Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp. nov. and reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov.

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Phenotypic data indicate that gliding, yellow/orange-pigmented, agar-digesting bacterial strains were members of the *Cytophaga*–*Flavobacterium*–*Bacteroides* (CFB) group. The strains were isolated from the surface of the marine benthic macroalga *Fucus serratus* L. and the surrounding seawater at three localities in Danish waters. The bacteria were Gram-negative, flexirubin-negative, aerobic, catalase-positive and oxidase-negative and were psychrophilic and halophilic. All strains utilized D-fructose, L-fucose and α -ketobutyric acid and degraded alginic acid, carrageenan, starch and autoclaved yeast cells. Amplification with primers specific for repetitive extragenic palindromic elements by PCR divided the strains of this study into two groups. Both groups showed unique PCR amplification patterns compared to reference strains of the CFB group. Phylogenetic analysis of 16S rDNA sequences showed association of these organisms and [*Cytophaga*] *lytica* at the genus level. Hybridization of total chromosomal DNA revealed that the new strains and [*Cytophaga*] *lytica* ATCC 23178^T were clearly distinct from each other and other previously described species of the CFB group. A new genus is described, *Cellulophaga* gen. nov. comprising two new species, *Cellulophaga baltica* gen. nov., sp. nov. (NN015840^T = LMG 18535^T) and *Cellulophaga fucicola* gen. nov., sp. nov. (NN015860^T = LMG 18536^T), as well as the emendation of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov.

Keywords: marine bacteria, *Cellulophaga baltica* gen. nov., sp. nov., *Cellulophaga fucicola* gen. nov., sp. nov., *Cellulophaga lytica* gen. nov., comb. nov., *Cytophaga*–*Flavobacterium*–*Bacteroides* group

INTRODUCTION

Descriptive studies of bacteria isolated from the surface of marine benthic macroalgae were reported as early as 1875 (Warming, 1875) and bacteria from

coastal seawater were reported in 1838 (Ehrenberg, 1838). However, few studies have investigated the taxonomic diversity of the cultivable bacteria on the surface of marine benthic macroalgae [see ZoBell (1946) for other earlier references]. Isolates from the *Cytophaga*–*Flavobacterium*–*Bacteroides* (CFB) group and the *Proteobacteria* branch are common members of the bacterial community in coastal sea regions and especially on the surface of marine benthic macroalgae (Chan & McManus, 1969; Shiba & Taga, 1980; Bolinches *et al.*, 1988; Duan *et al.*, 1995; Pinhassi *et al.*, 1997). The ability to degrade different biomacromolecules probably favours species of the CFB group, since cell wall compounds and release of exudates from macroalgae can act as a nutrient source and therefore

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Abbreviations: CFB group, *Cytophaga*–*Flavobacterium*–*Bacteroides* group; REP-PCR, PCR amplification with primers specific for repetitive extragenic palindromic elements.

The EMBL accession numbers for the 16S rDNA sequences of *Cellulophaga baltica* (NN015840^T) and *Cellulophaga fucicola* (NN015860^T) are AJ005972 and AJ005973, respectively.

make the surface an excellent habitat (Laycock, 1974; Kong & Chan, 1979; Hollohan *et al.*, 1986; Ramaiah & Chandramohan, 1992; Reichenbach, 1992; Rheinheimer, 1992). Isolates of [*Cytophaga*] *lytica* are extremely common in tide pools, on the surface of macroalgae and in other coastal habitats all over the world (Reichenbach, 1989).

The description of the order *Cytophagales* has had a turbulent history since the proposal of the genus by Winogradsky (1929) as covering all aerobic, cellulolytic and gliding bacteria. Polyphasic studies including phenotypic characterization, fatty acid composition, protein patterns and, not least, phylogenetic characterization have demonstrated a huge degree of biological diversity (Christensen, 1977; Bauwens & De Ley, 1981; Nakagawa & Yamasato, 1993). In recent years, the understanding and acceptance of the diversity of the CFB group have led to several reclassifications and descriptions of new genera. This was promoted by the emended description of the genus to include *Cytophaga hutchinsonii* and *Cytophaga aurantiaca* as the only valid species. The nomenclature of *Cytophaga agarovorans* and *Cytophaga salmonicolor* was emended to *Marinilabilia agarovorans* and *Marinilabilia salmonicolor*, respectively. The remaining species that were previously designated *Cytophaga* were hereafter invalid (Nakagawa & Yamasato, 1996). Marine, gliding bacteria from the Antarctic were assigned to three genera covering the species *Psychroserpens burtonensis*, *Gelidibacter algens* and *Psychroflexus torquis* [the latter also including emendation of [*Flavobacterium*] *gondwanense* to *Psychroflexus gondwanense* (Bowman *et al.*, 1997, 1998)]. *Polaribacter* is another genus comprising species isolated from marine environments in the Arctic and Antarctic. The description of this genus included two species, *Polaribacter irgensii* and *Polaribacter franzmannii*, as well as emendation of [*Flectobacillus*] *glomeratus* into *Polaribacter glomeratus* (Gosink *et al.*, 1998).

