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Flexibacter ovolyticus sp. nov., a Pathogen of Eggs and Larvae of Atlantic Halibut, *Hippoglossus hippoglossus* L.

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A psychrotrophic *Flexibacter* sp., *Flexibacter ovolyticus* sp. nov., was isolated from the adherent bacterial epiflora of Atlantic halibut (*Hippoglossus hippoglossus* L.) eggs and was shown to be an opportunistic pathogen for halibut eggs and larvae. The strains which we isolated had the enzymatic capacity to dissolve both the chorion and the zona radiata of the egg shells. A total of 35 isolates were characterized by using morphological and biochemical tests. These strains were rod shaped, gram negative, Kovacs oxidase positive, and pale yellow and exhibited gliding motility. They did not produce acid from any of the wide range of carbohydrates tested. Our isolates had the ability to degrade gelatin, tyrosine, DNA, and Tween 80. Starch, cellulose, and chitin were not degraded. The strains were catalase and nitrate reductase positive, did not produce H_2S, and did not grow under anaerobic conditions. *F. ovolyticus* resembles *Flexibacter maritimus*, but differs from the latter species in several biochemical and physiological characteristics. DNAs from *F. ovolyticus* strains had guanine-plus-cytosine contents which ranged from 30.3 to 32.0 mol% (strains EKC001, EKD002^T [T = type strain], and VKB004), and DNA-DNA hybridization studies revealed levels of relatedness between *F. ovolyticus* EKD002^T and *F. maritimus* NCMB 2154^T and NCMB 2153 of 42.7 and 30.0%, respectively. Compared with previously described *Cytophaga* and *Flexibacter* spp. with low guanine-plus-cytosine contents, *F. ovolyticus* constitutes a new species. Strain EKD002 (= NCIMB 13127) is the type strain of the new species.

Fish-pathogenic bacteria belonging to the order *Cytophagales* are well-known agents of disease in both freshwater and seawater fish species (23, 27, 34, 35), often causing high levels of mortality and economic losses in aquaculture operations. Increased interest in bringing new fish species into aquaculture has resulted in the expenditure of great effort to mass produce marine fish larvae, and Atlantic halibut (*Hippoglossus hippoglossus* L.) has been one of the most promising candidates for cold-water aquaculture (45). However, commercial success has been hampered by substantial mortality in the early stages of life (37, 38).

The existence of bacteria that colonize the surfaces of marine fish eggs has been recognized for a long time (36, 43). The mucosal surfaces of marine fish eggs are a good substrate for the adhesion and colonization of bacteria (21), and many of these colonizing bacteria are opportunistic in nature. Such colonization eventually results in disease and increased levels of mortality when various environmental stresses cause suboptimal conditions for eggs and larvae. Recently, bacteria belonging to the genus *Flexibacter* have been shown to play an important role in this process (4, 5). Egg groups in which *Flexibacter* sp. was present as a major component of the adherent egg epiflora exhibited very high levels of mortality on the days around hatching compared with egg groups in which *Flexibacter* sp. could not be detected or was present only as a minor part of the epiflora. Experimental infection trials in which *Flexibacter* sp. and *Vibrio* spp. were incubated with individual halibut eggs and larvae revealed that the pathogenesis and mortality patterns obtained with these taxa were quite different (5). *Flexibacter* sp. caused very high levels of mortality on the last few days before hatching and during the first few days after hatching. In contrast, various *Vibrio* spp. did not cause mortality at the egg stage, but did cause increasing levels of mortality throughout the yolk sac stage.

The nomenclature of the nonphotosynthetic, nonfruiting, gliding bacteria has been somewhat confused (40, 41, 44). Gliding bacteria isolated from diseased fish belong to the order *Cytophagales* and to either the genus *Flexibacter* or the genus *Cytophaga* (9). Problems concerning differentiation between the genera *Cytophaga* and *Flexibacter* and the taxonomic relationship of these taxa to the genus *Flavobacterium* have been recognized (24, 26, 28, 41), as indicated by references to the "*Flavobacterium-Cytophaga* group" by several authors. The names *Flexibacter columnaris*, *Flexibacter psychrophilus* (9), and *Flexibacter maritimus* (48) are validly published names for fish-pathogenic species belonging to the order *Cytophagales*.

In this study we characterized and described a new *Flexibacter* sp. that causes disease and high levels of mortality in the egg and larval stages of Atlantic halibut.

