魚病研究 Fish Pathology, 35 (2), 91–92, 2000. 6

A Brown Pigment-producing Strain of *Pseudomonas plecoglossicida* Isolated from Ayu with Hemorrhagic Ascites

Se Chang Park[1], Ichiro Shimamura[2], Masaru Hagihira[2] and Toshihiro Nakai[1]*

[1]*Fish Pathology Laboratory, Faculty of Applied Biological Science, Hiroshima University, Higashihiroshima, Hiroshima 739-8528, Japan*

[2]*Tokushima Prefectural Fisheries Experiment Station, Hiwasa, Tokushima 779-2304, Japan*

(Received November 24, 1999)

Key words: *Pseudomonas plecoglossicida*, bacterial hemorrhagic ascites, brown pigment, ayu, *Plecoglossus altivelis*

Bacterial hemorrhagic ascites causing mass mortalities has spread among cultured population of ayu *Plecoglossus altivelis* in Japan[1,2]. The causative becterium of the disease is closely related to *Pseudomonas putida* but differs from it in some biochemical characteristics[1,2], and a new species name *P. plecoglossicida* was proposed for the bacterium on the basis of phenotypic characteristics, 16S rRNA gene analysis and DNA-DNA hybridization[3]. During epidemiological research work of the disease in Tokushima Prefecture, Japan, in 1999, we isolated a phenotypic variant of *P. plecoglossicida* which produces brown diffusible pigment on agar plate. This paper describes isolation and characterization of the brown pigment-producing strain of *P. plecoglossicida*.

Materials and Methods

Brown pigment-producing becteria were isolated from ayu with hemorrhagic ascites during disease outbreaks at a fish farm in Tokushima Prefecture. Two isolates, BPTH-9903 and BPTH-9906, which were isolated in April 1999, were used in the following characterization tests. Four strains of *P. plecoglossicida*, 2 motile (FPC951=ATCC700383^T, PTH-9801) and 2 non-motile (AK-9510, PTH-9802), were used as reference strains (Table 1). These bacteria were cultured on Trypto-soy agar (TSA, Nissui) at 25°C overnight prior to experiments.

Morphological and biochemical characterization tests were carried out by the standard methods and commercially produced kits (API 20NE, API ZYM; BioMérieux). Motility test was done by a wet mount method. In electron microscopy, bacterial cell suspension was placed on a carbon-coated grid and negatively stained with 2% uranyl acetate, and examined with Hitachi-H600A electron microscope at 80 kV. A rabbit antiserum raised against *P. plecoglossicida* FPC941 strain[3] was used in slide agglutination.

BPTH-9903 isolate was used to examine pathogenicity to ayu weighing average 2.4 and 3.2 g. Groups of 10 fish were injected intramuscularly with doses of 6.5–6.9×10^{-1} to 10^1 colony forming units (CFU) per fish and then kept in 40 L plastic tanks with flow-through water at 22°C. Saline injections (0.05 mL / fish) were given to control groups. Mortalities were recorded daily for 2 weeks, and kidneys of dead fish were subjected to bacterial isolation using TSA to confirm that the death was due to the *P. plecoglossicida* infection. The 50% lethal dose (LD_{50}) was determined by the method of Reed and Meunch[4].

Table 1. *Pseudomonas plecoglossicida* strains used in this study and some differential characteristics

Stain	Source (year)	Pigment[1]	Motility	Agglutination[2]
Present isolates				
BPTH–9903	Tokushima Pref. (1999)	+	−	+
BPTH–9906	〃 (1999)	+	−	+
Reference strains				
FPC951[3]	Tokushima Pref. (1994)	−	+	+
AK–9510[4]	Kyoto Pref. (1995)	−	−	+
PTH–9801	Tokushima Pref. (1998)	−	+	+
PTH–9802	〃 (1998)	−	−	+

[1] Brown diffusible pigment.
[2] With rabbit anti–*P. plecoglossicida* (FPC941) serum.
[3] =ATCC700383. See ref. 3).
[4] See ref. 2).

* Corresponding author
E-mail: nakaitt@hiroshima-u.ac.jp

Results and Discussion

The diseased ayu was characterized by the heavy accumulation of hemorrhagic ascites. A single type of colony with brown diffusible pigment was isolated from all the examined fish (Fig. 1). Two isolates examined (BPTH-9903, BPTH-9906) were Gram-negative, aerobic, non-motile straight rod, catalase- and oxidase-positive, and other phenotypic characteristics were identical to those of *P. plecoglossicida*[3]. The API 20NE profile was 1-140-457. No flagellum was found by electron microscopy. Both isolates reacted positively with the anti-*P. plecoglossicida* serum. These characteristics were consistent with those of non-melanogenic *P. plecoglossicida* described in previous papers[1–3].

