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Relationship between *in vitro* Motility of *Pseudomonas plecoglossicida* and Clinical Conditions in Affected Ayu

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ABSTRACT—This study was conducted to verify the previous contrasting results on the motility of *Pseudomonas plecoglossicida* and clinical conditions (bloody ascites) in affected ayu *Plecoglossus altivelis*. In the present field surveys at Tokushima Prefecture in 1999 and 2001, all *P. plecoglossicida* isolates from dead ayu, except one isolate from a fish with ascites, were non-motile irrespective of the presence of bloody ascites. When ayu were injected intramuscularly with non-motile strains, either motile or non-motile isolates were obtained from the kidney of dead fish, while only motile ones were isolated after injection of motile strains. Bloody ascites manifested in most of the affected fish after injection of either type of the bacterium. In *in vitro* broth culture conditions, the non-motile strains altered into motile ones during two-week incubation. These results indicate that there are no relationship between *in vitro* motility of *P. plecoglossicida* and the presence of bloody ascites in affected ayu, and that the phenotypic alteration in the motility of *P. plecoglossicida* occurs under both *in vitro* and *in vivo* conditions.

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

There have been two serious bacterial diseases among cultured population of ayu *Plecoglossus altivelis* in Japan since around 1990; cold water disease caused by *Flavobacterium psychrophilum* (Wakabayashi *et al.*, 1994; Iida and Mizokami, 1996) and bacterial hemorrhagic ascites caused by *Pseudomonas plecoglossicida* (Nakatsugawa and Iida, 1996; Wakabayashi *et al.*, 1996; Nishimori *et al.*, 2000). Some antimicrobial agents such as florfenicol and sulfisozole are available to treat the former disease but not for the latter disease. Furthermore, as *P. plecoglossicida* infection emerges abruptly after such chemotherapy for cold water disease followed by heavy mortality, treatments for these two diseases must be considered inseparably.

P. plecoglossicida is homogeneous with respect to physiological, biochemical, and serological characteristics (Nakatsugawa and Iida, 1996; Wakabayashi *et al.*, 1996), and also consists of a single phage type (Park *et al.*, 2000). However, in two previous papers the authors described contrasting results for the motility of the bacterium and the presence of bloody (hemorrhagic) ascites in affected fish; both of these characteristics were negative in one study (Nakatsugawa and Iida, 1996), and both were positive in the other study (Wakabayashi *et al.*, 1996). We reconfirmed that both motile and non-motile types existed in strains of *P. plecoglossicida* which had been isolated from diseased ayu in geographically and chronologically different places (Park *et al.*, 2000).

In order to solve this discrepancy, the relationship between the motility of *P. plecoglossicida* and different clinical conditions (bloody ascites) was investigated by isolation and characterization of bacteria from naturally infected fish with or without the clinical sign, and by *in vitro* and *in vivo* experiments using some selected motile and non-motile strains.

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Materials and Methods

Field survey

Isolation of *P. plecoglossicida* from naturally affected ayu was performed at a private farm in Tokushima Prefecture in October 1999 (average fish weight: 56 g) and February 2001 (3.6 g). Kidney tissues of fish were stamped on Trypto-Soya Agar (TSA, Nissui) and incubated at 25°C for 2 days. The isolates were identified by agglutination test with an anti-FPC951 rabbit serum. The motility of the isolates was examined directly by a wet mount method using a microscope.

Bacterial strains

Six strains of *P. plecoglossicida* were used for the *in vivo* and *in vitro* studies; three strains, FPC951 (=ATCC 700383^T), FPC941 (ATCC 700384), and PTH-9801 were motile, and the other three, AK-9510, PH-9501, and PTH-9802 were non-motile (Table 1). A lophotrichous flagellation was observed in motile strains, but not in non-motile strains. These bacteria were cultured on TSA at 25°C overnight prior to experiments. All *in vitro* experiments were carried out at 25°C unless cited otherwise.

Infection of ayu with P. plecoglossicida

Groups of 20 ayu (average 4.2 g) were injected intramuscularly (IM) with motile or non-motile strains at a dose of 10^3 CFU/fish and then kept in 40 L plastic tanks with flow-through water at 20±1°C. Mortalities and the presence of bloody ascites in dead fish were recorded daily for 2 weeks, and the kidneys of dead fish were subjected to bacterial isolation to confirm that death was due to *P. plecoglossicida* infection. Re-isolated bacteria from dead fish were tested for their motility by the wet mount method.