MATERIALS AND METHODS

Bacterial strains. A number of phenotypically similar *Flexibacter*-like bacterial strains (designated *Flexibacter* sp. below) were isolated from the adherent epiflora of halibut eggs obtained from different egg batches (strains EKC001 to EKC015 [5 April 1989] and strains EKD001 to EKD005 [27 April 1989]) and from the water in egg incubators (strains VKB001 to VKB015 [5 April 1989]). On the basis of the characteristic colony morphology and key differential characteristics (Tables 1 through 3), *Flexibacter* sp. strains were isolated from various egg groups in two consecutive hatching seasons (1990 and 1991). Eggs were washed five times in autoclaved 70% seawater, homogenized, and plated onto Difco marine agar (MBA) and cytophaga agar (2) based on 70% seawater (CYTA). A total of 30 isolates obtained from the egg epiflora and 5 water isolates were subjected to

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TABLE 1. Biochemical and physiological characteristics of *F. ovolyticus* and *F. maritimus* NCMB 2154^T

Characteristic	*F. ovolyticus* (*n* = 35)[a]	*F. maritimus* NCMB 2154^T
Degradation of:		
Agar	−	−
Carboxymethyl cellulose	−	−
Cellulose	−	−
Chitin	−	−
DNA	+	+
Gelatin	+	+
Starch	−	−
Tween 80	+	+
Tyrosine	+	+
Urea	−	−
Nitrogen sources		
Tryptone	+	+
Casamino Acids	+	+
Yeast extract	+	+
Sodium glutamate	+	−
Potassium nitrate	−	−
Production of:		
β-Galactosidase	−	−
Catalase	+	+
Nitrate reductase	+	+
Ammonia	−	+
Hydrogen sulfide	−	−
Pigment on tyrosine	+	+
Growth in tryptic soy broth	−	−
Congo red adsorption	−	+
Production of EHVP in liquid culture[b]	−	+
Growth in pellicle in liquid culture	−	+
Stimulation or inhibition of growth by halibut egg homogenate	−	−
G+C content (mol%)[c]	31.0	30.1

[a] The *F. ovolyticus* isolates were very homogeneous and responded identically in all of the tests. +, 100% of the isolates were positive; −, 100% of the isolates were negative. *n* is the number of strains tested.

[b] EHVP, extracellular high-viscosity polysaccharide.

[c] The value given for *F. ovolyticus* is the mean of the results for three strains (strain EKC001, 30.8 mol%; strain EKD002^T, 30.3 mol%; strain VKB004, 32.0 mol%).

phenotypic characterization. Strains equivalent to *F. maritimus* NCMB 2154^T (T = type strain) and NCMB 2153 (National Collection of Marine Bacteria, Aberdeen, Scotland) were used as reference strains.

Morphology and growth. Cell morphology was determined by using phase-contrast microscopy of live specimens, scanning electron microscopy of infected halibut eggs, and transmission electron microscopy of cultured strains. Gliding motility was verified by using phase-contrast microscopy of hanging-drop preparations. Descriptions of pigmentation and colonial morphology were based on observations of MBA and CYTA cultures that were incubated at 15°C for 3 to 5 days. Microcyst formation was tested by using the method of Dworkin and Gibson (17). The production of flexirubin pigments was determined by using the KOH test described by Mudarris and Austin (35), and the production of an extracellular galactosamine glycan was examined by using Congo red adsorption (25). Anaerobic growth was tested by incubating MBA cultures under an N_2 atmosphere. Production of high-viscosity extracellular polysaccharides was examined in Difco marine broth (MB) after incubation for 3 days at 20°C. We also examined growth on thiosulfate-citrate-bile-sucrose agar and tryptic soy agar.

Physiological and biochemical characteristics. All of the tests were carried out at 15°C unless stated otherwise. When solid media were used, 1.5% Bacto Agar (Difco) was added prior to autoclaving. The agar was washed five times in distilled water, centrifuged, frozen as a wet paste with a water content of approximately 90%, and kept at −20°C until it was used.