In the present study, eleven isolates with phenotypic characteristics of 'Cytophaga-like bacteria' (e.g. gliding motility, flame-like edged colony, yellow/orange pigmentation, agarolytic activity and cell shape) were selected for taxonomic characterization. The isolates were selected from a collection of more than 500 bacterial strains isolated from Danish coastal waters and physiologically characterized (Johansen, 1996). We propose two new species of the CFB group associated with the brown alga *Fucus serratus* L. The two new species are classified into a new genus (*Cellulophaga* gen. nov.) as *Cellulophaga baltica* sp. nov. (NN015840^T) and *Cellulophaga fucicola* sp. nov. (NN015860^T). Moreover, we propose reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* comb. nov.

METHODS

Sample collection, isolation and bacterial strains. The collection sites of sampling were three Danish localities: Hirsholm 57°29', 1 N, 10°37', 3 E, Ellekilde Hage 56°02', 6 N,

12°37', 0 E and Svaneke 55°08', 2 N, 15°08', 9 E. The three localities have a mean salinity of approximately 28, 20 and 8‰, respectively (Sparre, 1984). At the three localities, the brown alga *Fucus serratus* L. was collected in the upper part of the sublittoral zone (1 m depth) and seawater samples were collected in the deeper part of the sublittoral zone (3–5 m depth).

The algae pieces were homogenized (for a maximum of 30 s) with an Ultra-Turrax in sterile deionized water containing 28.0, 20.0 or 8.5‰ sea salts (Sigma) (locality dependent). The homogenates and the seawater samples were diluted, plated in triplicate directly onto CYT agar (1.0 g casein, 0.5 g yeast extract, 0.5 g CaCl₂·H₂O, 0.5 g MgSO₄·H₂O, 15.0 g agar, 1000 ml deionized water, pH 7.3) with the addition of either 8.5, 20.0 or 28.0 g sea salts l⁻¹. The agar plates were incubated at 12 °C for 25 d in darkness. Single colonies were subcultured several times to ensure purity of the culture. The strains were preserved at -80 °C in a suspension in tryptic soy broth (TSB) containing 20‰ sea salts and 10 % glycerol. Investigated strains and type strains included in this study are listed in Table 1.

Biochemical and physiological characterization. For general maintenance of the bacteria, tryptic soy agar (TSA) with 20‰ sea salts [15.0 g tryptone (Difco), 5.0 g soytone (Difco) 20.0 g sea salts (Difco), 15.0 g agar, 1000 ml deionized water, pH 7.3 (adjusted with HCl/NaOH)] or TSB (as TSA but without agar) were used.

For utilization of carrageenan (2 %, w/v), alginic acid (2 %, w/v) and starch (1 %, w/v), mineral medium according to van der Meulen (1974) was used. For hydrolysis studies, agar (1 %, w/v; Difco), casein (0.5 %, w/v; Sigma), cellulose (0.5 %, w/v, AZCL-HE-cellulose; MegaZyme), elastin (0.1 %, w/v; Sigma), fibrinogen (0.1 %, w/v; Sigma), gelatin (0.6 %, w/v; Difco) or haemoglobin (0.1 %, w/v; Sigma) was added to TSA with 20‰ sea salts. Degradation of dead yeast cells was tested on VY/2 substrate containing 20‰ sea salts (Reichenbach, 1989). For determination of the Gram-reaction, the non-staining method (3 % KOH; Buck, 1982) was used. Catalase and oxidase production was also investigated. The ability to grow under anaerobic conditions was tested with the Oxoid Anaerobic system.

Temperature, salinity and pH profiles. To test growth at different temperatures, the isolates were incubated on TSA substrate at 2.5, 5, 12, 18, 26, 30, 32, 35, 37 and 40 °C. To test salt tolerance, TSA substrate containing 0, 2.5, 10, 15, 20, 25, 30, 40, 50 and 60‰ sea salts (Sigma) or NaCl was used. Growth at different pH (4, 5, 6, 7, 8 and 9) was performed on TSA substrate.

