Characteristics of Myxobacteria Associated with Some Freshwater Fish Diseases in Japan

Hisatsugu WAKABAYASHI and Syuzo EGUSA

(Received May 25, 1974)

Morphological, cultural and biochemical characteristics of 12 cultures of myxobacteria isolated mainly from eels with 'branchionephritis' and cyprinids with 'ulcer disease' were studied.

These organisms were gram-negative, slender rods which exhibited gliding motility on agar medium. All cultures were able to grow at 5°, 10°, 20° and 27°C, and tolerated a sodium chloride concentration of 1% for growth. They were uniform in their biochemical properties, though diverse in the production of cytochrome oxidase and in the reduction of nitrate. Some distinguishing features of the organisms were as follows: Degradation of starch, esculin and chitin; utilization of glucose, galactose and citrate; decomposition of tyrosine; nonproduction of hydrogen sulfide; negative reaction to Congo red. They are physiologically similar to the myxobacteria isolated from the Amerian hatchery-raised salmonids with gill disease, tail rot or other myxobacterial infections.

The histopathological features of experimentally infected eels resembled those of salmonids with 'hemorrhagic type of gill disease' described by WOOD and YASUTAKE.

Myxobacterial diseases have been poorly studied in Japan, where only columnaris disease have been reported,¹⁾ though widely studied in U. S. A. with both natural and reared populations of fishes. Included among the important myxobacterial disease well known in U. S. A. are columnaris disease caused by *Chondrococcus columnaris*,²⁾ cold-water disease due to *Cytophaga psychrophila*,³⁾ bacterial gill disease probably associated with several types of unidentified myxobacteria³⁾ and so on. During the course of our studies on branchionephritis of eel,⁴⁾ ulcer disease of carp or its relatives⁵⁾ and some cases of other diseases of unknown cause, we could often isolated a type of myxobacteria from the gills of affected fishes. The present paper reports preliminary studies on characteristic and pathogenicity of the isolates.

Materials and Methods

Materials Twelve isolates were used; six from eels, two from carps, two from crucian carps and two from goldfishes. The source of each culture investigated is shown in table 1. A strain of *Chondrococcus columnaris* was also included in this study for comparative purposes. Isolations of myxobacteria were carried out by using modified ANACKER and RUCKER's *Cytophaga* agar, which contained tryptone 0.2%, yeast extract 0.05%, beef extract 0.02%, sodium acetate 0.02%, calcium chloride 0.02%, and agar 1.5%. A bit of tissues taken from gills, skin or other organs was streaked onto the agar plate, which

* Dept. of Fish., Univ. of Tokyo, Tokyo, Japan (若林久嗣・江草周三: 東京大学農学部水産学科)

Table 1. Sources of the isolates studied

Group and culture number	Source of isolation	Location
Branchionephritis of eel (Erajin-en)		
M 26.....	Gills, eel	Yoshida, Shizuoka, Nov., 1972
M 27.....	Gills, eel	Yoshida, Shizuoka, Nov., 1972
M 40.....	Gills, eel	Yoshida, Shizuoka, Dec., 1972
M 41.....	Gills, eel	Yoshida, Shizuoka, Jan., 1973
M 56.....	Gills, eel	Yoshida, Shizuoka, Feb., 1973
M 81.....	Gills, eel	Yoshida, Shizuoka, May, 1973
Ulcer disease of cyprinids (Ana-aki-byo)		
M 84.....	Skin lesion, carp	Shioda, Nagano, June, 1973
M 68.....	Skin lesion, goldfish	Mizumoto, Tokyo, Mar., 1973
M 90.....	Skin lesion, goldfish	Mizumoto, Tokyo, June, 1973
M 34.....	Fin, crucian carp	Lake Tega, Chiba, Nov., 1972
M 60.....	Fin, crucian carp	Lake Imba, Chiba, Feb., 1973
Diseased carp		
M 75.....	Gills, color carp	Chiyoda, Tokyo, Mar., 1973

was then incubated at room temperature or 20°C for at least a week.