Growth at different temperatures was examined by using MB, and growth in the presence of different NaCl concentrations was assayed in MB (Difco formula) containing K salts substituted for Na salts. The impact of different seawater concentrations on growth was assayed in cytophaga broth containing appropriate seawater concentrations. We examined utilization of the following nitrogen sources in a medium that was based on artificial seawater: tryptone, Casamino Acids, yeast extract, sodium glutamate, and potassium nitrate. The medium was made by adding sterile filtered solutions of 2.0% sodium acetate, 20% glucose, and an N source (final concentrations, 0.02, 0.1, and 0.1%) to 1,000 ml of boiled, filtered, autoclaved artificial seawater (Ultramarin; Waterlife Ltd., Middlesex, England). We examined the possible inhibitory or stimulating effects of a homogenate of surface-disinfected (Buffodine; Evans Vanodine Ltd., Preston, England) (6) halibut eggs on the growth of *Flexibacter* sp. by adding 50-μl portions of homogenate to 5-mm wells in MBA plates onto which the bacterial strains were spread.

Gram staining was performed as described by Buck (12), and Kovacs oxidase and catalase activities were tested by using standard procedures. The following tests were performed as described by Hansen and Sørheim (22): oxidative-fermentative metabolism of glucose; aerobic acid production from fructose, sucrose, galactose, mannose, mannitol, glycerol, ribose, and *N*-acetylglucosamine; arginine dihydrolase; lysine decarboxylase; ornithine decarboxylase; gelatinase; amylase; chitinase; β-galactosidase; urease; DNase; lipase (Tween 80); Simmons citrate; indole; and nitrate reductase. In addition, the following characteristics were examined: pigment production on and degradation of tyrosine; hydrolysis of cellulose and carboxymethyl cellulose; degradation of agar; and H_2S and NH_3 production (30). The H_2S test was modified by using MB as a base and supplementing it with 0.01% L-cysteine. H_2S was detected by using lead acetate paper.

TABLE 2. Growth of *F. ovolyticus* and *F. maritimus* under various environmental conditions

Environmental variable	*F. ovolyticus* (*n* = 35)[a]	*F. maritimus* NCMB 2154^T
Growth at:		
4°C	+	−
10°C	+	+
25°C	+	+
30°C	−	+
Growth in[b]:		
20% Seawater	−	−
30% Seawater	−	+
50% Seawater	+	+
Growth in the presence of[c]:		
0.5% NaCl	−	−
1.0% NaCl	+	+
3.0% NaCl	+	+
6.0% NaCl	−	−

[a] *n* is the number of strains tested.

[b] Seawater-based cytophaga broth.

[c] MB (Difco formula) contained the appropriate amounts of NaCl. The remaining sodium salts were replaced by the corresponding potassium salts.

TABLE 3. Some characteristics that differentiate *F. ovolyticus* from previously described *Cytophaga* and *Flexibacter* species that have low DNA G+C contents[a]

Characteristic	*Cytophaga lytica*	*Cytophaga saccharophila*	*Cytophaga aquatilis*	*F. columnaris*	*F. psychrophilus*	*F. maritimus*	*F. ovolyticus*
Length of cells (μm)	1.5–3.5	2.5–6	2–15	2–12	1.5–7.5	2–5	2–20
Flexirubin pigment	–	+	+	+	+	–	–
Congo red adsorption	–	N[b]	–	+	–	+	–
Kovacs oxidase	+	–	–	+	+	+	+
Catalase	+	–	+	+	–	+	+
NO_3^- used as N source	–	N	+	–	N	–	–
Acid produced from carbohydrates aerobically	N	N	+	–	–	–	–
Degradation of:							
Starch	+	+	+	–	–	–	–
Carboxymethyl cellulose	+	+	+	–	–	–	–
Agar	+	+	–	–	–	–	–
Chitin	–	–	+	–	–	–	–
Tyrosine	+	N	+	–	+	+	+
DNase	N	N	+	N	N	+	+
β-Galactosidase	+	N	+	–	–	–	–
NO_3^- reduced	–	+	+	+	–	+	+
NH_3 produced	+	+	+	+	+	+	–
H_2S produced	+	+	–	+	–	–	–
Highest NaCl concn tolerated (%)	6	2	2	0.5	0.8	>3	>3
Growth on seawater media	+	–	–	–	–	+	+
Growth at 4°C	N	+	–	+	+	–	+
Maximum temp (°C)	<40	<37	30	37	<25	<37	<30
G+C content (mol%)	33 (32–34)[c]	32 (32–36)	32 or 34	30	32	31 (31–32)	30 (30–32)
Habitat	Marine	Freshwater	Freshwater	Freshwater	Freshwater	Marine	Marine

[a] Data for *Cytophaga lytica*, *Cytophaga saccharophila*, *Cytophaga aquatilis*, *F. columnaris*, *F. psychrophilus*, and *F. maritimus* from references 7 through 9, 40, and 48 and this study.
[b] N, no information available.
[c] G+C content of the type strain and, in parentheses, range of G+C contents for the species.