BIOLOG characterization. All isolates were physiologically characterized by the BIOLOG GN MicroPlate method. Strains were grown for 24 h at 20 °C on TSA containing 20‰ sea salts. Inocula were prepared in saline water. The optical densities of inocula were determined by a BIOLOG spectrophotometer at 590 nm and were diluted to an OD₅₉₀ of around 0.26 in 0.9 % NaCl. Each well in the BIOLOG GN MicroPlate was inoculated with 150 µl cell suspension and the microplates were incubated at 20 °C for 72 h. The microplates were read in a BIOLOG MICROSTATION plate reader at 690 nm.

Phase-contrast microscopy. Photomicrographs of early stationary phase cultures (17 h) of isolates were taken with an Olympus BH-60 microscope fitted with an Olympus OM-2 camera.

Table 1. Strains used in this study

Taxon and culture collection no.	Original source of strain	Reference
[<i>Cytophaga</i>] <i>lytica</i> ATCC 23178 ^T	Beach mud, Limon (Costa Rica)	Lewin (1969)
[<i>Cytophaga</i>] <i>marinoflava</i> DSM 3653 ^T	Open North Sea off Aberdeen (UK)	Colwell <i>et al.</i> (1966)
[<i>Cytophaga</i>] <i>uliginosa</i> DSM 2061	Marine bottom sediment	ZoBell & Upham (1944)
<i>Flavobacterium johnsoniae</i> ATCC 17061 ^T	Soil near Rothamsted (UK)	Reichenbach (1989)
<i>Flavobacterium flevense</i> ATCC 27944 ^T	Freshwater and wet soil, IJsselmeer area (The Netherlands)	van der Meulen <i>et al.</i> (1974)
NN015860 ^T	Surface of <i>Fucus serratus</i> *	This study
NN016046	Seawater†	This study
NN016048	Seawater†	This study
NN014845	Surface of <i>Fucus serratus</i> ‡	This study
NN014847	Surface of <i>Fucus serratus</i> ‡	This study
NN014850	Surface of <i>Fucus serratus</i> ‡	This study
NN014871	Surface of <i>Fucus serratus</i> ‡	This study
NN014873	Surface of <i>Fucus serratus</i> ‡	This study
NN015839	Surface of <i>Fucus serratus</i> ‡	This study
NN015840 ^T	Surface of <i>Fucus serratus</i> ‡	This study
NN016038	Surface of <i>Fucus serratus</i> ‡	This study

* Collected on 22 July 1994 at Hirsholm. The seawater temperature was 19.9 °C and salinity was 25‰.

† Collected on 7 September 1994 at Ellekilde Hage. The seawater temperature was 16.4 °C and salinity was 18‰.

‡ Collected on 2 August 1994 at Hullehavn. The seawater temperature was 19.4 °C and salinity was 8‰.

Characterization by amplification with primers specific for repetitive extragenic palindromic elements by PCR (REP-PCR). The method used was according to Johnsen *et al.* (1996).

DNA isolation. All isolates were grown at 20 °C overnight in TSB + 20‰ sea salts and 7 ml of each culture was harvested by centrifugation at 6000 *g*. The cells were resuspended in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, 10 mM NaCl, pH 8) plus 50 mg lysozyme ml⁻¹ (Sigma) and incubated for 30 min at 37 °C. Thiocyanate (500 µl) was added and the mixture was incubated at room temperature for 10 min, then for 10 min on ice, followed by the addition of 250 µl cold 3 M sodium acetate and incubation on ice for 10 min. The mixture was extracted with 500 µl chloroform/isoamyl alcohol/pentanol (1:24:25, by vol.), twice with chloroform/isoamyl-alcohol (24:1, v/v), and the DNA was precipitated with 0.54 vol. cold 2-propanol. The DNA was spooled on an inoculation loop and washed in 70% ethanol for 10 min. The spooled DNA was resuspended in 100 µl TE buffer. Each DNA sample was purified by the addition of 1.25 ml 96% ethanol and 50 µl 3 M sodium acetate and incubated for 30 min at -20 °C. The DNA was recovered by centrifugation at 20000 *g* for 10 min at 5 °C. The supernatant was discarded, the DNA pellet was washed by addition of 500 µl 70% ethanol. After centrifugation at 20000 *g* for 10 min at 5 °C, the supernatant was discarded and the DNA was resuspended in 100 µl TE buffer. The quality and purity of each DNA preparation were measured by the absorbance ratio (A_{260}/A_{280}) with a Beckman DU 650 spectrophotometer.

DNA base composition. Determinations of the G + C content (mol%) were made by reverse phase HPLC as described by Mesbah *et al.* (1989). The values are means of triple determinations.

