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Characteristics of *Cytophaga psychrophila* (Borg) Isolated During Outbreaks of Bacterial Cold-Water Disease

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Characteristics of 10 strains of *Cytophaga psychrophila* isolated during a number of epizootics of bacterial cold-water disease were compared. Morphological, cultural, biochemical, and serological data showed that the organisms were very closely related. The isolates exhibited gliding motility, formed neither fruiting bodies nor microcysts, were actively proteolytic, and grew only at low temperatures. Data presented extend the description of *C. psychrophila*.

The etiological agent of bacterial cold-water disease was originally isolated by Borg (Ph.D. Thesis, University of Washington, Seattle, 1948). This organism was found to be a nonfruiting myxobacterium which was unable to grow at temperatures above 25 C. On the basis of pathogenicity and low optimal growth temperature, the organism was considered to be a new species of myxobacterium. The name *Cytophaga psychrophila* was proposed. The description of this organism is somewhat incomplete, and it is not included in *Bergey's Manual of Determinative Bacteriology*, 7th ed.

Bacterial cold-water disease is generally found in young silver salmon in early spring when water temperatures are low. In some hatcheries, up to 30% of the fry have been lost as a result of this disease. Generally, when water temperatures increase to 13 C the disease abates.

Because of the importance and widespread distribution of this organism among the hatchery populations of fish in the Pacific Northwest, it was of interest to compare cultures isolated from different outbreaks of disease to determine whether one or several strains of the organism were involved. This report describes the results of these studies and extends the description of *C. psychrophila*.

MATERIALS AND METHODS

Source of strains. A total of 10 strains of *C. psychrophila*, isolated from silver and chinook salmon taken from various hatcheries in the Pacific Northwest, were studied. The code numbers and source of each are shown in Table 1. The strains isolated at various locations in the State of Washington were provided from the culture collection at the University of Washington through the courtesy of E. J. Ordal. Strains 143a and 144a are two of the original cultures isolated by Borg.

The cultures were maintained on *Cytophaga* agar deeps. This medium is composed of tryptone, 0.05%; yeast extract, 0.05%; beef extract, 0.02%; sodium acetate, 0.02%; and agar (Difco), 0.4%. The stock cultures were incubated at 18 C for about 1 week and then stored at 4 C.

Liquid cultures used as inocula for the various tests performed were grown in *Cytophaga* broth. The composition of this medium is the same as that described above except that the agar is omitted. All culture media were cooled to refrigeration temperatures before use.

Morphological characteristics. After 24 hr of incubation in *Cytophaga* broth at 18 C, the organisms were Gram-stained. Cell morphology and motility were determined by examination of wet mounts with a phase-contrast microscope. Colony appearances on *Cytophaga* agar were noted after 36 hr of incubation. In an attempt to induce fruiting-body and microcyst formation, the organisms were placed on sterile fish tissues submerged in tap water. This procedure was first described by Ordal (6).

Environmental characteristics. The ability of strains to grow in *Cytophaga* broth at 37, 30, 23, 18, and 4 C was recorded after incubation periods ranging from 1 day to 2 weeks, depending on the temperature employed. Growth was determined by examining tubes for turbidity.

The NaCl tolerance of the organisms was determined by use of *Cytophaga* broth containing various amounts of NaCl. Cultures were examined for growth within 2 weeks. The NaCl concentrations employed included 0, 0.1, 0.2, 0.4, 0.8, 1.0, and 2.0%.

Susceptibility to antibacterial agents. Susceptibility to antibiotics was determined by placing Sensi-discs of the antibiotics (BBL) on agar plates streaked with the

TABLE 1. *Source of isolates*

Strain	Source	Fish host
Simp 2	Simpson Fish Hatchery, Washington State Department of Fisheries	Silver salmon
L. Kal. 1	Kalama Fish Hatchery, Washington State Department of Fisheries	Chinook salmon
LMC 6	Lower Minter Creek Fish Hatchery, Washington State Department of Fisheries	Silver salmon
Si-5	Siletz Salmon Hatchery, Oregon Fish Commission	Silver salmon
A1-2	Alsea Salmon Hatchery, Oregon Fish Commission	Silver salmon
A1-3	Alsea Salmon Hatchery, Oregon Fish Commission	Silver salmon
Dun 1	Dungeness Fish Hatchery, Washington State Department of Fisheries	Silver salmon Silver salmon
143a	Minter Creek Fish Hatchery, Washington State Department of Fisheries	Silver salmon
144a	Minter Creek Fish Hatchery, Washington State Department of Fisheries	Silver salmon
Is-9	Issaquah Fish Hatchery, Washington State Department of Fisheries	Silver salmon

test organism. The strains were tested for susceptibility to chlortetracycline, 30 μg; bacitracin, 10 units; chloramphenicol, 30 μg; dihydrostreptomycin, 10 μg; erythromycin, 2 μg; neomycin, 30 μg; penicillin, 10 units; polymyxin B, 30 μg; sulfadiazine, 1 mg; and tetracycline, 30 μg.

