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Characteristics of Myxobacteria Isolated from the Surface of Freshwater Fish[1]

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A study was made of 32 nonpathogenic myxobacterial isolates obtained from a variety of fish taken in the Pacific Northwest. Morphological, cultural, biochemical, and serological studies were carried out on these strains. All were found to be members of the genus *Cytophaga*. Two myxobacterial strains pathogenic to fish were also included in this study for comparative purposes. These pathogenic organisms were found to be culturally and physiologically similar to some of the nonpathogenic strains. Antiserum against the pathogenic species, however, showed no cross agglutination when tested against the other myxobacterial isolates. As a result, serological procedures appear promising as a rapid means for distinguishing pathogenic myxobacteria from one another and from saprophytic myxobacteria commonly found on fish.

Myxobacteria are important agents of disease in both natural and hatchery populations of fish. The diseases caused by these organisms have been discussed by various workers (3, 9, 16, 17). Included among the important myxobacterial fish pathogens are *Chondrococcus columnaris*, the etiological agent of columnaris disease, *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease, and probably several types of unidentified myxobacteria which are associated with gill disease in fish. Both freshwater and anadromous fish are highly susceptible to infection by these organisms, and severe economic losses have occurred in populations of fish as a result of the diseases they cause (4, 14, 15).

Nonpathogenic myxobacteria have been found to be abundant on the skin and gills of freshwater fish. On the basis of their colony morphology, some of these organisms could be easily confused with the pathogenic species. The present investigation was undertaken to characterize saprophytic myxobacteria occurring on the surface of fish and to determine the degree of similarity between these organisms and known pathogenic species. It is hoped that this study will stimulate interest in this group of bacteria and contribute to the development of adequate procedures for the identification of myxobacteria pathogenic to fish.

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Materials and Methods

Source of isolates. We studied 32 strains of nonpathogenic myxobacteria. The organisms were isolated over a period of 3 years from fish taken at various locations in the Pacific Northwest. One culture of *C. columnaris* and one culture of *C. psychrophila* were also included in this study for comparative purposes. The source of each strain investigated is shown in Table 1. All of these cultures were preserved by lyophilization at the time of isolation.

The organisms were maintained on *Cytophaga* medium containing 0.4% agar (1). The stock cultures were incubated at 18 C for 1 week and then stored at 4 C.

Morphological characteristics. The organisms were stained by Gram's method after incubation for 24 hr at 18 C in *Cytophaga* broth. Cell morphology and motility were determined by examination of wet mounts by use of a phase contrast microscope. Colony morphology was observed on *Cytophaga* agar after 72 hr of incubation at 18 C. The ability of the organisms to produce fruiting bodies and microcysts was tested with the procedure described by Ordal (13).

Environmental characteristics. The ability of the strains to grow in *Cytophaga* broth at 37, 30, 25, 18, and 4 C was noted. The incubation periods extended from 1 day to 2 weeks depending on the incubation temperature used.

The method of Anderson and Ordal (2) was followed to test for anaerobic growth. The medium used consisted of 0.18% peptone, 0.09% yeast extract, 0.09% beef extract, 0.9% glucose, and 0.18% $NaHCO_3$. The glucose and $NaHCO_3$ were filter sterilized and added to the basal medium aseptically. Tubes containing 10 ml of this medium were inocu-

TABLE 1. *Source of myxobacteria studied*

Strain	Source	Host
1	Alsea Trout Hatchery, Oregon Game Commission	Rainbow trout
2, 3	Wizard Falls Fish Hatchery, Oregon Fish Commission	Atlantic salmon
4, 5	Yakima River, Prosser, Wash.	Sucker
6	Hanford slough, Richland, Wash.	Bass
7, 8	Hanford slough, Richland, Wash.	Sucker
9	Hanford slough, Richland, Wash.	Carp
10, 11, 12, 13, 14, 15, 16, 17, 18	Chilko Lake, British Columbia	Sockeye salmon
19	Chilko Lake, British Columbia	Whitefish
20	Oregon State University	Catfish
21, 22, 23, 24	Siletz Salmon Hatchery, Oregon Fish Commission	Silver salmon
25, 26	Snake River, Ice Harbor Dam	Chinook salmon
27	Snake River, Ice Harbor Dam	Sucker
28	Columbia River, Rock Island Dam	Whitefish
29	Columbia River, Rock Island Dam	Sunfish
30, 31	Columbia River, Rock Island Dam	Sucker
32	Columbia River, Rock Island Dam	Sockeye salmon
Chondrococcus columnaris	Chilko Lake, British Columbia	Sockeye salmon
Cytophaga psychrophila	Siletz Salmon Hatchery, Oregon Fish Commission	Silver salmon

lated with two drops of a *Cytophaga* broth culture and were overlayed with sterile Vaspar to exclude air.