DNA-DNA hybridization. Slot blotting and DNA hybridization were performed using a modification of the method described previously (Nielsen *et al.*, 1995). The radiolabelled probes were made using the Random Primed DNA Labelling kit (Boehringer Mannheim) and Hybond N membrane (0.45 µm; Amersham Life Science) was used in the hybridization.

16S rDNA sequencing and data analysis. PCR amplification of the 16S rRNA genes was performed as described by Rainey *et al.* (1992). PCR products were purified using a PCR purification kit (Qiagen) and used as templates for sequencing with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Perkin Elmer). Sequencing was performed on a 373 prism DNA sequencer (ABI). Sequences were aligned against a ribosomal database by using the aligning tool of the ARB program package (Strunk & Ludwig, 1995) and final adjustments of the alignment were made manually. Corrected pairwise evolutionary distances were computed using the corrections of Jukes & Cantor (1969), modified by J. Felsenstein to allow for unequal base frequencies as described by Kishino & Hasegawa (1989). The phylogenetic tree was constructed by using neighbour-joining (Saitou & Nei, 1987). Bootstrap values were calculated for 1000 phylogenetic trees (Felsenstein, 1985). The phylogenetic analysis was made using facilities collected in the ARB program package (Strunk & Ludwig, 1995).

RESULTS

Morphological and physiological characteristics

During isolation and cultivation, all strains showed gliding motility and were Gram-negative, flexirubin-negative, non-spore-forming, non-motile rods. In TSB + 20‰ sea salts after 20 h at 26 °C, single cells of

Fig. 1. Phase-contrast micrographs of the brown-alga-associated strains grown in TSB + 20‰ sea salts at 26 °C for 17 h. (a) Strain NN015840^T; (b) strain NN015860^T. Bar, 5 µm.

NN015840^T measured 2.2–4.5 µm in length and 0.6–0.8 µm in diameter (Fig. 1a) and those of NN015860^T were 2.5–4.7 µm in length and 0.7–0.8 µm in diameter (Fig. 1b).

Growth of all strains occurred between 2 and 30 °C. With optimal growth around 26–30 °C. No growth was observed at or beyond 35 °C. Growth was inhibited at 32 °C and no growth was detected after 24 h incubation. However, when the plates were taken from 32 to 26 °C, growth appeared within 24 h. The colony morphology of the two new species varied significantly depending on the temperature and substrate.

The colony colours of the NN015840 group and NN015860^T were yellow and orange, respectively, at all temperatures (2–30 °C). At 18 and 26 °C, the colonies of both strains had a pronounced metallic tinge. At temperatures of 2–12 °C and at 30 °C, the colony morphology of the NN015840 group and NN015860^T was round, smooth, weakly convex with a regular edge, whereas at temperatures of 18–26 °C, the colony morphology of the two new species changed significantly depending on the substrate. All observations were made at 20 °C after 4 d incubation. Colonies of the NN015840 group were about 7 mm in diameter, yellow, slightly convex, sunken into the agar, had a flame-like edge and showed gliding motility on TSA substrate, whereas on CYT, the colonies were 0.5–1.0 mm in diameter, pale yellow, flat and showed a narrow zone of gliding motility. Colonies of NN015860^T were about 5 mm in diameter, yellow, slightly convex and demonstrated gliding motility on TSA substrate, whereas on CYT, the colonies were 0.5 mm in diameter, very pale yellow (nearly white), flat and showed a narrow zone of gliding motility.

All strains grew on TSA supplemented with 10–50‰ sea salts. Weak growth was observed at 2.5‰, whereas no growth was observed without sea salts and at 60‰ sea salts. Addition of NaCl (2.5–60‰) to the TSA substrate generally only supported weak or no growth of the strains. All strains grew well on TSA at pH 7 and

Table 2. Characteristics of the brown-alga-associated strains isolated from Danish waters

Strains: 1, NN015860^T; 2, NN015840 group (NN014845, NN014847, NN014850, NN014871, NN014873, NN015839, NN016038, NN016046, NN016048 and NN015840^T). All strains were negative in the utilization of α -cyclodextrin, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, cellobiose, *i*-erythritol, D-mannitol, D-melibiose, xylitol, acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, DL-lactic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyrroglutamic acid, D-serine, L-serine, DL-carnitine, γ -amino butyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol and DL- α -glycerol phosphate.