API ZYM (Analytab Products, Montalieu-Vercieu, France) tests were used to detect various constitutive enzymes in *Flexibacter* sp. strains EKC001, EKD002^T, and VKB004 and *F. maritimus* NCMB 2154^T and NCMB 2153. The preparations were incubated for 12 h at 20°C as described by Bernardet and Grimont (9).

Gel electrophoresis of bacterial proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29) was performed with *Flexibacter* sp. strains EKC001 and EKD002^T (isolated from egg epiflora), *Flexibacter* sp. strain VKB004 (isolated from water from one of the incubating units), and *F. maritimus* NCMB 2153 and NCMB 2154^T. The bacteria were grown for 48 h at 15°C in prefiltered MB until the early stationary phase of growth and then were inactivated by using formalin (final concentration, 0.5% [vol/vol]) and pelleted by centrifugation for 20 min at 6,198 × *g* (Minifuge T; Heraeus Crist, Osterode, Germany). The resulting pellet was washed twice in 0.9% NaCl and suspended in 1 ml of distilled water, and this preparation was stored as 200-μl aliquots at −80°C. The bacteria were diluted three times in 2-mercaptoethanol containing 2× sample buffer (29), heated for 5 min at 95°C, and applied to 12% polyacrylamide gels. The gels were run by using a Bio-Rad gel (16-cm) system according to the manufacturer's recommendations. Proteins were visualized by silver staining as described by Marshall and Latner (33).

DNA isolation and purification. DNAs from strains EKC001, EKD002^T, VKB004, NCMB 2154^T, and NCMB 2153 were isolated and purified. The bacteria were grown for 48 h at 20°C in prefiltered MB, which yielded 2 to 3 g (wet weight) of cells in the late exponential phase of growth. The cells were harvested by centrifugation, washed in saline-EDTA (0.15 M NaCl and 0.1 M EDTA), and frozen at −80°C. The cells were lysed, and the nucleic acids were extracted and purified by using the method of Marmur (32). The DNA was purified until the ratio of A_{260} to A_{230} and the ratio of A_{260} to A_{280} were between 1.8 and 2.1 and between 1.8 and 1.9, respectively.

G+C contents of DNAs. Guanine-plus-cytosine (G+C) contents were determined for *Flexibacter* sp. strains EKC001, EKD002^T, and VKB004 and *F. maritimus* NCMB 2154^T and NCMB 2153. The G+C contents of DNAs were determined by thermal denaturation (15), using 0.5× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the solvent and DNA concentrations corresponding to an initial A_{260} of 0.7, representing approximately 35 μg of DNA per ml (31). A Shimadzu model UV-240 spectrophotometer equipped as described by Torsvik et al. (46) was used. G+C contents were calculated as described by De Ley (15). DNA from *Escherichia coli* ATCC 11775 (G+C content, 51.5 mol%) was used as a standard.

DNA-DNA hybridization. The genetic relationship between *Flexibacter* sp. strains EKC001, EKD002^T, and VKB004 and *F. maritimus* NCMB 2154^T and NCMB 2153 was determined on the basis of spectrophotometrically recorded initial renaturation rates of the DNA types and mixtures of DNAs (16, 19, 49). The DNA was sheared in a French pressure cell at 20,000 lb in^{-2}, giving DNA fragments with a mean molecular mass of 420,000 daltons (19, 46). DNA concentrations of 56 μg/ml (A_{260}, 1.2) were used in the reassociation experiments; this is well below the upper limit of 80 μg of DNA per ml recommended by Gillis et al. (19). Reassociations were performed in 2× SSC. In the interval between 0.125× SSC and 4× SSC, the DNA melting tem-

perature (T_m) and the optimal reassociation temperature (T_{OR}) both increase by approximately 4°C as a result of a doubling of the Na^+ concentration (19). According to Gillis et al. (19), the T_{OR} at a given SSC concentration is 20 to 26°C below the T_m at that SSC concentration. The velocity of reassociation is approximately independent of temperature from the T_{OR} to about 15°C below the T_{OR} (19). The T_{OR} was calculated by using the T_m in 2× SSC, which was calculated from the T_m in 0.5× SSC as described by De Ley (15). This led to T_m values (in 2× SSC) of 94.5°C for *E. coli* and about 86°C for the *Flexibacter* sp. and *F. maritimus* strains. The reassociation experiments were performed by using a T_{OR} of 60°C for the two *Flexibacter* species and a T_{OR} of 70°C for *E. coli*. The percentage of hybridized DNA was calculated as described by De Ley et al. (16). The equipment used in the reassociation experiments has been described previously (46).