Physiological tests. The basal medium used to test the ability of the isolates to degrade gelatin, starch, casein, tributyrin, tyrosine, xanthine, and albumin consisted of tryptone, 0.1%; yeast extract, 0.05%; beef extract, 0.05%; and agar (Difco), 1.1%.

Gelatinase production was tested by use of the basal medium supplemented with 0.5% gelatin. After 1 week of incubation at 18 C, the plates were flooded with acid mercuric chloride to show areas of gelatin liquefaction (3).

Starch hydrolysis was detected by use of a medium containing 0.2% soluble starch. Plates of this medium were inoculated, incubated for 1 week, and then flooded with an iodine solution. Colonies with the ability to hydrolyze starch produced clear areas in an otherwise blue medium.

The ability to hydrolyze casein was tested by inoculating the cultures onto the basal medium containing 10% skim milk. After 1 week, the plates were examined for clear areas of hydrolysis in the opaque medium.

Tributyrin hydrolysis was determined by clearing produced on 0.05% tributyrin incorporated in the basal medium. Plates were read after 2 weeks of incubation.

Tyrosine breakdown was measured by the ability of cultures to dissolve 0.5% tyrosine suspended in basal medium within 2 weeks.

The ability to decompose xanthine was tested by use of basal medium supplemented with 0.4% xanthine. The cultures were examined for periods up to 1 month for the disappearance of crystals around the areas of growth.

Albumin hydrolysis was detected by use of the basal medium containing 0.25% egg albumin. Evidence of hydrolysis was detected after 1 week incubation by the addition of acid mercuric chloride to the plates.

The method of Emerson and Weiser (2) was used to detect cellulose digestion. The mineral salt medium devised by Stanier (8) was supplemented with 1.5% agar and used as a basal medium. The plates were examined every 2 days for 1 month for depressions in the overlay surrounding the colonies.

Chitin decomposition was tested with a suspension of chitin prepared according to the procedure described by Stanier (9). A 5-ml amount of agar (containing 0.1% peptone, 0.5% chitin, and 0.7% Difco agar) was overlaid on non-nutrient agar plates and allowed to dry 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.

Non-nutrient agar (1%), containing 1% (wet weight) autoclaved, washed cells of *Escherichia coli*, was used to determine the ability of the strains to lyse bacterial cells. Evidence of lysis was indicated by the appearance of clear zones around the areas of growth.

A slight modification of the Hugh-Leifson procedure (5) was used to test for the production of acid from carbohydrates. The basal medium consisted of peptone, 0.2%; sodium chloride, 0.2%; K_2HPO_4, 0.03%; bromothymol blue, 0.0015%; and agar (Difco), 0.3%. The carbohydrate solution was filter-sterilized and added to the basal medium at a final concentration of 1.0%.

A modified litmus milk medium was used to test the action of the organisms on milk. The medium consisted of 5.0% skim milk and 0.04% litmus.

Hydrogen sulfide production was tested by use of lead acetate strips over tryptone broth cultures of the test organisms.

The medium used for the Voges-Proskauer and methyl red tests consisted of 0.7% peptone and 0.5% glucose. Tests for acid production with the use of

methyl red indicator and for acetylmethylcarbinol production with 40% KOH and α-naphthol reagent were carried out.

Tests for the reduction of nitrate to nitrite, for indole formation, and for catalase were carried out according to the procedures described in *Manual of Microbiological Methods* (7).

The presence of cytochrome oxidase was determined by the method of Gaby and Hadley (4). The appearance of a blue color within 2 min after addition of the reagent was considered a positive reaction.

Nutritional requirements of the organisms were tested by use of a casein hydrolysate medium containing 0.1% $(NH_4)_2HPO_4$, 0.02% KCl, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.2% vitamin-free acid-hydrolyzed casein. The ability to utilize citrate as a sole source of carbon was tested by use of a mineral base medium containing 0.02% $MgSO_4 \cdot 7H_2O$, 0.1% $NH_4H_2PO_4$, 0.1% K_2HPO_4, 0.2% NaCl, 0.2% sodium citrate, 1.5% agar (Difco), and 0.001% bromothymol blue.

Serological methods. Immune sera were obtained from rabbits injected intravenously with saline-washed suspensions of cells in successive doses of 0.2, 0.4, 0.8, 1.5, and 2.0 ml on every 3rd day. The rabbits were bled from the marginal ear vein 2 weeks after the last injection. The sera were stored in the frozen state.