The tolerance to NaCl was determined by inoculating *Cytophaga* broth containing various amounts of NaCl and examining for growth within a period of 2 weeks. The salt concentrations used were 0, 0.5, 1.0, 2.0, 3.0, and 4.0%.

Physiological tests. Cytophaga agar was used as the basal medium to test the ability of the isolates to degrade starch, gelatin, casein, tributyrin, and tyrosine.

Gelatin liquefaction. Gelatinase production was tested with the basal medium supplemented with 0.4% (w/v) gelatin. After 2 to 3 days of growth, the plates were flooded with acid mercuric chloride to show areas of gelatin liquefaction (6).

Casein hydrolysis. The ability to hydrolyze casein was determined with a medium containing 2% (v/v) skim milk. After 48 hr of growth, the plates were examined for areas of hydrolysis in an otherwise opaque medium.

Starch hydrolysis. The medium used to detect starch hydrolysis contained a 0.2% (w/v) solution of potato starch. The plates were flooded with an iodine solution after 2 to 3 days of incubation. Colonies with the ability to hydrolyze starch produced clear areas in an otherwise blue medium.

Tyrosine degradation. Decomposition of tyrosine was measured by the disappearance, within 2 weeks, of 0.5% (w/v) tyrosine suspended in the basal medium.

Esculin hydrolysis. Hydrolysis of esculin was checked with a medium containing 1.0% peptone, 0.1% esculin, 0.05% ferric ammonium citrate, and 1.5% agar (Difco). The ability to split esculin was demonstrated by the formation of a black precipitate around the colonies within 7 days.

Digestion of chitin. Chitin decomposition was tested with a suspension of chitin prepared according to the procedure of Stanier (20). A 5-ml amount of agar containing 0.1% peptone, 0.5% chitin, and 1.0% Difco agar was overlayed on non-nutrient agar plates and allowed to dry at 37 C for 1 day. Cultures to be tested were spotted onto the surface of the overlay agar, and hydrolysis was detected by the dissolution of chitin around the areas of growth.

Decomposition of cellulose. The method of Emerson and Weiser (5) was used to detect cellulose digestion. The mineral salts medium devised by Stanier (19) was supplemented with 1.5% agar and used as a basal medium. The plates were examined at regular intervals for 1 month for depressions in the overlay surrounding the colonies.

Lysis of bacterial cells. Bacterial cell agar was prepared with washed cell suspensions of five selected species (Table 2). The growth obtained from 250 ml of nutrient broth was washed three times in distilled water, suspended in 160 ml of distilled water containing 1.5% Difco agar, and autoclaved. To test for lytic activity, non-nutrient agar plates were overlayed with 5 ml of bacterial cell agar and inoculated. Evidence of lysis was indicated by the appearance of clear zones around the areas of growth.

Nitrate reduction. The ability to reduce nitrates was examined according to the procedures described in the *Manual of Microbiological Methods* (18). Zinc powder was used to detect false negatives.

Indole formation. Indole production in 1.0% (w/v) tryptone broth was determined by the method of Kovács as described in the *Manual of Microbiological Methods* (18).

Hydrogen sulfide formation. The production of hydrogen sulfide was tested with lead acetate strips over tryptone broth cultures of the test organisms.

Citrate utilization. The utilization of citrate as a sole source of carbon was tested with Koser's citrate medium (12).

Catalase production. The presence of catalase was determined by mixing a loopful of organisms grown on *Cytophaga* agar in 2.0% hydrogen peroxide.

Cytochrome oxidase formation. The cytochrome oxidase test of Gaby and Hadley (8) was used to determine the presence of an oxidase.

Carbohydrate utilization. A modification of the Hugh-Leifson procedure (10) was used to test for the production of acid from carbohydrates. The basal medium consisted of 0.2% peptone, 0.03% K_2HPO_4, 0.0015% bromothymol blue, and 0.3% Difco agar. All carbohydrates were filter sterilized and added to the basal medium at a final concentration of 0.5%.

Serological procedures. Antisera for *C. psychrophila* and *C. columnaris* were prepared by injecting rabbits subcutaneously with triply washed cells suspended in Freund's adjuvant (7). A slide agglutination procedure was used to examine the serological relationships among the isolates.