Scanning electron microscopy. Halibut eggs from an egg batch in which about 99% of the egg epiflora consisted of *Flexibacter* sp. (measured as the number of colony-forming units on MBA) were examined by scanning electron microscopy. The eggs were washed in sterile filtered (pore size, 0.22 μm) seawater and fixed in formaldehyde-glutaraldehyde (final concentrations, 2.5 and 2.0% [vol/vol], respectively) in 0.05 M cacodylate buffer (pH 7.2) (20). The eggs were postfixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in ethanol, critical-point dried, and coated with gold-palladium. A JEOL model JSM-6400 scanning electron microscope operated at 6 kV was used to examine the specimens.

Transmission electron microscopy. Strain EKC001 axenic bacterial growth from a 4-day-old MBA culture (15°C) was examined by transmission electron microscopy. A drop of particle-free (autoclaved and ultracentrifuged), distilled water was placed on the bacterial growth, and the preparation was carefully stirred with a sterile toothpick. Samples (30 μl) of the resulting bacterial suspension were applied to carbon- and Formvar-coated 400-mesh copper grids, and the bacteria were allowed to adhere for 5 min at room temperature. Superfluous liquid was gently removed by using a piece of filter paper. The grids were allowed to air dry before they were stained three times (30 s each) in a 1% uranyl acetate solution. Electron microscopy was performed at 80 kV with a JEOL model 100 CX electron microscope.

RESULTS

Phenotypic characteristics. The cells were long, slender rods (0.4 by 2 to 20 μm) with gliding motility. Occasionally, filaments that were 70 to 100 μm long were formed. Colonies were light yellow and flat with irregular edges on CYTA. Typical spreading growth was not observed on CYTA. However, on solidified N source-containing media spreading growth was observed. On MBA, colonies were light brownish yellow, flattened, and elevated with regular edges. Because of a rapid decrease in viability, the *Flexibacter* sp. strains had to be subcultured every 5 to 7 days. Bacterial growth on agar plates exhibited a characteristic pattern, with lysis in the center and viable cells toward the edges of each colony. Transmission electron microscopy confirmed the nonflagellated morphology and also revealed the presence of bacteriophages.

The KOH test used to determine the production of flexirubin pigments was negative. No microcyst formation was observed, but spherical cells (diameter, 0.5 to 0.7 μm) were found in the late exponential phase of growth. No growth occurred under anaerobic conditions or on tryptic soy agar or thiosulfate-citrate-bile-sucrose agar. The bacteria were gram negative and Kovacs oxidase and catalase positive. No pH change was observed in the medium when the strains were tested for oxidative-fermentative metabolism of glucose. Acid was not produced from any of the carbohydrates tested. Arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase activities were not detected, the bacteria were not able to utilize citrate as a sole carbon source, and they did not produce indole from tryptophan. Additional biochemical and physiological characteristics are shown in Tables 1 and 2. The homogeneity of the *Flexibacter* sp. isolates is reflected in the fact that they responded identically in the tests which we performed.

