Flavobacterium branchiophila sp. nov., a Causative Agent of Bacterial Gill Disease of Freshwater Fishes

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The name *Flavobacterium branchiophila* is proposed for a group of 16 bacterial strains that were isolated in Japan (5 isolates), Oregon (10 isolates), and Hungary (1 isolate) from cultured salmonids or sheatfish suffering from bacterial gill disease. These bacteria grow in cytophaga medium at temperatures between 10 and 25°C. The isolates are gram-negative, nonmotile, slender rods measuring 0.5 by 5 to 8 µm. They utilize gelatin, casein, starch, glucose, fructose, sucrose, maltose, and trehalose. The mean guanine-plus-cytosine content of the deoxyribonucleic acids of three selected strains was 30 mol%. Strain BGD-7721 (= ATCC 35035) is designated the type strain of the new species.

Bacterial gill disease (BGD), which results in high mortality in salmonid fishes, is characterized by the presence of numerous filamentous bacterial cells on the surfaces of the gills (16). Such gram-negative bacteria have been isolated from some salmonids with BGD in Japan and Oregon and have been classified as *Flavobacterium* sp. (7, 17). This bacterium appeared abundantly on the surfaces of the gills of juvenile trout within 18 to 24 h after waterborne exposure of the fish to a bacterial suspension of cells in an aquarium. The infection caused irritation to the gills and impaired their respiratory function (18). The fish eventually died from suffocation in water containing levels of dissolved oxygen much higher than the incipient lethal levels for uninfected control fish (19). Farkas (3) isolated filamentous, nongliding bacteria from certain freshwater fishes afflicted with BGD in Hungarian fish farms. These isolates were identified as *Flavobacterium* sp. and were reported to exhibit positive slide agglutination with the antiserum prepared against our isolates. In this paper, we present evidence that BGD isolates from Japan, Oregon, and Hungary constitute a new species, for which the name *Flavobacterium branchiophila* is proposed.

MATERIALS AND METHODS

Bacterial strains. The 16 bacterial strains isolated from diseased fish and used in this study included the following organisms: strain BGD-7501, which was isolated in 1975 in Gunma, Japan, from rainbow trout; strains BGD-7721^T (= ATCC 35035^T) (T = type strain), BGD-7736, BGD-7737, and BGD-7738, which were isolated in 1977 in Gunma, Japan, from Yamame (*Oncorhynchus masou*); strain FDL-1, which was isolated in 1978 at the Corvallis Fish Disease Laboratory in Oregon from steelhead trout; strains BV-1 (= ATCC 35036), BV-4, BV-5, BV-6, BV-8, and FDL-2, which were isolated in 1978 at the Bonneville Hatchery in Oregon from chinook salmon; strain FDL-3, which was isolated in 1978 at the Corvallis Fish Disease Laboratory in Oregon from sockeye salmon; strains OS-1 and OS-2, which were isolated in 1978 at the Oak Springs Hatchery in Oregon from steelhead trout; and strain FL-15, which was isolated in 1983 in Hungary from sheatfish and was supplied by J. Farkas (3). The strains were isolated by streaking portions of gill tissues on cytophaga agar (CA) (1). The bacteria were stored in

cytophaga broth (CB) containing 10% glycerol at -80°C or were preserved by lyophilization. All of our results were based on cultures incubated at 18°C unless otherwise noted.

Morphology and growth studies. Cell morphology was determined by examining gram-stained smears by light microscopy or by electron microscopy. The bacterial smears were stained with Sudan black (2) to detect intracellular granules of poly-β-hydroxybutyrate. Motility was determined by mixing small amounts of CA colony growth in water and examining the suspension by phase-contrast microscopy. The method of Perry (12) was also used to test for gliding movement.