Utilization of:	1	2*
Dextrin	–	1 (–)
Glycogen	–	1 (–)
Tween 40	+	0 (–)
L-Arabinose	+	4 (–)
D-Arabitol	–	1 (+)
D-Fructose	+	10 (+)
L-Fucose	+	10 (+)
D-Galactose	–	8 (+)
Gentiobiose	–	5 (+)
α -D-Glucose	–	10 (+)
<i>m</i> -Inositol	–	1 (+)
α -D-Lactose	–	1 (–)
Lactulose	–	1 (–)
Maltose	–	2 (–)
D-Mannitol	–	1 (–)
D-Mannose	–	6 (+)
Methyl β -D-glucoside	–	1 (–)
Psicose	–	4 (–)
D-Raffinose	–	1 (–)
L-Rhamnose	+	2 (–)
D-Sorbitol	+	0 (–)
Sucrose	–	10 (+)
D-Trehalose	–	10 (+)
Turanose	–	1 (–)
Methyl pyruvate	+	10 (+)
Mono-methyl-succinate	–	1 (–)
Formic acid	–	3 (–)
D-Galacturonic acid	–	1 (–)
D-Gluconic acid	–	1 (–)
β -Hydroxybutyric acid	–	1 (+)
γ -Hydroxybutyric acid	–	2 (–)
α -Ketobutyric acid	+	10 (+)
α -Ketoglutaric acid	–	2 (–)
α -Ketovaleric acid	–	1 (–)
Malonic acid	–	1 (–)
Propionic acid	–	1 (–)
Succinic acid	–	1 (–)
Glucuronamide	–	7 (+)
L-Glutamic acid	–	8 (+)
L-Ornithine	–	1 (–)
L-Proline	–	3 (–)
L-Threonine	–	2 (–)
Glucose 1-phosphate	–	1 (–)
Glucose 6-phosphate	–	1 (–)

* Number of positives out of a total of 10; the phenotype of NN015840^T is indicated in parentheses.

Table 3. Selected physiological and biochemical characteristics of strains NN015840^T and NN015860^T and some CFB strains

Strain: 1, [*Cytophaga*] *lytica*; 2, [*Cytophaga*] *marinoflava*; 3, [*Cytophaga*] *uliginosa*; 4, *Flavobacterium flevense*; 5, NN015840^T; 6, NN015860^T. +, Positive; –, negative; NT, not tested.

Character	1*	2*	3*	4*	5	6
Cell size (L × W μm)	1.5–3.5 × 0.4	1–3 × 0.5–0.6	1.2–4 × 0.4	2–5 × 0.5–0.7	2.2–4.5 × 0.6–0.8	2.5–4.7 × 0.7–0.8
Colour of cell mass	Yellow	Yellow	Orange	Yellow	Yellow	Yellow
Colony morphology (on TSA + 20‰ sea salts)	Gliding	Round	Gliding	Round	Gliding	Gliding
Flexirubin reaction (20% KOH)	–	–	+	–	–	–
Catalase	+	+	+	–	+	+
Oxidase	+	+	+	+	–	–
Degradation of:						
Agar	+	–	+	+	+	+
Alginic acid	–	–	+	NT	+	+
Autoclaved yeast cells	–	–	–	–	+	+
Carrageenan	+	+	+	+	+	+
Casein	–	–	+	–	+	–
Cellulose (<i>endo</i> -cellulase)	+	NT	+	+	+	+
Elastin	–	–	–	–	+	+
Fibrinogen	–	–	–	–	+	–
Gelatin	–	–	–	–	+	–
Starch	+	+	+	–	+	+
Anaerobic growth	–	–	–	–	–	–
Growth in 60‰ sea salts	+	NT	NT	NT	+	+
Optimum temperature (°C)	22–30	20–30	20–30	25	~ 25	~ 25
Maximum temperature (°C)	35–40	< 37	NT	< 35	< 32	< 32
Habitat	Marine	Marine	Marine	Freshwater	Marine	Marine

* Data partly obtained from Reichenbach (1989).

8, whereas no growth was observed after 4 d at pH 4, 5, 6 or 9. However, after 10 d incubation, very weak growth was found at pH 9. All strains created craters in the TSA substrate, which was scored as positive for agar degradation. No positive flexirubin reactions were observed. All strains were catalase-positive and oxidase-negative. Results of the utilization of carbon sources are presented in Table 2. Results of the biochemical and physiological tests are listed in Table 3 with data from other species of the CFB group.

REP-PCR genomic fingerprints

The diversity of the macroalgae-associated *Cytophaga*-like bacteria was determined by REP-PCR. Results of REP-PCR are shown in Fig. 2. Ten strains (NN015840^T, NN014845, NN014847, NN014850, NN014871, NN014873, NN015839, NN016038, NN016046 and NN016048) showed similar banding patterns. These strains are referred to as the NN015840 group. With strains NN014847 and NN016038, a single band failed to appear compared to the other species of this group. In contrast, NN015860^T showed a unique banding pattern distinguishing it from the other isolates and the type strains tested in this study (Fig. 2).