API ZYM tests. The following substrates were hydrolyzed by all of the *Flexibacter* sp. strains which we tested: 2-naphthyl-phosphate (acid and alkaline phosphatase), 2-naphthyl-butyrate, 2-naphthyl-caprylate, L-leucyl-2-naphthylamide, L-valyl-2-naphthylamide, and naphthol-AS-BI-phosphate. The strains did not hydrolyze 2-naphthyl-myristate, L-cystyl-2-naphthylamide, *N*-benzoyl-DL-arginine-2-naphthylamide, *N*-glutaryl-phenylalanine-2-naphthylamide, 6-Br-2-naphthyl-α-D-galactopyranoside, 2-naphthyl-β-D-galactopyranoside, naphthol-AS-BI-βD-glucuronide, 2-naphthyl-α-D-glucopyranoside, 6-Br-2-naphthyl-β-D-glucopyranoside, 1-naphthyl-*N*-acetyl-β-D-glucosaminide, 6-Br-2-naphthyl-α-D-mannopyranoside, and 2-naphthyl-α-L-fucopyranoside. The *Flexibacter* sp. strains differed from *F. maritimus* with respect to hydrolysis of L-cystyl-2-naphthylamide and *N*-benzoyl-DL-arginine-2-naphthylamide, which were hydrolyzed by *F. maritimus* but not by the *Flexibacter* sp. strains.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Silver staining of the polyacrylamide gels clearly revealed differences between the protein pattern of strain VKB004, which was isolated from tank water, and the protein patterns of the two isolates obtained from egg epiflora, strains EKC001 and EKD002^T (Fig. 1, lanes 5, 3, and 4, respectively). In both the high- and low-molecular-weight regions strain VKB004 produced prominent bands that were not present in the strain EKC001 and EKD002^T patterns. In contrast, strains EKC001 and EKD002^T produced almost identical electrophoretic patterns in both high- and low-molecular-weight regions. However, small differences between these two isolates were observed. The protein pattern of strain VKB004 was completely different from the patterns of the reference strains, *F. maritimus* NCMB 2153 and NCMB 2154^T (lanes 5, 1, and 2, respectively). Only minor similarities between strain NCMB 2153 and strains EKC001 and EKD002^T were observed (lanes 1, 3, and 4, respectively). In contrast to strain NCMB 2153, the protein pattern of strain NCMB 2154^T was similar in some ways to the patterns of strains EKC001 and EKD002^T. However, differences in the band patterns were observed both in the high-molecular-weight region (approximately 70, 86, 60, and 50 kDa, as determined by comparison with the migration of standard proteins) and in the low-molecular-weight region (approximately 32 kDa). Minor differences between strains EKC001 and EKD002^T were also observed, but these differences were much less than the differences between these isolates and strain NCMB 2154^T.

DNA base compositions. The DNA T_m values (19) for strains EKC001, EKD002^T, and VKB004 were 78.0, 77.8, and 78.5°C, respectively, in 0.5× SSC. The T_m values for strains NCMB 2153 and NCMB 2154^T were 77.8 and 77.7°C, respectively. The G+C contents of strains EKC001, EKD002^T, and VKB004 were 30.8, 30.3, and 32.0 mol%,

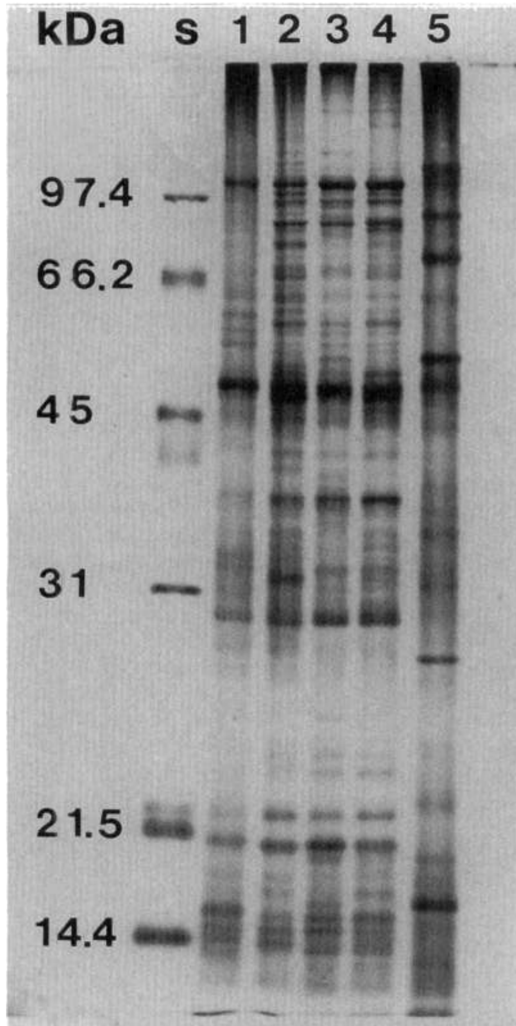

FIG. 1. Sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis of *F. ovolyticus* and *F. maritimus*. Lanes 1 and 2, *F. maritimus* NCMB 2153 and NCMB 2154^T, respectively; lanes 3 through 5, *F. ovolyticus* EKC001, $EKD002^T$ (from egg epiflora), and VKB004 (from tank water), respectively. Samples of 5 μl per well were used. A 10-μl portion of a 100×-diluted protein standard (low range; Bio-Rad) was also used (lane S). The protein bands were visualized by silver staining.

respectively. The G+C contents of *F. maritimus* NCMB 2154^T and NCMB 2153 were 30.3 and 30.1 mol%, respectively. The values given by Wakabayashi et al. (48), who used calf thymus DNA (G+C content, 42.9 mol%) as a standard, were 31.6 mol% for strain NCMB 2154^T and 31.3 to 32.5 mol% for *F. maritimus*.

