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 H2S, 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 EKCO01, EKD002T (= type strain), and VKB004), and DNA-DNA hybridization studies revealed levels of relatedness between F. ovolyticus EKD002T and F. maritimus NCMB 2154T and NCMB 2153 of 42.7 and 30.0%, respectively. Compared with previously described Cytophaga and Flexibacter sp. 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 Cytophagaales 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. The mucosal surfaces of marine fish eggs are a good substrate for the adhesion and colonization of bacteria (21), and the egg epiflora and seawater fish species (23, 27, 34, 35), often causing high mortality and economic losses in aquaculture 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 Cytophagaales.

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 EKCO01 to EKCO15 [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...
Degradation of: Production of timus 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.

Nitrogen sources Production was assayed by incubating MBA cultures under an N₂ atmosphere. The production of EHVP was determined by using the KOH test modified by using MB as a base and supplementing it with 0.01% L-cysteine. H₂S was detected by using lead acetate paper.

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 Kovačs 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, glyc erol, 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 H₂S and NH₃ production (30). The H₂S test was modified by using MB as a base and supplementing it with 0.01% L-cysteine. H₂S was detected by using lead acetate paper.

Phenotypic characterization. Strains equivalent to F. maritimus NCMB 2154T (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 N₂ 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 thiourea-sulfate 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.

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>F. ovolyticus (n = 35)</th>
<th>F. maritimus NCMB 2154T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degradation of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cellulose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chitin</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tween 80</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urea</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nitrogen sources</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptone</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Casamino Acids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sodium glutamate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Reduction of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ammonia</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hydrogen sulfide</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pigment on tyrosine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth in tryptic soy broth</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Congo red adsorption</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Production of EHVP in liquid culture</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Growth in pellicle in liquid culture</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stimulation or inhibition of growth</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>31.0</td>
<td>30.1</td>
</tr>
</tbody>
</table>

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, 30.3 mol%; strain VKB004, 32.0 mol%).

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

<table>
<thead>
<tr>
<th>Environmental variable</th>
<th>F. ovolyticus (n = 35)</th>
<th>F. maritimus NCMB 2154T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>10°C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25°C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth in a:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20% Seawater</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>30% Seawater</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>50% Seawater</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth in the presence of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% NaCl</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1.0% NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.0% NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6.0% NaCl</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Cytophaga lytica</em></th>
<th><em>Cytophaga saccharophila</em></th>
<th><em>Cytophaga aquatilis</em></th>
<th><em>F. columnaris</em></th>
<th><em>F. psychrophilus</em></th>
<th><em>F. maritimus</em></th>
<th><em>F. ovolyticus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of cells (µm)</td>
<td>1.5–3.5</td>
<td>2.5–6</td>
<td>2–15</td>
<td>2–12</td>
<td>1.5–7.5</td>
<td>2–5</td>
<td>2–20</td>
</tr>
<tr>
<td>Flexirubin pigment</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Congo red adsorption</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Kovac oxidase</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NO₃⁻ used as N source</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>−</td>
<td>−</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Acid produced from carbohydrates aerobically</td>
<td>N</td>
<td>N</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Degradation of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Agar</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Chitin</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>N</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNase</td>
<td>N</td>
<td>N</td>
<td>+</td>
<td>N</td>
<td>N</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduced</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H₂S produced</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Highest NaCl concn tolerated (%)</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
<td>0.8</td>
<td>&gt;3</td>
<td>&gt;3</td>
</tr>
<tr>
<td>Growth on seawater media</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 4°C</td>
<td>N</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maximum temp (°C)</td>
<td>&lt;40</td>
<td>&lt;37</td>
<td>30</td>
<td>37</td>
<td>&lt;25</td>
<td>&lt;37</td>
<td>&lt;30</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>33 (32–34)†</td>
<td>32 (32–36)</td>
<td>32 or 34</td>
<td>30</td>
<td>32</td>
<td>31 (31–32)</td>
<td>30 (30–32)</td>
</tr>
<tr>
<td>Habitat</td>
<td>Marine</td>
<td>Freshwater</td>
<td>Freshwater</td>
<td>Freshwater</td>
<td>Freshwater</td>
<td>Marine</td>
<td>Marine</td>
</tr>
</tbody>
</table>

*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.