Morphological Characteristics Cell morphology was studied using smears from cultures and lesions. The smears were stained with LÖFFLER's methylene blue. Examinations for motility and their ability to glide on agar plate were made with the phase contrast microscope by wet-mount method and the slide culture method, respectively.

Fruiting body formation and microcyst production of cultures were examined by the same methods as BULLOCK⁽⁶⁾ adopted: Fruiting body formation was observed by inoculating the isolates onto small pieces of autoclaved fish tissue and submersing the pieces in a large quantity of sterile tap water; the presence of microcysts was studied with cultures grown in a modified DWORKIN and GIBSON's medium containing casitone 1.0%, magnesium sulfate 0.008 M, glycerol 0.5 M.

Environmental Characteristics The ability of isolates to grow on the modified *Cytophaga* agar at 5°, 10°, 20°, 27°, 32° and 37°C were observed for 2 weeks. Tolerance to sodium chloride was determined by using the modified *Cytophaga* broth containing sodium chloride 0%, 0.5%, 1.0%, 2.0%, 3.0%, or 4.0%. Dependency of the growth on pH was examined with the broth of which the initial pH was adjusted at 5.0, 5.4, 6.0, 6.5, 7.0, 8.0, 8.9, 9.3 and 9.6 by using 4N HCl or 4N NaOH. In both the pH and sodium chloride tolerance tests, growth was determined by visible turbidity after 5 day incubation at 20°C.

Physiological Characteristics Unless otherwise stated, methods for the various tests were essentially those of PACHA and PORTER,⁽⁷⁾ and ANACKER and RUCHER's *Cytophaga* medium⁽⁸⁾ containing tryptone 0.05%, yeast extract 0.05%, beef extract 0.02% and sodium acetate 0.02% was used as basal medium. The presence of cytochrome oxidase was

determined by using test paper 'Nissui'. The Congo red test consisted of flooding colonies with 0.01 % aqueous solution of Congo red. Appearance of red color in the margin of colonies was considered positive.⁹⁾

Sensitivity of the isolates to some antimicrobial agents was examined by the disk method on the modified *Cytophaga* agar. 'Mono Disk Showa' was used for the tests with polymyxin B and furazolidone. 'Tri Disk Eiken' was applied to penicillin, chloramphenicol, tetracycline, kanamycin, colistin, naldixic acid, novobiocin, sulfadimethoxine and sulfisoxazole.

Pathogenicity Attempts artificially were made to infect eel (about 150 g) by adding bacteria to the aquarium water. Pure cultures of myxobacteria recently isolated from the gills of diseased eels were grown on the modified *Cytophaga* agar for 24 hours and were then added to the aquarium water to give a density of about 1×10^8 cells/ml. Each aquarium received two eels; one was intact and the gills of the other mechanically injured by rubbing with tooth brush.

Carp, goldfishes and weatherfishes were also challenged in the same way.

Results

Morphology All of the isolates were gram-negative, slender rods which showed a swinging movement in the wet-mount preparations and exhibited gliding motility on agar. The cells were about 0.5μ wide and 2 to 5μ long in the smears of 18 hour old cultures on agar (Fig. 1). In general, the organisms tended to become somewhat shorter at high incubation temperatures or as the culture aged.

Colonies grown on the modified *Cytophaga* agar were yellow-orange in color, thin, flat and spreading with irregular edges (Fig. 2). No fruiting bodies were observed after a month incubation in any of the isolates tested. Microcyst-like organisms were observed in the cultures grown in the modified DWORKIN and GIBSON's medium (Fig. 3). These round cells were observed to scatter among vegetative cells. They could have been spheroplasts or involution forms, because conversion of cells to microcysts was found to occur synchronously with fruiting myxobacteria.¹⁰⁾

Environmental Characteristics All of the isolates could grow in media with 0% to 1.0% sodium chloride and some tolerated 2% for growth. They could grow in media the initial pH of which ranged 7.0 to 9.3, and could not survive at pH 6.0. Growth was observed at 5° to 27°C.