RESULTS

Morphological characteristics. The organisms studied in this investigation corresponded to the classical definition of myxobacterial cells. All of the isolates were gram-negative, slender, weakly refractile, rod-shaped bacteria which exhibited gliding motility. The dimensions of the cells were found to vary with the age of the culture. Actively growing cells of the organisms were approximately 0.5 μm in diameter and from 2 to 7 μm long. In general, the organisms tended to become somewhat shorter and thicker as the cultures aged. Numerous involution forms were also noted in older cultures. These structures consisted of spherical organisms with small round or rod-like structures usually visible within the cell. Rod-shaped organisms with a spherical extrusion also occurred in these cultures.

On *Cytophaga* agar, the organisms produced colonies characteristic of myxobacteria. Colonies produced were yellow-orange in color, thin, flat, and spreading with irregular edges. A photograph of a typical colony is shown in Fig. 1.

Fruiting bodies were produced on bits of fish tissue by the strain of *C. columnaris* studied in this investigation. However, none of the other isolates formed these structures. Microcysts were never observed in any of the cultures studied. Hence, except for the strain of *C. columnaris*, all of the organisms included in this study appeared to be members of the genus *Cytophaga*.

Cultural and physiological characteristics. All of the isolates grew readily between the temperatures of 4 and 18 C. At temperatures above 18 C, the number of organisms capable of growing was inversely proportional to the increase in temperature. At 25 C, *C. psychrophila* and 5 of the

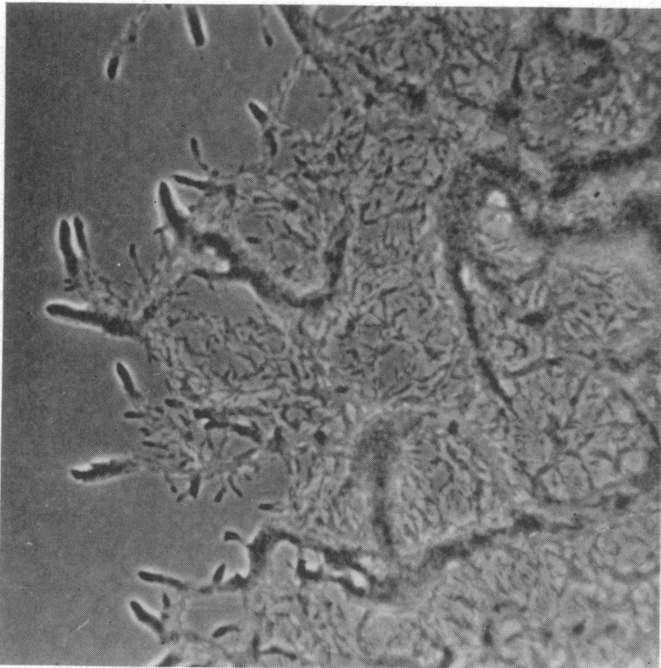

FIG. 1. *Phase contrast micrograph of the edge of a typical myxobacterial colony.* × *140.*

TABLE 2. *Summary of characteristics of myxobacteria isolated from fish*[a]

Characteristic	Group A		Group B			Group C				Group D								*Cytophaga psychrophila*	*Chondrococcus columnaris*
	10, 14, 26[b]	11, 30	4, 17, 8	1, 8, 29	28	13, 15	22, 23	9, 21	18	20	27	2, 6, 7	16	3, 5	12, 19, 32	25	31		
Growth at 25 C	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Growth anaerobically	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NaCl tolerance																			
Growth in 0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in 0.5%	−	−	+	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	+
Growth in 1.0%	−	−	±	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−
Growth in 2.0%	−	−	−	+	+	+	+	±	+	+	−	+	−	±	−	−	−	−	−
Growth in 3.0%	−	−	−	±	−	−	−	−	−	+	−	±	−	−	−	−	−	−	−
Growth in 4.0%	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Degradation of																			
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Esculin	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Chitin	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Cellulose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Tributyrin	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−
Carbohydrate utilization																			
Glucose oxidized	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
Glucose fermented	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Lactose oxidized	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Lactose fermented	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Galactose oxidized	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Galactose fermented	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose oxidized	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose fermented	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Nitrate reduced	−	−	−	+	+	+	+	−	−	+	+	−	−	−	−	−	−	−	−
Hydrogen sulfide produced	−	−	+	+	+	+	+	+	−	+	−	+	+	−	−	−	−	−	+
Tyrosine decomposed	+	+	±	±	−	−	+	+	+	+	−	+	+	+	+	+	+	+	−
Citrate utilized	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−
Indole produced	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Catalase produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cytochrome oxidase produced	+	+	−	−	−	+	−	−	−	−	−	−	+	−	+	+	−	−	+
Lysis of dead bacterial cells																			
Escherichia coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus	+	+	±	±	−	−	+	+	+	−	−	−	+	±	+	+	+	+	−
Bacillus subtilis	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mycobacterium smegmatis	±	−	±	+	+	+	−	±	−	+	−	±	−	±	+	+	−	−	+