The ability of the strains to grow at 5, 10, 18, 25, and 30°C was tested in CB; the incubation periods ranged from 1 week to 1 month depending on the temperature. The tolerance to NaCl was determined by inoculating CB containing 0, 0.025, 0.05, 0.075, 0.1, or 0.2% NaCl and recording the amount of growth for more than 2 weeks. The strains were tested for anaerobic growth by incubating CB cultures for 3 weeks in an anaerobic jar (GasPak; BBL Microbiology Systems, Cockeysville, Md.).

Physiological characteristics. Catalase was detected by applying hydrogen peroxide to growth on CA and observing evolution of gas bubbles. Cytochrome oxidase was detected by using commercially prepared test paper (Nissui Co., Tokyo, Japan). Production of flexirubin was assayed by detecting brown pigment after 20% KOH was added to young colonies on CA. Hydrogen sulfide and indole were tested in medium containing 0.5% tryptone and 0.5% yeast extract. Production of indole was determined by the method of Kovacs (8). Hydrogen sulfide was detected by inserting a strip of paper saturated with a lead acetate solution into tubes. Nitrate reduction was tested in CB containing 0.1% KNO₃. Hydrolysis of starch was detected by covering bacterial surface growth on CA containing 0.1% soluble starch with a gram iodine solution, and hydrolysis of gelatin was detected by flooding growth on CA containing 0.4% gelatin with 8 to 10 ml of a solution containing 15 g of HgCl₂ in 100 ml of distilled water and 20 ml of concentrated HCl; positive hydrolysis was recorded when a clear halo surrounding agar colonies was observed. Hydrolysis of casein was determined on CA containing 0.05% skim milk; a positive response was recorded when a clear zone surrounding agar colonies was observed. Esculin degradation was detected by the presence of a distinct dark brown precipitate in a culture grown in CB containing 0.1% esculin following

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FIG. 1. Electron micrograph of negatively stained cells of *Flavobacterium branchiophila* BGD-7721^T. Bar = 1 μ m.

addition of a few drops of a 1% ferric citrate solution. Chitin decomposition was determined by using a modification of the method of Skerman (15). Cellulose digestion was demonstrated by disintegration of a strip of Toyo no. 2 filter paper in a tube of CB inoculated with the bacterium. Deoxyribonuclease activity was determined on a slant of DNase medium (Eiken, Tokyo, Japan). Carbohydrate acidification was tested in broth medium containing 0.05% tryptone, 0.05% yeast extract, 0.0018% phenol red, and filter-sterilized carbohydrate at a concentration of 0.2%. All cultures were incubated for 1 week, and color changes (red to yellow) were observed for acidification of the carbohydrate.

Tests for the detection of 19 specific enzymes were performed with a commercially produced kit system (API ZYM, La Balme-les-Grottes, Montalieu-Vercieu, France).

DNA homology and DNA base composition. Deoxyribonucleic acid (DNA) relatedness was determined for three strains of *Flavobacterium branchiophila*, strains BGD-7721^T (= ATCC 35035^T), BV-1 (= ATCC 35036), and FL-15. The following reference strains were also included: *Flavobacterium aquatile* IAM 12316 (= ATCC 11947) and *Flexibacter columnaris* EK-28 (20). DNA was isolated and purified by using the method of Saito and Miura (14). The DNAs from the four strains other than strain FL-15 were labeled with ³H by nick translation (in vitro) essentially by the method of Rigby et al. (13) and in accordance with instructions furnished with a commercial nick translation reagent kit (kit N.5000; Amersham International plc, Buckinghamshire, England). DNA-DNA hybridization experiments were done on sodium dodecyl sulfate at 42°C (10).

The guanine-plus-cytosine (G+C) contents of three of the strains were determined by the thermal denaturation method (9). DNA from *Escherichia coli* K-12 (G+C content, 51.6 mol%; Sigma Chemical Co., St. Louis, Mo.) was used as a control.

RESULTS AND DISCUSSION

Morphology and phenotypic characteristics. The cells of all of the strains were gram-negative slender rods measuring 0.5 by 5 to 8 μ m, and they usually occurred in chains of two or three cells in CB (Fig. 1). They were nonmotile and showed neither gliding movement nor swarming growth on agar media.