* 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, EKD002T, and VKB004 and *F. maritimus* NCMB 2154T and NCMB 2153. The preparations were incubated for 12 h at 20°C as described by Bernardet and Grimont (9).

**DNA isolation and purification.** DNAs from strains EKC001, EKD002T, VKB004, NCMB 2154T, 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 A₂₆₀ to A₂₃₀ and the ratio of A₂₆₀ to A₂₃₀ 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, EKD002T, and VKB004 and *F. maritimus* NCMB 2154T and NCMB 2153. The G+C contents of DNAs were determined by thermal denaturation (15), using 0.5x standard saline citrate (SSC; 1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the solvent and DNA concentrations corresponding to an initial A₂₆₀ 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, EKD002T, and VKB004 and *F. maritimus* NCMB 2154T 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 (A₂₆₀, 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 2x SSC. In the interval between 0.125 x SSC and 4x SSC, the DNA melting tem-
temperature \( (T_m) \) 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 \textit{Flexibacter} sp. and \textit{F. maritimus} strains. The reassociation experiments were performed by using a \( T_{OR} \) of 60°C for the two \textit{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 \textit{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 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 \textit{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 \textit{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 \textit{Flexibacter} sp. strains which we tested: 2-naphthyl-phosphosphate (acid and alkaline phosphatase), 2-naphthylbutyrate, 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-lysyl-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-β-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 \textit{Flexibacter} sp. strains differed from \textit{F. maritimus} with respect to hydrolysis of L-cystyl-2-naphthylamide and N-benzoyl-DL-arginine-2-naphthylamide, which were hydrolyzed by \textit{F. maritimus} but not by the \textit{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, \textit{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%,
**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* EKCO01, EKD002T, and VKB004 and *F. maritimus* NCMB 2154T and NCMB 2153

<table>
<thead>
<tr>
<th>Strain used as recipient DNA</th>
<th>% Relatedness with donor DNA prepared from:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain</td>
<td>Strain</td>
</tr>
<tr>
<td>EKCO01</td>
<td>100a</td>
<td>96.7</td>
</tr>
<tr>
<td>EKD002T</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>VKB004</td>
<td>42.9</td>
<td>42.7</td>
</tr>
<tr>
<td>NCMB 2154T</td>
<td>30.3</td>
<td>30.0</td>
</tr>
</tbody>
</table>

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 2154T 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 EKCO01 and EKD002T) 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 EKCO01 and EKD002T, 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.](image-url)
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* (Övölytica 1. 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 purple to brown, pink, orange, or 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. H₂S 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 EKCO01, EKDO002, 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 EKDO02 (= NCIMB 13127) was isolated in 1987 from the adherent epidermis 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 EKCO01 and VKB004 have been deposited in the National Collection of Industrial and Marine Bacteria as strains NCIMB 13128 and NCIMB 13129, respectively.

**Acknowledgments**


**References**


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**Flexibacter ovolyticus** Sp. nov.

*Flexibacter ovolyticus* is a new species of flexibacter found in halibut eggs. It is aerobic, gram-negative, and grows at 4°C, but not at 30°C. The cell wall contains 32.0 mol% G+C. The type strain, EKDO02 (= NCIMB 13127), was isolated from halibut eggs in western Norway. This species is not related to *Flexibacter maritimus* or *Flexibacter psychrophilus*. It is distinguished by its ability to grow at 4°C and its lower G+C content. This species has been deposited in the National Collection of Industrial and Marine Bacteria under the strain number NCIMB 13128.


34. **Masumura, K., and H. Wakabayashi.** 1977. An outbreak of gliding bacterial disease in hatchery-born red seabream (Pomatoschistus microps) and gilthead (Acanthopagrus schlegeli) fry in Hiroshima. Fish Pathol. 12:171-177.