[a] Symbols: + = organisms possess characteristic; − = organisms lack characteristic; ± = characteristic possessed by some organisms and not by others.
[b] Strain number.

nonpathogenic strains failed to grow; at 30 C, 10 of the remaining isolates were unable to grow. Only one isolate, in addition to *C. columnaris*, was capable of growing at 37 C. Of the 32 nonpathogenic myxobacteria studied, 7 were capable of growing anaerobically. Neither of the pathogenic species included in this study could grow in the absence of oxygen. On the basis of arbitrarily selected cultural and physiological characteristics, the organisms were separated into groups. Group A grew readily at 20 C, but not at 25 C; group B grew anaerobically; group C oxidized glucose, but did not grow anaerobically; and group D grew readily at 25 C and neither oxidized glucose nor grew anaerobically. Subdivision of these groups was made on the basis of characteristics held in common by various strains. A summary of the characteristics of the organisms is presented in

Table 2. For comparison, the properties of *C. psychrophila* and *C. columnaris* are also included in the table.

Serological analyses. Each of the 32 nonpathogenic myxobacterial isolates was tested against antisera prepared against *C. psychrophila* and *C. columnaris*. None of the heterologous strains were agglutinated by either of these antisera, whereas the homologous strains were agglutinated at antisera dilutions of 1:320 and 1:640. From these results, it appeared that the nonpathogenic myxobacteria associated with fish are serologically distinct from the pathogenic species.

DISCUSSION

In this study, we demonstrated that several types of freshwater cytophagas occur on the surface of fish. Most of the organisms grew readily at 25 C and were obligately aerobic; however, a number of psychrophilic strains and several facultative anaerobes were also noted. The ability to degrade macromolecules appears to be characteristic of these organisms. All of the isolates were found to be actively proteolytic. The majority were also amylolytic, lipolytic, and chitinoclastic. In addition, they were able to attack dead bacterial cells.

Proteolytic microorganisms which grow at low temperatures have been considered to be of primary importance in fish spoilage (11). Since the myxobacteria obtained from freshwater fish grew at low temperatures and exhibited proteolytic activity, it is possible that these organisms could be associated with the spoilage of fish.

One of the objectives of this study was to compare the characteristics of pathogenic and nonpathogenic species of myxobacteria isolated from fish. On the basis of cultural and physiological characteristics, the pathogenic strains are similar to some of the nonpathogenic species. *C. psychrophila* can be placed into group A on the basis of its temperature range of growth, and *C. columnaris* can be placed into group D since it grows readily at 25 C and neither oxidizes glucose nor grows anaerobically. One notable difference was the fact that the nonpathogenic strains decomposed complex carbohydrates more readily than the pathogenic strains. Nearly all of the nonpathogenic isolates hydrolyzed starch, esculin, and chitin, whereas the pathogenic species did not. Whether these tests would be of value in distinguishing myxobacteria pathogenic to fish would require additional studies involving a larger number of strains.

The formation of fruiting bodies by *C. columnaris* served to distinguish this organism from the myxobacteria in group D. However, since this property does not appear to be a stable characteristic of the organism (16), it could not be relied upon as a means of identification.

Both *C. psychrophila* and *C. columnaris* were found to be serologically distinct from each other and from other myxobacteria associated with fish. Antisera prepared against these two pathogens agglutinated the homologous strains, but failed to cross-react with any of the other organisms investigated in this study. These findings indicate that serological procedures might provide a rapid and useful means for identifying the pathogenic myxobacteria.

The occurrence of fermentative myxobacteria on fish was not an unexpected finding. Borg (3) reported isolating organisms of this type from young salmon infected with gill disease. Anderson and Ordal (2) also reported the occurrence of fermentative myxobacteria on fish. As a result of their studies, a new species of cytophaga, *C. succinicans*, was described. *C. succinicans* differed from the other fermentative cytophagas which have been described in that CO_2 was required by the organism for anaerobic growth. Only one of the strains of myxobacteria studied in the present investigation, strain 28, required CO_2 for anaerobic growth and could be considered to be related to *C. succinicans*.

It is likely that many of the myxobacteria isolated in this study represent undescribed species. However, since speciation was not the objective of this investigation, no attempt was made to assign specific names to any of the isolates.

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