DNA-DNA hybridization. The levels of DNA relatedness among *Flexibacter* sp. strains EKC001, $EKD002^T$, and VKB004 and *F. maritimus* NCMB 2154^T and NCMB 2153 are shown in Table 4. The DNA-DNA hybridization experiments revealed high levels of homology among the three strains of *Flexibacter* sp., but strain VKB004 differed slightly from strains EKC001 and $EKD002^T$. Our results clearly separated the *Flexibacter* sp. strains from *F. maritimus* and demonstrated the relatedness of the three *Flexibacter* sp. strains. The reassociation value for *F. maritimus* was somewhat higher than the value of 73% reported by Bernardet and Grimont (9), which may have been in part due to differences in methodological approaches.

***Flexibacter*-infected halibut eggs.** The new *Flexibacter* species was isolated as part of the adherent bacterial epiflora of eggs obtained from different egg batches; the percentage of this organism in the epiflora ranged from a few percent to 99%. When the bacterium formed less than 25 to 30% of the epiflora, the eggs hatched and developed normally, while eggs on which *Flexibacter* sp. constituted the major part of the bacterial epiflora exhibited a dramatic increase in the level of mortality at the hatching stage. In the latter cases, *Flexibacter* sp. accounted for as much as 60 to 70% of the colony-forming units in water samples obtained from the egg and larval incubators, while in the former cases, *Flexibacter* sp. was scarcely detectable when the viable count method was used. These results emphasize the opportunistic nature of *Flexibacter* sp. with respect to disease in halibut larvae.

Scanning electron microscopy of infected halibut eggs revealed that a very high level of proteolytic activity was caused by the *Flexibacter* sp. strains. Colonization resulted in wounds or ulcerations of varying severity on otherwise clean egg surfaces (Fig. 2). The chorion (the outermost layer of the egg shell) was completely dissolved, and the underlying zona radiata was severely damaged by bacterial exoenzymatic activity, possibly resulting in egg puncture, leakage of cell constituents, and larval death (5).

DISCUSSION

Various *Flexibacter* spp. are known to cause diseases in different fish species (1, 7, 8, 23, 47). The *Flexibacter* sp. which we studied is an opportunistic pathogen that causes high levels of mortality in halibut eggs and larvae when it is present in high numbers or when larval resistance is weakened because of different environmental stresses (37). This is a common characteristic of many of the fish-pathogenic bacteria (10, 13, 14, 18, 42). Scanning electron microscopy of infected halibut eggs revealed that our *Flexibacter* sp. had the enzymatic capacity to dissolve both the chorion and the zona radiata of egg shells (Fig. 2). As far as we know, this characteristic has not been reported for any of the previously described fish-pathogenic bacteria, although bacteria that dissolve the chorion of cod (*Gadus morhua* L.) eggs have been observed (21). It is likely that exoenzymatic bacterial activity is the cause of the characteristic mortality pattern observed in infected eggs.

All of our *Flexibacter* sp. strains exhibited a growth pattern that was characterized by a rapid decrease in viability after 5 to 7 days both in liquid media and on solid media. This might have been the result of lysogenic induction of temperate phages by some unknown factor because we detected bacteriophages in all of the strains. The factors responsible for the lysogenic induction are unknown, and we were not able to cure any of the strains of their phages. Recent studies have shown that bacteriophages are abundant in natural aquatic environments and are closely coupled to microbial trophodynamics (3, 11, 39). Thus, it is possible that bacteriophages are involved in the rapid decrease in viability that is often encountered during primary isolation of bacterial populations from various environmental sources (e.g., soil and seawater).

The strains which we isolated form a very homogeneous group, and they responded identically to morphological and biochemical tests. Bernardet and Grimont (9) included *Cytophaga psychrophilus* and *Cytophaga columnaris* in the genus *Flexibacter*, mainly on the basis of the inability of

TABLE 4. Levels of DNA relatedness for *F. ovolyticus* EKC001, EKD002^T, and VKB004 and *F. maritimus* NCMB 2154^T and NCMB 2153

Strain used as recipient DNA	% Relatedness with donor DNA prepared from:				
	Strain EKC001	Strain EKD002^T	Strain VKB004	Strain NCMB 2154^T	Strain NCMB 2153
EKC001	100[a]				
EKD002^T	96.7	100			
VKB004	92.7	91.5	100		
NCMB 2154^T	42.9	42.7	40.4	100	
NCMB 2153	30.3	30.0	26.4	85.6	100

