counted under an optical microscope.[4,6,10]

Cultures and centrifugation of strains: All strains were incubated in LB medium with 10% seawater exudate for 24 h and the culture medium was centrifuged at 7,000 rpm at 10°C for 10 min to obtain supernatant and biomass respectively.

Kinetic and morphological observations of *Paramecium caudatum*: In each experiment, paramecium motility was observed by using a single well test apparatus. The apparatus consists of a microscopic slide with 5mm-diameter well in its center and a silicon sheet (0.5mm thickness). Fifty microliters of the paramecium cell suspension in the logarithmic phase were placed in the well and kinetic state and morphology were observed under optical microscopy. Five replicates were performed for each sample.[3,14,15]

Evaluation of toxicity intensity of strains: The culture supernatant and the biomass of the strains obtained by centrifugation were added to *P.caudatum* culture and the morphology and motility of paramecium cells were observed at 5, 20, 40 and 60 min. If *P.caudatum* was motile but degraded morphologically, it was regarded as "dead". If more than 70% of *P. caudatum* died at 5, 20 and 40 min, it was evaluated as high toxic, median toxic and weak toxic respectively. If no abnormal motility and morphology changes were observed at 60 min wiithout any dead cell, it was evaluated as nontoxic.[2,8]

Experimental design for application to the Pollack larvae of isolates: The fertilized eggs of pollack were collected in the East Sea of the DPR Korea. The hatchery (40L) was sterilized with 25ppm nano-antimicrobial agent for 40min and washed with sea water. The water flow rate was 0.7L/min initially and 1.2L/min finally at fourth day after incubation. After 11 days from fertilization, nano-antimicrobials (25ppm) were added to aqueous water at every 3h intervals per day. The treatment of isolates was carried out in separate experimental tanks on 4~11 days after fertilization. On day 13 after fertilization, the pollack larvae were transferred to the feed resin bath (100L) as soon as the eggs were hatched. Three treatments were set up and each of them consisted of four baths (100fish/L).

Four plastic baths were treated with isolates at the egg and the post-larvae stage and eight plastic baths were untreated (control). The temperature of the seawater was maintained at 6°C under good aeration and the flow rate was gradually increased from 0.7 to 1.5L/min. Light intensity was provided at 500lx and a concentration diet of algae was added three times a day. The fortified rotifers and shrimps were fed to Walleye pollack post-larva and the exudate was stirred three times daily. The isolates were added to the treatments at 2, 4, 15, 25 and 35 days-old of post-larvae.[5,7,9,11]

Survival rate and specific growth rate of pollack larvae: Survival rate and specific growth rate (SGR) of all post larva were measured by following equation.

Survival rate (%) = (living population/initial individual) × 100

SGR (mg/day) = $(\ln Wt - \ln W_0) \times 100/t$

where Wt=weight of pollack at a given time and W0=initial weight of pollack and t=duration(day).

Data analysis: All data were expressed as mean ± SE or mean ± SD.

Results and Discussion

Toxicity evaluation of isolates by *Paramecium caudatum*: First, the toxicity of the culture supernatant of the strains was examined (Table 1). The supernatant of the strain *Pseudomonas fragi* KCCC 10110 showed no linear movement of fast speed while *P. caudatum* spread evenly without a complicated and rotational motion. In addition, no dead cells were present after 180 min. The probiotic *Bacillus subtilis* KCCC 11214 and *Lactobacillus lactis* KCCC 13208 were homogenized in the culture supernatant. However, in the culture supernatant of pathogens, *P. caudatum* was concentrated at edge through rotational motion and their morphology and motility gradually disappeared. This is a typical reaction of *P. caudatum* against toxic substances.

The toxicity of the isolates was then examined (Table 2). In the case of the strain *Pseudomonas fragi* KCCC 10110, the probiotic strains *Bacillus subtilis* KCCC 11214 and *Lactobacillus lactis* KCCC 13208, the cell counts of *P. caudatum* were higher and it showed normal motility whereas the numbers of *P. caudatum* decreased significantly and very show motion or no motion was observed in the treatments with pathogens.

From tables 1 and 2, it can be seen that the responses of *P. caudatum* to the culture supernatant and biomass of probiotics and pathogens are quite different. Moreover, in the case of probiotics, it can be drawn that the *P. caudatum* cell counts increased and showed no motility change. From these result, we can conclude that the isolates from the Walleye pollack intestine are not toxic.

Effect of isolates on the special growth rate, survival rate, body weight and body length of walleye pollack larvae: We conducted experiments to determine whether isolate *P. fragi* had a positive effect on the special growth rate, survival rate and body weight of post-larvae walleye pollack larvae or not (Table 3).

Table 1
Kinetic status and 100% lethal time of *Paramecium caudatum* (medium)

		Kinetic status	100% lethal time (min)
Test	*Pseudomonas fragi* KCCC 10110	normal	-
Probiotics	*Bacillus subtilis* KCCC 11214	normal	-
	Lactobacillus lactis KCCC 13208	normal	-
Pathogens	*Clostridium tetani* 473	rotary motion, dead	3.3±0.5
	Salmonella typhosa 901	rotary motion, dead	44.7±2.1
	Aeromonas punctata 823	rotary motion, dead	15.3±1.2

Table 2
Kinetic status and 100% lethal time of *Paramecium caudatum* (biomass)

		Mobility	Cell/ml (Paramecium)
Test	*Pseudomonas fragi* KCCC 10110	normal	185.3±4.1
Probiotics	*Bacillus subtilis* KCCC 11214	normal	190.0±5.7
	Lactobacillus lactis KCCC 13208	normal	165.1±7.1
Pathogens	*Clostridium tetani* 473	slow straight and rotary motion	25.6±4.2
	Salmonella typhosa 901	-	-
	Aeromonas punctata 823	-	-