Fig. 2. Ethidium bromide post-stained 3% NuSieve agarose gel with REP-PCR fingerprints. Lane: L, 0.1K ladder; 1, [*Cytophaga*] *lytica*; 2, *Flavobacterium johnsoniae*; 3, *Flavobacterium flevense*; 4, [*Cytophaga*] *uliginosa*; 5, [*Cytophaga*] *marinoflava*; 6, NN014845; 7, NN014847; 8, NN014850; 9, NN014871; 10, NN014873; 11, NN015839; 12, NN015840^T; 13, NN016038; 14, NN016046; 15, NN016048; and 16, NN015860^T.

Fig. 3. Phylogenetic tree, constructed by neighbour-joining, showing the relationship between strains NN015840^T and NN015860^T with organisms within the CFB group. Bootstrap values indicated at the branches were calculated from 1000 trees. The following 16S rRNA gene sequences were used in the analysis (EMBL accession nos are given in parentheses): strain NN015840^T (AJ005972); strain NN015860^T (AJ005973); [*Cytophaga*] *lytica* DSM 2039^T (M28058); [*Cytophaga*] *uliginosa* ATCC 14397^T (M28238); [*Cytophaga*] *marinoflava* ATCC 19326^T (M58770); [*Cytophaga*] *latercula* ATCC 23177^T (M58769); *Psychroserpens burtonensis* ATCC 700359^T (U62913); *Gelidibacter algens* ATCC 700364^T (U62914); *Polaribacter glomeratus* ATCC 43844^T (M58775); *Polaribacter irgensii* ATCC 49675^T (M61002); *Flexibacter maritimus* ATCC 43398^T (M64629); *Flavobacterium flevense* ATCC 27944^T (M58767); *Flavobacterium johnsoniae* DSM 425 (M59053); *Flavobacterium aquatile* DSM 1132^T (M28236); and *Cytophaga hutchinsonii* DSM 1761^T (M58768).

Table 4. Results of DNA–DNA hybridization between the two new brown-alga-associated species and other species from the CFB group

Strain	DNA homology (%)	
	NN015840 ^T	NN015860 ^T
[<i>Cytophaga</i>] <i>lytica</i> ATCC 23178 ^T	12	18
[<i>Cytophaga</i>] <i>marinoflava</i> DSM 3653 ^T	9	9
[<i>Cytophaga</i>] <i>uliginosa</i> DSM 2061	3	15
<i>Flavobacterium flevense</i> ATCC 27944 ^T	3	7
<i>Flavobacterium johnsoniae</i> ATCC 17061 ^T	0	0
NN015840 ^T	100	0
NN014845	100	6
NN014847	102	4
NN014850	100	3
NN014871	106	7
NN014873	102	5
NN015839	100	6
NN016038	100	5
NN016046	78	5
NN016048	71	0
NN015860 ^T	0	100

DNA composition

The G+C contents of strains NN015840^T and NN015860^T were 33.0 and 32.4 mol %, respectively.

Phylogenetic characterization

The majority of the 16S rDNA sequences were recovered by PCR amplification of rDNA. Full sequences of the PCR products were obtained. These

comprised 1474 nt from strain NN015840^T and 1472 nt from strain NN015860^T, which includes about 98 % of the full 16S rDNA sequence. The sequences were manually aligned and were most homologous to sequences from organisms of the CFB group. The phylogenetic tree in Fig. 3 shows the position of strains NN015840^T and NN015860^T among other related organisms using *Cytophaga hutchinsonii* as an out-group. The tree demonstrates that the two new brown-

macroalga-associated bacteria are positioned in a branch that also comprises [*Cytophaga*] *lytica*. The similarities between NN015840^T, NN015860^T and [*Cytophaga*] *lytica* are 93.3–98.6%. The closest relative to this branch is [*Cytophaga*] *uliginosa*, to which sequence similarities are 90.4–91.1%.

DNA–DNA hybridization

For determination of DNA homology, radiolabelled DNA probes of strains NN015840^T and NN015860^T were prepared. Results of hybridization against DNA of all isolates plus reference strains are listed in Table 4. With strain NN015840^T, DNA homologies of 71–106% were obtained against all strains of the NN015840 group. Moreover, the DNA homologies between NN015840^T, the type strains of related species and NN015860^T were all below 12%. This shows that NN015840^T represents a new species. The DNA homologies between NN015860^T and the other strains were no higher than 18%, indicating that NN015860^T also represents a new species.