[a] The levels of hybridized DNA are expressed as percentages that are based on spectrophotometrically determined initial renaturation rates. The levels of renaturation for homologous DNAs were normalized to 100%.

these organisms to degrade polysaccharides. Much work will need to be done to develop a proper taxonomy for the *Cytophaga-Flexibacter-Flavobacterium* phylogenetic branch. Taking into account the current state of knowledge, we agree with Bernardet and Grimont that polysaccharide degradation should be emphasized and propose that the strains which we isolated belong to the genus *Flexibacter*. The name *Flexibacter maritimus* (= *Cytophaga marina* in *Bergey's Manual of Systematic Bacteriology* [40]) has been validly published previously (24, 48). Thus, fish-pathogenic, gliding bacteria from both marine and freshwater environments have been placed in the genus *Flexibacter*. These organisms have DNA G+C contents ranging from 30 to 34 mol% and do not have the capacity to degrade polysaccharides. On the basis of their low G+C contents, their resemblance in phenotypic characteristics to *F. maritimus*, and the descriptions of Bernardet and Grimont of *F. columnaris* and *F. psychrophilus*, we propose that our egg-damaging strains should be named *Flexibacter ovolyticus* sp. nov.

That this decision is appropriate is shown by the DNA-DNA hybridization results, which revealed close relationships among the three *F. ovolyticus* strains examined (91.5 to 96.7%) (Table 4). Levels of relatedness of 40.4 to 42.9 and 26.4 to 30.3% between *F. ovolyticus* and *F. maritimus* NCMB 2154^T and NCMB 2153, respectively, qualify these organisms for positions in different species (28). The differences in sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns between the two egg isolates (strains EKC001 and EKD002^T) and the water isolate (strain VKB004) (Fig. 1) may reflect differences in protein expression. The divergent characteristics of strain VKB004 are underlined by its somewhat higher G+C content (Table 1) and its levels of DNA relatedness (about 90%) with strains EKC001 and EKD002^T, which were related to each other at

FIG. 2. Scanning electron microscopy of the surface of a *F. ovolyticus*-infected halibut egg. The chorion was dissolved by bacterial exoenzymatic activity, and the bacteria attacked the underlying zona radiata. The micrograph shows the edge of a wound, with undamaged egg surface to the left and severe attack on the zona radiata to the right. Bar = 10 μm.

a level of about 97% (Table 4). Characteristics that differentiate *F. ovolyticus* from previously described *Cytophaga* and *Flexibacter* species that have G+C contents between 30 and 36 mol% are shown in Table 3.

Description of *Flexibacter ovolyticus* sp. nov. *Flexibacter ovolyticus* (ō'vō.lyt.ic'us. L.n. *ovum*, egg, Gr.n. *lysis*, loosening or dissolution; *ovolyticus*, egg damaging). The cells are gram-negative, long, slender rods (0.4 by 2 to 20 μm) which occasionally grow to filaments that are 70 to 100 μm long. Colonies are Kovacs oxidase positive and pale yellow. Microcysts are not formed. The cells exhibit gliding motility, do not adsorb Congo red, and do not possess a flexirubin type of pigment. *F. ovolyticus* is strictly aerobic and does not produce acid from carbohydrates. It degrades gelatin, tyrosine, DNA, and Tween 80, but starch, cellulose, and chitin are not degraded. *F. ovolyticus* possesses catalase and nitrate reductase activities. H_2S is not produced, and 50% seawater is required for growth. Alternatively, media based on artificial seawater containing at least 1% NaCl may be used. *F. ovolyticus* grows at 4°C, but not at 30°C. The G+C contents of the DNAs of strains EKC001, EKD002^T, and VKB004 range from 30.3 to 32.0 mol%. Additional characteristics of *F. ovolyticus* are shown in Tables 1, 2, and 4.

Type strain EKD002 (= NCIMB 13127) was isolated in 1989 from the adherent epiflora of halibut eggs at the Austevoll Aquaculture Research Station in western Norway. The description of the type strain corresponds to the species description. The G+C content of the DNA of the type strain is 30.3 mol%. In addition to the type strain, strains EKC001 and VKB004 have been deposited in the National Collection of Industrial and Marine Bacteria as strains NCIMB 13128 and NCIMB 13129, respectively.

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