Table 3
Comparison of specific growth rate (SGR), survival rate, weight and body length of post-larvae walleye pollack before and after treatment

	After hatching (days)	Control	Test
SGR(mg/day±SE)	4	$2.39±0.02^a$	$2.69±0.04^b$
	15	$3.51±0.06^b$	$3.61±0.05^b$
	25	$2.86±0.06^c$	$2.92±0.03^c$
	35	$2.26±0.03^a$	$2.68±0.06^b$
ASR(%±SD)	35	20.3±5.7	24.4±6.1
Body weight(mg ± SD)	35	4.64±0.46	5.38±0.49
Body length(mm ± SD)	35	9.4±0.3	10.1±0.6

Different superscript letters within same rows indicate significant differences between control and test.

The specific growth rate of isolate treatment was significantly higher and reached 2.68 ± 0.06 at 35 days after hatching. The survival rates of the control and treatment groups were significantly different with 20.3% and 24.4% respectively. Also, the average survival rate of the isolate-treated larvae was 1.2 times higher than control group. The weight and length of treated group were significantly higher than that of the control. Overall, the isolates from the pollack digestive tract were found to be beneficial for growth.

Conclusion

The main objective of this study was to develop a reliable, simple and rapid method to evaluate the toxicity of candidate probiotics in laboratory conditions. The toxicity test method using a protozoan is simpler, faster and more sensitive than using higher animals. We conclude that the evaluation method of toxicity using paramecium is a new method to examine the toxicity of probiotic candidates. However, more detailed and in-depth research is needed in this field.

Acknowledgement

We thank Dr. Ryu Myong-chol for his kind help in elaborating our manuscript and in encouraging our further study.

References

1. Brutkowska M., The effect of certain salt solutions and osmotic stimuli on the ciliary movement and food intake in *Paramecium caudatum*, *Acta Protozoologica*, **4**, 353-364 **(1967)**

2. Elanskii S.N., Petrunina Y.V. and Lavrova O.I., A comparative analysis of *Stachybotrys chartarum* Strains isolated in Russia, *Microbiology*, **73**, 60-65 **(2004)**

3. Epstein S.S.M., The Photodynamic effect of the Carcinogen, 3,4-Benzpyrene on *Paramecium caudatum*, *Cancer Research*, **23**, 35-44 **(1963)**

4. Hussain M.M. and Amanchi N.R., Low cost microbioassay test for assessing cytopathological and physiological responses of ciliate model *Paramecium caudatum* to carbofuran pesticide, *Pesticide Biochemistry and Physiology*, **90**, 66-70 **(2008)**

5. Jorunn S., Ingrid B., Stine W.D. and Olav V., Probiotic strains introduced through live feed and rearing water have low colonizing success in developing Atlantic cod larvae, *Aquaculture*, **438**, 17-23 **(2015)**

6. Kaza M.J., Boczek M., Izydorczyk K. and Sawicki J., Toxicity assessment of water samples from rivers in Central Poland using a battery of microbio tests a pilot study, *Polish Journal of Environmental Studies*, **16(1)**, 81-89 **(2007)**

7. Lauzon H.L., Gudmundsdottirc S. and Pedersend M.H., Isolation of putative probionts from cod rearing environment, *Veterinary Microbiology*, **132**, 328-339 **(2008)**

8. Miyoshi N., Kawano T., Tanaka M., Kadono T., Kosaka T., Kunimoto M., Takahashi T. and Hosoya H., Use of paramecium species in bioassays for environmental risk management: determination of IC50 values for water pollutants, *Journal of Environmental Health Science and Engineering*, **49**, 429-435 **(2003)**

9. Paweł M.R. and Marcin M.C., Influence of Selected Fluorescent Dyes on Small Aquatic Organisms, *Acta Geophysica*, **59**, 91-109 **(2011)**

10. Rao J.V., Srikanth K., Arepalli S.K. and Gunda V.G., Toxic effects of acephate on *Paramecium caudatum* with special

emphasis on morphology, behaviour and generation time, *Pesticide Biochemistry and Physiology*, **86**, 131-137 **(2006)**

11. Reid H.I., Treasurer J.W. and Birkbeck T.H., Analysis of bacterial populations in the gut of developing cod larvae and identification of *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus* as pathogens of cod larvae, *Aquaculture*, **288**, 36-43 **(2009)**

12. Rouabhi R., Berrebbah H. and Djebar M.R., Toxicity evaluation of flucycloxuron and diflubenzuron on the cellular model *Paramecium* sp., *African Journal Biotechnology*, **5(1)**, 045-048 **(2006)**

13. Sako F., Taniguchi N., Kobayashi N. and Takakuwa E., Effects of food dyes on *Paramecium caudatum*: toxicity and inhibitory effects on leucine aminopeptidase and acid phosphatase activity, *Toxicology and Applied Pharmacology*, **39**, 11-17 **(1977)**

14. Takahashi T., Yoshii M., Kawano T., Kosaka T. and Hosoya H., A new approach for the assessment of acrylamide toxicity using a green paramecium, *Toxicology In Vitro*, **19**, 99-105 **(2005)**

15. Takiguchi N., Tajima T., Asayama K., Ikeda T., Kuroda A., Kato J. and Ohtake H., Behavioral responses of the ciliated protozoan *Paramecium caudatum* to 2,4-dichlorophenoxyacetic acid and its analogues, *Journal of Bioscience and Bioengineering*, **93**, 416-420 **(2002)**.

(Received 26th October 2022, accepted 30th December 2022)