A tube agglutination procedure was used to titrate the antisera. The antigen suspension consisted of doubly washed saline suspension of cells adjusted to an optical density of 0.22 at a wavelength of 525 mμ. The tubes were incubated at 52 C for 2 hr and for approximately 18 hr at 4 C before being read. A slide agglutination procedure was used to determine the relationships between the test isolates. The antigen suspension employed was the same as that used in the tube agglutination studies.

RESULTS

Morphological characteristics. All of the isolates were weakly refractile, slender, gram-negative, flexible rods (Fig. 1). Actively growing cells of the organisms were about 0.75 μ in diameter and 1.5 to 7.5 μ long. As the cultures aged, the cells tended to become somewhat shorter than those found in log-phase cultures.

On *Cytophaga* agar, the colonies of the strains of *C. psychrophila* studied were bright yellow in color and generally showed thin, spreading margins. No diffusible pigment was apparent. A photograph of an edge of a typical colony is shown in Fig. 2. Although the colony shown in Fig. 2 is characteristic for this organism, variations may occur. In some cases, deeply colored colonies with entire edges were formed. The fact that several colony types appeared on the same plate indicates that colony morphology is a variable characteristic for this organism. No attempt was made to determine the effect of various environmental factors on colony appearance.

Neither fruiting-body formation nor microcyst production occurred on any of the culture media. Attempts to induce fruiting bodies on bits of fish tissue also were unsuccessful. Thus, these organisms appear to be typical representatives of the genus *Cytophaga* as defined by Stanier (8).

Environmental characteristics. All of the test isolates grew readily at temperatures between 4 and 23 C. As would be expected, growth at lower

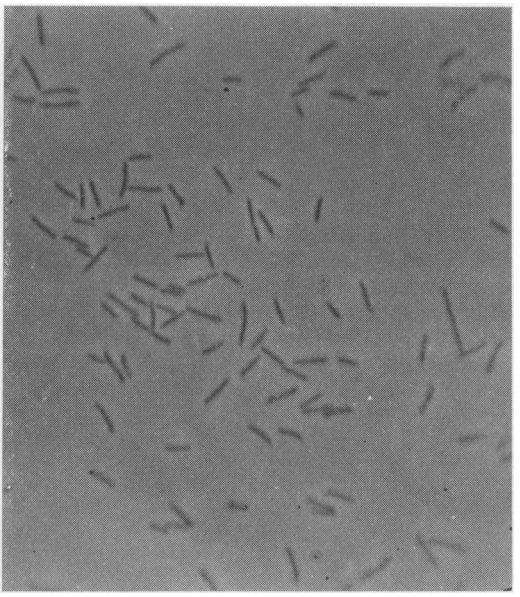

FIG. 1. *Phase-contrast micrograph of a culture of Cytophaga psychrophila.* $\times$ *1,100.*

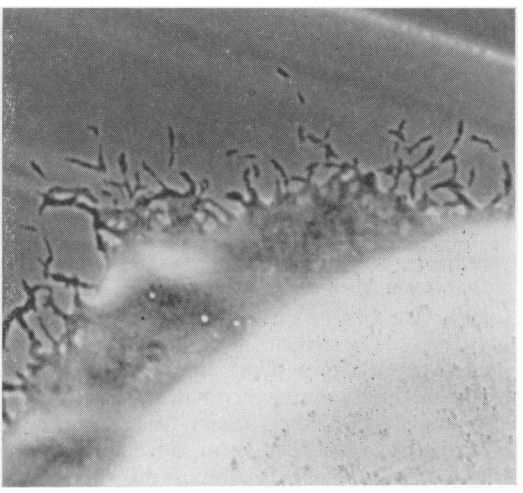

FIG. 2. *Phase-contrast micrograph of the edge of a colony of Cytophaga psychrophila on Cytophaga agar.* $\times$ *140.*

TABLE 2. *Selected physiological characteristics of Cytophaga psychrophila*

Characteristic	Percentage of strains positive
Gelatin degradation	100
Casein hydrolysis	100
Albumin digested	100
Tributyrin hydrolyzed	100
Tyrosine decomposed	20
Digestion of autoclaved cells of *Escherichia coli*	100
Starch hydrolyzed	0
Chitin degraded	0
Cellulose decomposed	0
Xanthine hydrolyzed	0
Litmus milk peptonized	100
Carbohydrate utilization:	
Glucose oxidized	0
Glucose fermented	0
Cellobiose oxidized	0
Cellobiose fermented	0
Hydrogen sulfide produced	0
Indole produced	0
Voges-Proskauer positive	0
Nitrate reduced to nitrite	0
Catalase produced	100
Cytochrome oxidase produced	0
Growth in casein hydrolysate medium	100
Growth on citrate medium	0

temperatures was much slower than that occurring in the vicinity of 20 C. None of the organisms was capable of growing at 30 or 37 C.