DISCUSSION

Fingerprinting of bacteria by REP-PCR was first described by Versalovic *et al.* (1991) as a method for the discrimination of Gram-negative enterobacteria. Later, the method was used to determine the clonal origin of Gram-negative enterobacteria (Versalovic *et al.*, 1994) and to discriminate between closely related environmental isolates of *Pseudomonas* (Johnsen *et al.*, 1996). In this study, the technique was successfully used for rapid primary genotypic differentiation of yellow/orange coloured and gliding bacteria from the CFB group. The NN015840 group and strain NN015860^T both showed a unique banding pattern, as did all the included type strains. The homogeneity of the NN015840 group was confirmed by hybridization of total chromosomal DNA. Using the suggested DNA–DNA hybridization values of 70% or higher for species identity (Stackebrandt & Goebel, 1994), the isolates all belong to the same bacterial species. This shows that the REP-PCR technique is a good method for fast and easy sorting in groups of related isolates in a primary screening for a collection comprising *Cytophaga*-like organisms.

Members of the CFB group are known to play a key role in the biodegradation of organic matter (e.g. from macroalgae) in seawaters (Paerl & Pinckney, 1996; Rheinheimer, 1992; Fuhrman, 1992). These bacteria participate in the food chain by transferring carbon in the form of components of macroalgae-like exudates and parts of cell walls to higher trophic levels in the microbial food chain (Fenchel & Jørgensen, 1977). The two new species of the CFB group could metabolize agar, alginic acid, carrageenan, cellulose and L-fucose. Moreover, strain NN015840^T and most members of the NN015840 group could utilize D-galactose, α -D-glucose and D-mannose. These compounds are all important components of marine

benthic macroalgae (Lobban & Harrison, 1994; Hellebust, 1974). In brown algae, α -D-glucose is a basic component of the storage product laminaran (Percival, 1968) and cell walls contain alginic acid and fucan (a polymer of L-fucose with varying proportions of D-galactose, D-mannose and D-glucuronic acid) (Percival, 1979). The macroalgae surface-associated bacteria isolated in this study are thus able to decompose highly polymeric material from the living brown alga *F. serratus*. For this reason, we suggest that the strains are involved in an active decomposition of the alga, where the bacteria use the macroalga not only as a substratum but also as a substrate. Whether the hydrolytic activities of the bacteria indicate a parasitism or whether they are part of a symbiotic relationship remains to be investigated.

The use of phylogenetic techniques has recently led to a partial reclassification of the disordered complex of species described as *Cytophaga* and *Flavobacterium*. Many strains previously designated *Cytophaga* were reclassified in *Flavobacterium* (Bernardet *et al.*, 1996). Later the same year, the genus *Cytophaga* was emended to comprise the species *Cytophaga hutchinsonii* and *Cytophaga aurantiaca* only (Nakagawa & Yamasato, 1996).

The results of REP-PCR divided the macroalga-associated bacteria included in this study into two groups (the NN015840 group and NN015860^T). DNA–DNA hybridization showed that isolates of the NN015840 group had a homology above 70%. For this reason, these isolates should be considered as members of the same species. Strains NN015840^T and NN015860^T did not show significant DNA homology to any phylogenetically closely related species. Thus, these strains represent two new species of the CFB group. 16S rDNA analysis clearly indicates a relationship at the genus level between strains NN015840^T, NN015860^T and [*Cytophaga*] *lytica*. However, the correct phylogenetic position of the next relative, [*Cytophaga*] *uliginosa*, is more unclear because of the relatively low bootstrap value. These four taxa share many phenotypic traits. However, [*Cytophaga*] *uliginosa* differs in colony colour and shows a difference in the flexirubin reaction. Moreover, the G+C composition of the closely related taxa comprising NN015840^T, NN015860^T and [*Cytophaga*] *lytica* are in the range 32–33 mol%, whereas that for [*Cytophaga*] *uliginosa* is 42 mol%.

This leads us to the conclusion that the NN015840^T, NN015860^T and [*Cytophaga*] *lytica* taxa should be assigned to the same genus for which we propose the name *Cellulophaga* gen. nov. A description of the new genus is given below. Some traits useful for discriminating species of the new genus from the closest relatives are listed in Table 5. On the basis of the results of REP-PCR, physiological characteristics, DNA–DNA hybridization and 16S rDNA gene sequence analysis, we propose two new bacterial species: *Cellulophaga baltica* gen. nov., sp. nov. (for strains NN015840^T, NN014845, NN014847, NN014850,

Table 5. Phenotypic characteristics differentiating the *Cellulophaga* species from other members of the *Cytophagaceae* and *Flavobacteriaceae*

Abbreviations: –, negative; +, positive; v, variable; Y, yellow; O, orange; M, marine; S, soil; FW, freshwater.