Growth on CA was usually slow, with colonies appearing only after 2 to 5 days of incubation at 18°C. The colonies were light yellow, round, transparent, smooth, and about 0.5 to 1 mm in diameter after incubation for 5 days. The agar colony pigments were not of the flexirubin type. On Trypticase soy agar, growth occurred only when the medium was diluted about 20-fold. All strains grew well at 10, 18, and 25°C and in the presence of 0.025% NaCl or in the absence of NaCl. Some strains were also able to grow at temperatures as low as 5°C and as high as 30°C and in the presence of higher concentrations of NaCl (0.05 to 0.1%). All of the strains failed to grow anaerobically.

Most of the bacterial strains were uniform in their physiological characteristics, although some strains did not grow in the test medium for nitrate reduction. Also, there was no growth on DNase medium. In the cellulose digestion test, the bacteria grew on the surface of the filter paper, but disintegration of the strip did not occur. A summary of the phenotypic properties is shown in Table 1.

DNA homology and DNA base composition. Labeled DNAs from strains BGD-7721^T (= ATCC 35035^T) and BV-1 (= ATCC 35036) were highly related to the DNAs of the isolates from fish with BGD but were not highly related to either *Flavobacterium aquatile* IAM 12316 (= ATCC 11947) or *Flexibacter columnaris* EK-28 (Table 2).

The G+C contents of three of the strains ranged from 29 to 31 mol% (mean, 30 mol%).

TABLE 1. Characteristics for differentiating *Flavobacterium branchiophila* from other *Flavobacterium* species^a

Characteristic	<i>Flavobacterium branchiophila</i>	<i>Flavobacterium aquatile</i> ^b	<i>Flavobacterium breve</i> ^b	<i>Flavobacterium balustinum</i> ^b	<i>Flavobacterium meningosepticum</i> ^b	<i>Flavobacterium odoratum</i> ^b	<i>Flavobacterium multivorum</i> ^b	<i>Flavobacterium spiritivorum</i> ^b
Yellow pigmentation	+	+	+	+	d	+	+	d
Growth at 37°C	—	—	d	+	+	+	+	+
Good growth on nutrient agar	—	—	+	+	+	+	+	+
Hydrolysis of:								
Gelatin	+	—	+	+	+	+	d	+
Casein	+	+	+	+	+	+	—	—
Esculin	—	—	—	+	+	—	+	+
Starch	+	NG	—	—	—	—	—	—
Acid produced from:								
Glucose	+	+	d	+	d	—	+	+
Fructose	+	—	—	+	d	—	+	+
Lactose	—	+	—	—	d	—	+	+
Sucrose	+	+	—	—	—	—	+	+
Maltose	+	+	d	—	+	—	+	+
Trehalose	+	—	—	—	d	—	+	+
Cellobiose	+w	—	—	—	—	—	+	+
Arabinose	—	—	—	—	—	—	+	d
Xylose	—	—	—	—	—	—	+	+
Rhamnose	—	—	—	—	—	—	d	—
Raffinose	+w	—	—	—	—	—	+	+
Mannitol	—	—	—	—	d	—	—	+
Salicin	—	—	—	—	—	—	+	+
Indole production	—	—	+	+	d	—	—	—
Nitrate reduction	—	NG	—	—	d	+	—	—
Mol% G+C	29-31	32	32	33	33	31-36	40	41

^a +, Positive; +w, weakly positive; —, negative; NG, no growth on test medium.^b Data modified from reference 5 with permission.

Evidence for a distinct taxon. On the basis of phenotypic characteristics, the strains which we examined were found to represent a very homogeneous group of bacteria thought to belong to a single species. The 67 to 96% homology range showed by the strains seemed to be reasonable for strains in the same species according to the concept suggested by Johnson (6).