All of the cultures grew in the presence of 0.8% NaCl. In 1% NaCl medium, only six of the organisms were capable of growing, and 2% NaCl inhibited growth of all of the isolates tested. No decrease in growth was noted in the basal medium alone without the addition of NaCl.

Antibiotic susceptibility. Antibiotics which proved to be inhibitory to the 10 strains of *C. psychrophila* were chlortetracycline, bacitracin, chloramphenicol, dihydrostreptomycin, erythromycin, neomycin, penicillin, and tetracycline. In addition, 4 of the 10 isolates were susceptible to polymyxin B.

Physiological properties. Results of 24 physiological tests carried out on the isolates are given in Table 2. These data indicate that *C. psychrophila* is actively proteolytic, but shows no ability to degrade either simple or complex carbohydrates. Tyrosine degradation appears to be a variable characteristic of this organism, since only 2 of the 10 isolates were able to decompose this compound. The nutritional requirements of the organism were not complex as indicated by the fact that the isolates were able to grow on a vitamin-free casein hydrolysate medium.

Serological analyses. Three strains of *C. psychrophila* isolated during different outbreaks of bacterial cold-water disease were selected and used to immunize rabbits. The three strains chosen were 144a, Dun 1, and LMC 6. The homologous titers of these antisera were 1:640.

To test the serological homogeneity of the isolates of *C. psychrophila*, slide agglutination tests were carried out on the organisms. It was found that each of the 10 cultures was agglutinated by each of the three antisera. Thus, the isolates are similar serologically.

The specificity of the antisera for *C. psychrophila* was tested by carrying out slide agglutination studies with 24 nonpathogenic cytophagas isolated from fish. None of these isolates was agglutinated by the three antisera available; hence, no serological evidence was found supporting a relationship between *C. psychrophila* and other myxobacteria associated with fish.

DISCUSSION

On the basis of a limited number of tests, Borg (1) described *C. psychrophila* as a gram-negative, flexible rod which exhibited creeping motility. The organism produced neither fruiting bodies nor microcysts, was strictly aerobic, and grew only at low temperatures. In addition, casein was hydrolyzed and catalase was produced by this bacterium. Two of the cultures originally isolated by Borg were available for comparison in the present investigation. These two isolates differed from the other organisms studied in that they were able to degrade tyrosine. No other significant characteristics were noted which would allow Borg's isolates to be distinguished from the other cultures studied.

The 10 strains of *C. psychrophila* studied in this investigation were found to represent a very homogeneous group of microorganisms. This finding is of interest since the organisms were isolated over a period of years in widely separated geographic areas and from different species of fish. The only cultural and physiological differences noted were with regard to sodium chloride tolerance, sensitivity to sulfadiazine, and the ability to decompose tyrosine. Morphologically and serologically the cultures were also very closely related.

The fact that antiserum prepared against *C. psychrophila* appears to be highly specific for this organism would suggest that somatic antigens might be used to distinguish *C. psychrophila* from other myxobacteria occurring on fish. Therefore, it may be possible to use serological procedures as a means for rapid identification of the organism. Before reliance can be placed on such a

test, however, a greater number of myxobacteria will have to be studied.

It is of interest to note that all of the isolates of *C. psychrophila* examined in the present study were actively proteolytic. Perhaps this activity plays an important role in the pathogenicity of the organisms.

On the basis of the results of this investigation, the description of *C. psychrophila* is extended as follows:

The cells are gram negative, rod-shaped and vary in length depending on the age of the culture. The average size of young cells is about 0.75 by 3.5 μ. Gliding motility is exhibited on solid surfaces.

Colonies on *Cytophaga* agar are yellow in color and generally exhibit a thin, spreading edge. Nonspreading variants occur. No diffusible pigment was produced.

Gelatin, casein, albumin, tributyrin, and autoclaved cells of *Escherichia coli* hydrolyzed. Starch, chitin, cellulose, xanthine not degraded. Tyrosine degradation is variable.

Litmus milk peptonized.

Glucose and cellobiose neither fermented nor oxidized.

Indole negative. Voges-Proskauer and methyl red negative.

Nitrate not reduced to nitrite.

Hydrogen sulfide not produced.

Catalase produced. Cytochrome oxidase not produced.

Growth on casein hydrolysate broth. No growth on citrate medium.

No growth in the presence of 2% NaCl.

Optimal temperature about 20 C. No growth at 30 C.

Source: Isolated from fish with bacterial coldwater disease.

Habitat: Freshwater. A pathogen of fish.

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