In vitro *culture of* P. plecoglossicida *under different conditions*

Each motile or non-motile strain was incubated under different temperatures (15, 20, 25 or 30°C) on TSA, or in serially diluted TSB (Trypto-Soy Broth, Eiken) at 25°C, or in TSB containing antimicrobial compounds (florfenicol 600 μg/mL, chloramphenicol 500 μg/mL, or sulfisozole sodium 2,000 μg/mL) at 25°C. *P. plecoglossicida* is highly resistant to these agents (Nakatsugawa and Iida, 1996). Each culture on TSA was successively subcultured on a fresh medium everyday for 10 days. In broth cultures, a loopful of culture was transferred on TSA everyday and their one-day cultures at 25°C were tested for the motility. In a separate experiment, a non-motile PTH-9802 strain was inoculated into filter-sterilized (0.45 μm membrane filter) supernatants which had been collected from each 20-day-culture (25°C) of *P. plecoglossicida* strains in TSB, incubated at 25°C, and then the motility of PTH-9802 strain was examined everyday as described above. A similar experiment was carried out using these spent culture fluids that had been previously heated at 100°C for 3 h.

Results

Clinical signs of fish and the motility of the isolates in field surveys

In the 1999 survey, bloody ascites was observed in 25 of 29 fish examined and almost all *P. plecoglossicida* isolates were non-motile (Table 2). Only one motile isolate was obtained from a dead fish with bloody ascites. There were no apparent clinical signs including bloody ascites in dead ayu in the 2001 survey and only non-motile *P. plecoglossicida* were isolated from the kidney of dead fish (n=10).

Clinical signs of fish and the motility of P. plecoglossicida *in infection experiments*

All of IM-injected fish with *P. plecoglossicida* strains, either motile or non-motile, were dead at 3rd to 5th day post-injection (Table 3). The characteristic bloody ascites was observed in the fish injected not only with the motile strains but also the non-motile strains at high incidence. Isolates from the fish injected with motile strains were all motile, while half of those from the fish injected with the non-motile strains were motile.

In vitro *alteration in the motility of* P. plecoglossicida

No change in motility was observed during 10 days successive subcultures on TSA at the various incu-

Table 1. *P. plecoglossicida* strains in this study and their motility

Strain	Isolation from ayu		Motility	References
	Location	Year		
FPC951[a]	Tokushima Prefecture	1994	+	Nishimori *et al.* 2000
FPC941[b]	Shiga Prefecture	1994	+	Nishimori *et al.* 2000
PTH-9801	Tokushima Prefecture	1998	+	Park *et al.* 2000
AK-9510	Kyoto Prefecture	1995	−	Nakatsugawa and Iida 1996
PH-9501	Hiroshima Prefecture	1995	−	Nakatsugawa and Iida 1996
PTH-9802	Tokushima Prefecture	1998	−	Park *et al.* 2000

[a] Type strain, ATCC 700383
[b] ATCC 700384

Table 2. Clinical conditions of naturally affected ayu and motility of isolated *P. plecoglossicida*

Year	Average fish weight	Bloody ascites in fish		Motility of isolate	
		+	−	+	−
1999	56.0 g	25	4	1	28
2001	3.6 g	0	10	0	10

bation temperatures or in TSB at the different nutritional conditions or in TSB supplemented with three drugs. However, longer incubation (12 or 14 days) in drug-free TSB changed all three non-motile strains into motile ones, but no change was noticed in the motility of the motile strains during 20-day incubation (Table 4). No significant changes of pH values were noticed throughout the incubation period. Such alteration in the motility was observed within 8 days in the non-motile PTH-9802 strain when it was incubated with each 20-day-spent culture fluid (Table 5). This alteration was also observed in the spent culture fluids previously treated at 100°C for 3 h (data not shown). Once the non-motile strains turned to motile ones, the motility was retained throughout subsequent cultures on agar media.

Discussion

The present bacterial isolation from dead ayu in a culture facility in Tokushima Prefecture in 1999 and 2001 shows that there are no clear relationship between the presence of bloody ascites in diseased fish and *in vitro* motility of the causal organism; some of diseased fish were characterized by ascites but others were not, and

Table 3. Re-isolation of *P. plecoglossicida* from dead ayu after intramuscular injection with motile or non-motile strains

Injected strain (dose : CFU/fish)		Number of fish			
		tested	dead	dead with bloody ascites	from which motile *P. plecoglossicida* was isolated
Motile strain	FPC951 (1.6×10^3)	20	20	16	20
	FPC941 (1.8×10^3)	20	20	20	20
	PTH-9801 (2.0×10^3)	20	20	12	20
Non-motile strain	AK-9510 (1.5×10^3)	20	20	16	10
	PH-9501 (3.4×10^3)	20	20	17	11
	PTH-9802 (1.2×10^3)	20	20	19	10

Table 4. Alteration in the motility of motile and non-motile *P. plecoglossicida* strains in TSB