Physiological Characteristics With the exceptions of nitrate reduction and cytochrome oxidase production, all the test cultures showed the same biochemical reactions, which are summarized in table 2.

Sensitivity of the isolates to antimicrobial agents is given in table 3.

Table 2. Summary of characteristics of myxobacteria isolated from eels, carps, etc., and comparison with closely related organisms

	This authors Eel	Carp, etc.	PACHA ⁽⁸⁾ Trouts, etc.	BULLOCK ⁽⁷⁾ Trouts, etc.	<i>Chondro- coccus columnna- ris</i> *	<i>Cyto- phaga psychro- phila</i> ⁽⁸⁾
Heat tolerance						
Growth at 5°C	+	+	+	+	—	+
Growth at 27°C	+	+	d	+	+	—
Growth at 32°C	—	—		d	+	—
NaCl tolerance						
Growth in 0%	+	+	+		+	+
Growth in 0.5%	+	+	d	+	d	+
Growth in 1.0%	+	+	d	d	—	—
Growth in 2.0%	+	d(4/6)	d	—	—	—
Growth in 3.0%	—	—	d	—	—	—
Degradation of						
Casein	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+
Starch	+	+	+	d	—	—
Esculin	+	+	d		—	—
Chitin	+	+	d		—	—
Cellulose	—	—	—	—	—	—
Tributylin	—	—	d	d	—	+
Carbohydrate utilization						
Glucose	+w	+w	d	d	—	—
Lactose	—	—	d		—	—
Galactose	+w	+w	d		—	—
Sucrose	—	—	d		—	—
Nitrate reduced	d(3/6)	d(4/6)	d	d	d	—
Hydrogen sulfide produced	—	—	d	—	+	—
Tyrosine decomposed	+	+	d	d	—	+
Citrate utilized	+	+	d		—	—
Indole produced	—	—	—		—	—
Catalase produced	+	+	+	+	+	+
Cytochrome oxidase produced	d(4/6)	d(3/6)	d	—	+	—
Lysis of dead bacterial cells						
Escherichia coli	+	+	+	+	+	+
Congo red test	—	—			+	

* This authors

+ = positive; +w = weakly positive; — = negative; d = diverse; () = Expressed as number of cultures positive divided by number of cultures tested.

Pathogenicity One of the two attempts to infect eels with the myxobacteria was successful (Table 4), though challenge tests with carps, goldfishes and weatherfishes were unsuccessful. The experimentally infected eels displayed the essentially same symptoms as the naturally occurred cases. They became gradually inactive without any externally visible lesions except for the gill, which was characterized grossly by many apparent minute hemorrhages in the gill filaments.

Table 3. Sensitivity of the isolates to antimicrobial agents
(Sensitivity Disks 'EIKEN' & 'SHOWA')

Antimicrobial agents	Sensitive			Resistant
	+++	++	+	—
Penicillin	0	0	0	12
Chloramphenicol	0	0	2	10
Tetracycline	0	11	1	0
Kanamycin	0	0	0	12
Colistin	0	0	0	12
Nalidixic acid	12	0	0	0
Novobiocin	1	9	2	0
Polymyxin B	0	0	0	12
Sulfadimethoxine	1	0	2	9
Sulfisoxazole	0	7	5	0
Furazolidone	0	2	7	3

Discussion

In this paper we demonstrated that a myxobacterium was associated with some freshwater fish diseases in Japan. On the basis of its cultural and physiological properties it is very similar to the isolates from the salmonids with bacterial gill disease, tail rot or other diseases,⁶⁾ and also resembles some of the nonpathogenic species from the body surface of freshwater fish⁷⁾ (Table 2), though there is no completely identical type among them. If the observation of microcysts in the test cultures is valid, they may be identified as a member of the genus *Sporocytophaga*. But the taxonomic position of the isolate must be the subject of future study as it is now debatable whether myxobacteria isolated from fishes, including *Chondrococcus columnaris*, be able to produce the true microcyst or not.¹¹⁾

The gill lesions of the experimentally infected eels showed very interesting histopathological features. In such acute cases as in the aquaria #4 and #5 (Table 4) a large

Table 4. Pathogenicity tests with the isolates to pond-cultured eels.