There have been no descriptions on brown pigment-producing strain of *P. plecoglossicida* in the previous papers[1–3]. The melanogenic feature, colony appearance and non-motility of the present isolates from ayu initially led us to identify them as *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids. Because that the present brown pigment-producing variant of *P. plecoglossicida* showed negative reaction against an rabbit anti-*A. salmonicida* (NCMB1102) serum, it can be distinguished from typical *A. salmonicida* by serological test. However, it is still necessary to examine serological relationships to other melanogenic strains of *A. liquefaciens* (=*A. hydrophila*)[5] or *P. fluorescens*[6], even if they are rarely encountered in fish disease diagnosis.

The previous reports[1,2] described contrary results on the motility of *P. plecoglossicida* isolates and presence of hemorrhagic ascites in diseased fish. According to our experience in Tokushima Prefecture, hemorrhagic ascites was always observed as a characteristic clinical sign in diseased fish from which usual non-melanogenic *P. plecoglossicida* was isolated. However, motility of the isolates was very variable; even if they were isolated from dead fish in the same outbreak, some were motile and others were non-motile. Polar multitrichous flagella were easily demonstrated in motile isolates by electron microscopy, but not in non-motile ones. Further examinations will be required to clarify this strange phenomenon.

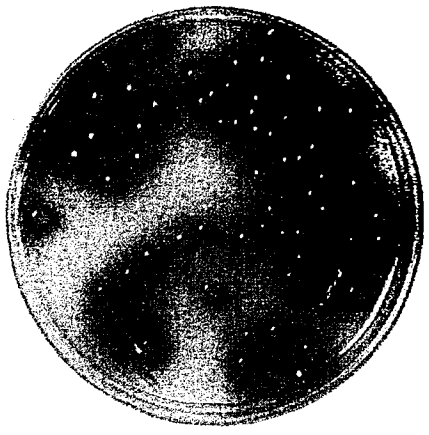

Fig. 1. Culture of *P. plecoglossicida* BPTH-9903 isolate showing diffusible brown pigmentation on TSA.

Table 2. Pathogenicity of *Pseudomonas plecoglossicida* BPTH-9903 isolate in ayu

Experiment (weight of fish)	Injection dose (CFU/fish)	No. of fish dead/tested
1 (2.4g)	6.9×10^{1}	9/10
	10^{0}	5/10
	10^{-1}	1/10
	Control	0/10
2 (3.2 g)	6.5×10^{1}	10/10
	10^{0}	5/10
	10^{-1}	3/10
	Control	0/10

The present brown pigment-producing variant was proved to be highly virulent to ayu by experimental infection (Table 2), the LD_{50} of BPTH-9903 isolate being 7×10^{0} CFU/fish. Fish died showing hemorrhagic ascites 5 to 10 days after being injected intramuscularly. Throughout epidemiological survey of the disease in Tokushima Prefecture in 1999, it was only one farm from which melanogenic *P. plecoglossicida* was isolated. Although this phenotypic variant seemed to be a minor type, it should be taken into consideration in diagnostic work of the disease.

Acknowledgments

We thank the staffs of the Hiroshima and Kyoto Prefectural Fisheries Experiment Stations and Dr. H. Wakabayashi (The University of Tokyo) for kindly providing *P. plecoglossicida* strains used in this study.

References

1) Wakabayashi, H., K. Sawada, K. Ninomiya and E. Nishimori (1996): *Fish Pathol.*, **31**, 239–240. 2) Nakatsugawa, T. and Y. Iida (1996): *Fish Pathol.*, **31**, 221–227. 3) Nishimori E., K. Kita-Tsukamoto and H. Wakabayashi (2000): *Int. J. Syst. Evol. Microbiol.*, **50**, 83–89. 4) Reed, L. J. and H. Meunch (1938): *Am. J. Hyg.*, **27**, 493–497. 5) Paterson, W. D. (1974): *J. Fish. Res. Board Can.*, **31**, 1259–1261. 6) Frerichs, G. N. and A. Holliman (1991): *J. Fish Dis.*, **14**, 599–601.