Strain		Days after incubation in TSB at 25°C									
		2	4	6	8	10	12	14	16	18	20
Motile strain	FPC951	+[a]	+	+	+	+	+	+	+	+	+
	FPC941	+	+	+	+	+	+	+	+	+	+
	PTH-9801	+	+	+	+	+	+	+	+	+	+
Non-motile strain	AK-9510	−[b]	−	−	−	−	−	+	+	+	+
	PH-9501	−	−	−	−	−	−	+	+	+	+
	PTH-9802	−	−	−	−	−	+	+	+	+	+

[a] motile
[b] non-motile

Table 5. Alteration in the motility of a non-motile *P. plecoglossicida* strain (PTH-9802) in spent-culture fluids

Spent-culture fluids from		Days after incubation in spent-culture fluid at 25°C									
		1	2	3	4	5	6	7	8	9	10
Motile strain	FPC951	−[a]	−	−	−	−	+[b]	+	+	+	+
	FPC941	−	−	−	−	+	+	+	+	+	+
	PTH-9801	−	−	−	−	−	+	+	+	+	+
Non-motile strain	AK-9510	−	−	−	−	−	+	+	+	+	+
	PH-9501	−	−	−	−	−	−	−	+	+	+
	PTH-9802	−	−	−	−	+	+	+	+	+	+

[a] non-motile
[b] motile

almost all isolates were non-motile though only one motile isolate was obtained from a fish with ascites (Table 2). Infection experiments using selected motile and non-motile strains also clearly demonstrated that there are no essential relationship between the motility of the organism and manifestation of bloody ascites in fish (Table 3); ascites was produced in the fish injected with the non-motile strains and then the motile cells were isolated from some of these dead fish, but the motility of the motile strains was never lost after fish-passages and long-term subcultivation on agar plates. Therefore, it is concluded that the variations in the bacterial motility and the presence of bloody ascites were just reflected in the previous contrasting observations (Nakatsugawa and Iida, 1996, Wakabayashi *et al.*, 1996).

It is known that the synthesis of bacterial flagella is affected by culture conditions such as temperature or antibiotics (Hasegawa *et al.*, 1982; Martinez and Gordee, 1966). In particular, we suspected that drugs such as florfenicol and sulfisozole frequently used for treatment of cold water disease may cause the variation in the motility of *P. plecoglossicida*. Contrary to our expectation, incubation of motile or non-motile strains of *P. plecoglossicida* with these drugs resulted in no changes in the motility during 10-day culture period, as well as the cultures at various temperatures or nutritional conditions. However, the prolonged cultivation in liquid medium induced alteration from non-motile to motile (Table 4). These results, together with field surveys and infection experiments described above, suggest that most of fresh isolates of *P. plecoglossicida* from naturally infected fish are non-motile and phenotypic alteration into motile one occurs during subsequent artificial manipulations. In the present study, unidentified heat-stable substance(s) produced during bacterial growth is possibly associated with alteration in the motility of *P. plecoglossicisa* (Table 5). The precise mechanism remains for the future study.

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References

Hasegawa, E., R. Kamiya and S. Asakura (1982): Thermal transition in helical forms of *Salmonella* flagella. *J. Mol. Biol.*, **160**, 609–621.

Iida, Y. and A. Mizokami (1996): Outbreaks of coldwater disease in wild ayu and pale chub. *Fish Pathol.*, **31**, 157-164.

Martinez, R. J. and E. Z. Gordee (1966): Formation of bacterial flagella. *J. Bacteriol.*, **91**. 870–875.

Nakatsugawa, T. and Y. Iida (1996): *Pseudomonas* sp. isolated from diseased ayu, *Plecoglossus altivelis*. *Fish Pathol.*, **31**, 221–227. (in Japanese with English abstract)

Nishimori, E., K. Kita-Tsukamoto and H. Wakabayashi (2000): *Pseudomonas plecoglossicida* sp. nov., the causative agent of bacterial hemorrhagic ascites of ayu, *Plecoglossus altivelis*. *Int. J. Syst. Evol. Microbiol.*, **50**, 83–89.

Park S. C., I. Shimamura, M. Fukunaga, K-I. Mori and T. Nakai (2000): Isolation of bacteriophages specific to fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. *Appl. Environ. Microbiol.*, **66**, 1416–1422.

Wakabayashi H., T. Toyama and T. Iida (1994): A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. *Fish Pathol.*, **29**, 101–104.

Wakabayashi, H., K. Sawada, K. Ninomiya and E. Nishimori (1996): Bacterial hemorrhagic ascites of ayu caused by *Pseudomonas* sp. *Fish Pathol.*, **31**, 239–240. (in Japanese with English abstract)