Test	Aquarium number	Culture number	Density (cells/ml)	Water temp. (°C)	Number of fish tested	Number of fish died	Lethal time (days)
No. 1	1	(control)	0	10	2	0	survived
	2	M26	1.5×10^6	10	2	2	10 & 12*
	3	M27	1.0×10^6	10	2	1	14 & survived
Nov. 23–Dec. 14	4	M26	1.5×10^6	20	2	2	2* & 3
	5	M27	1.0×10^6	20	2	2	2* & 2
							Myxobacteria in aquarium (Dec. 9)
No. 2	6	M26	1.3×10^6	15	2	0	+
Nov. 29–Dec. 14	7	M27	1.0×10^6	15	2	0	+
	8	M27	1.3×10^6	20	2	0	+

* Moribund fish were sacrificed for histological examinations.

number of slender, rod shaped bacteria were observed on the surface of the lamellae, and part of the capillaries expanded into globose masses of blood (**Fig. 4**) which gave the gills a gross appearance of minute hemorrhages in its filaments. In such chronical cases as in the aquaria #2 and #3, in addition to a congestive gross appearance the occurrence of remarkable hyperplasia was observed, and myxobacterial colonies were occasionally found in a vacant space of the fused lamillae (**Fig. 5**). It was noticeable that these gill lesions resembled rather to those of 'hemorrhagic gill disease' of hatchery-raised fingerling salmonids¹²⁾ than those of 'bacterial gill disease', especially in the acute cases.

References

- 1) H. WAKABAYASHI and S. EGUSA: This Bull., **32**, 1015-1022 (1966), (in Japanese).
- 2) E. J. ORDAL and R. R. RUCKER: Proc. Soc. exp. Biol. Med., **56**, 15-18 (1944).
- 3) A. F. BORG: Wildlife Disease No. 8, 85 p. (1960).
- 4) S. EGUSA: Fish Pathol., **5**, 51-66 (1970), (in Japanese).
- 5) T. MIYAZAKI and S. EGUSA: *ibid.*, **7**, 115-121 (1973), (in Japanese).
- 6) G. L. BULLOCK: Tech. Paper No. 80, 30 p. (1972).
- 7) R. E. PACHA and S. PORTER: Appl. Microbiol., **16**, 1901-1906 (1968).
- 8) R. L. ANACKER and E. J. ORDAL: J. Bact., **78**, 25-32 (1959).
- 9) J. L. JOHNSON and W. S. CHILTON: Science **152**, 1247-1248 (1966).
- 10) M. DWORKIN and H. VOELZ: J. gen. Microbiol., **28**, 81-85 (1962).
- 11) T. G. MITCHELL, M. S. HENDRIE and J. M. SHEWAN: J. appl. Bact., **32**, 40-50 (1969).
- 12) E. M. WOOD and W. T. YASUTAKE: Prog. Fish-Cult., **19**, 7-13 (1957).

Fig. 1. Eighteen hour old culture on modified ANACKER and ORDAL's *Cytophaga* agar (stained with LÖFFLER's methylene blue). $\times 1200$

Fig. 2. Comparison of the colonies between the myxobacteria isolated from 'branchionephritis' (right) and *Chondrococcus columnaris* (left), grown five days on the modified *Cytophaga* agar after spotting inoculation.

Fig. 3. Ten day old culture on modified DWORKIN and GIBSON's medium showing vegetative cells and what appear to be microcysts (stained with LÖFFLER's methylene blue). $\times 1200$

Fig. 4. Gill lesion of the experimentally infected eel. The myxobacteria are on the lamellae (arrow), and the capillaries expand into globose masses of blood (stained with hematoxylin and eosin). $\times 250$

Fig. 5. A mass of the myxobacteria (arrow) shut in a vacant space of the fused lamellae (stained with GIEMSA's stain). $\times 600$