It has been generally recognized that there is considerable biochemical and chemotaxonomic similarity between *Flavobacterium* and *Cytophaga* species (4, 11). In *Bergey's Manual of Systematic Bacteriology* (5), the genus *Flavobacterium* is differentiated from the genus *Cytophaga* primarily by the presence or absence of gliding motility. For now, it appears that the nongliding bacterium described here most probably belongs to the genus *Flavobacterium*, although the current description of the genus *Flavobacterium* defines the

morphology of this taxon as rods not exceeding 3 µm long and having a minimum G+C content of 31%. The bacterium isolated from fish with BGD described here should be recognized as a new species, for which we proposed the name *Flavobacterium branchiophila* (bran.chi.o'phi.la. Gr. *branchia*, gill; Gr. adj. *philos*, loving; M.L. adj. *branchiophila*, gill loving).

The characteristics that are useful for differentiating *Flavobacterium branchiophila* from other *Flavobacterium* species are summarized in Table 1.

Description of *Flavobacterium branchiophila* sp. nov. The type strain is strain BGD-7721; this strain has been deposited with the American Type Culture Collection, Rockville, Md., as strain ATCC 35035.

Gram-negative, nonsporeforming, rod-shaped cells approximately 0.5 by 5 to 8 µm with parallel sides and rounded ends.

Intracellular granules of poly-β-hydroxybutyrate are absent.

Nonmotile. Does not glide or spread.

Colonies on CA are light yellow, smooth, round, raised, entire, and 0.5 to 1 mm in diameter after incubation for 5 days at 18°C.

Cultures in CB are uniformly turbid.

Chemoorganotrophic; metabolism is oxidative.

Growth occurs at temperatures between 10 and 25°C.

Growth occurs in the presence of 0 to 0.075% (wt/vol) NaCl.

Catalase and cytochrome oxidase are produced.

Gelatin, casein, and starch are degraded, but esculin, chitin, agar, and cellulose are not.

Acid but no gas is produced from glucose, fructose, sucrose, maltose, trehalose, cellobiose, melibiose, raffinose, and inulin, but not from galactose, lactose, arabinose, xy-

TABLE 2. DNA relatedness among the strains of *Flavobacterium branchiophila* and between *Flavobacterium branchiophila* and other similar bacteria

Source of unlabeled DNA	% Relatedness to labeled DNA from:			
	<i>Flavobacterium branchiophila</i>	<i>Flavobacterium aquatile</i>	<i>Flexibacter columnaris</i>	
	Strain BGD-7721 ^T	Strain BV-1	IAM 12316	EK-28
<i>Flavobacterium branchiophila</i>				
BGD-7721 ^T	100	68	11	10
BV-1	79	100	20	8
FL-15	96	67	22	13
<i>Flavobacterium aquatile</i> IAM 12316	17	12	100	15
<i>Flexibacter columnaris</i> EK-28	32	18	15	100

lose, rhamnose, adonitol, mannitol, dulcitol, sorbitol, inositol, or salicin.

The following enzymes are produced (determined by using API ZYM galleries): alkaline phosphatase, esterase (C_4), esterase-lipase (C_8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, and phosphoamidase.

The following enzymes are not produced (determined by using API ZYM galleries): lipase (C_{14}), trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, and α -fucosidase.

The G+C content of the DNA ranges from 29 to 31 mol%; for the type strain, the G+C content is 30 mol% (as determined by thermal denaturation).

The type strain was isolated in 1977 from a diseased fingerling Yamame (*Oncorhynchus masou*) from a fish hatchery in Gumma Prefecture, Japan.

One additional strain of *Flavobacterium branchiophila*, strain BV-1, has been deposited in the American Type Culture Collection as strain ATCC 35036.

ACKNOWLEDGMENTS

We express our appreciation to S. Egusa, University of Tokyo, Tokyo, Japan, and J. L. Fryer, Department of Microbiology, Oregon State University, Corvallis, for their constant interest and encouragement during the course of this work.

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