Species	Gliding motility	Flexirubin	Colour of cell mass	Catalase	Hydrolysis			G + C (mol %)	Habitat
					Agar	Starch	Casein		
<i>Cellulophaga baltica</i>	+	–	Y	+	+	+	+	33.0	M
<i>Cellulophaga fucicola</i>	+	–	Y	+	+	+	–	32.4	M
<i>Cellulophaga lytica</i>	+	–	Y	+	+	+	–	32	M
[<i>Cytophaga</i>] <i>uliginosa</i>	+	+	O	+	+	+	+	42	M
[<i>Cytophaga</i>] <i>marinoflava</i>	–	–	Y	+	–	+	–	37	M
<i>Cytophaga</i> group*	–	+	Y–O	–	–	–	–	39–42	S
<i>Flavobacterium flevense</i>	+	–	Y	–	+	–	+	33/35	FW
<i>Flavobacterium johnsoniae</i>	+	+	Y	+	+	–	+	33	S, FW
<i>Psychroflexus</i> group†	v	–	O	+	–	+	–	32–36	M, sea ice

* Includes *Cytophaga hutchinsonii* and *Cytophaga aurantiaca*.† Includes *Psychroflexus torquis* and *Psychroflexus gondwanense*.

NN014871, NN014873, NN015839, NN016038, NN016046 and NN016048) and *Cellulophaga fucicola* gen. nov., sp. nov. (type strain NN015860^T). Finally, [*Cytophaga*] *lytica* becomes *Cellulophaga lytica*.

Description of *Cellulophaga* gen. nov.

Cellulophaga (Cel.lu.lo'pha.ga. M.L. n. *cellulosum* cellulose; *phaga* Gr. v. *phagein* to eat; M.L. fem. n. *Cellulophaga* eater of cellulose, referring to the *endo*-cellulase enzyme activity).

Gram-negative, rod-shaped cells, 1.2–4.7 µm long and 0.4–0.8 µm wide. Resting stages are absent. Motility by gliding. Colony colour is yellow to orange with a metallic tinge on TSA + 20‰ sea salts. Colony morphology changes with temperature, substrate and salinity. No bathochromic shift is observed on addition of 20 % KOH to colonies. Slightly or moderately halophilic. Strict aerobe. Chemo-organotroph. Catalase-positive. Metabolism is respiratory. All strains hydrolyse agar, carrageenan, starch and cellulose (*endo*-cellulase activity). Optimum conditions for growth are around 25 °C and pH 7–8. The DNA G + C content is 32–33 mol % (determined by HPLC). Habitat: the marine environment. Member of the family *Cytophagaceae*. The genus contains three species: *Cellulophaga baltica*, *Cellulophaga fucicola* and the type species, *Cellulophaga lytica*.

Description of *Cellulophaga baltica* gen. nov., sp. nov.

Cellulophaga baltica (bal'ti.ca. L. adj. *balticus* of the Baltic Sea, isolated at Bornholm, an island at the entrance to the Baltic Sea).

Cells are rod-shaped, occur singly, in pairs or sometimes in chains. Cell length is 2.2–4.5 µm and width is

0.6–0.8 µm. Cells are Gram-negative and non-motile and have aerobic metabolism, do not grow under (facultative) anaerobic conditions. Colonies on TSA + 20‰ sea salts at 20 °C are round, yellow with flame-like edge, demonstrate gliding motility and have a pronounced metallic tinge at temperatures between 18 and 26 °C. Growth occurs at pH 7–8 and 2–30 °C (optimally at 26–30 °C); no growth observed over 32 °C. Colony morphology changes significantly depending on the temperature, substrate and salinity. Members of this species are weakly halophilic and grow on substrates containing 10–50‰ sea salts (optimally around 20‰). Elevated sea salt concentration is necessary for good growth. No bathochromic shift is observed on addition of 20 % KOH to colonies. The DNA G + C content is 33.0 mol % (type strain). Catalase-positive and oxidase-negative. The potential to metabolize different carbon sources is presented in Tables 2 and 3. Source and habitat: isolated from the surface of the brown alga *Fucus serratus* L. Hullehavn in Svaneke, a town on the island of Bornholm at the entrance to the Baltic Sea. The type strain is NN015840^T and has been deposited in the BCCM/LMG Bacteria Collection as LMG 18535^T.

Description of *Cellulophaga fucicola* gen. nov., sp. nov.

Cellulophaga fucicola (fu.ci.co'la. L. *Fucus* seaweed, genus name of a brown alga; L. *colus* dweller; L. n. *fucicola* living on *Fucus serratus* L.)

Cells are rod-shaped, occurring singly, in pairs or sometimes in chains. Cell length is 2.5–4.7 µm and width is 0.7–0.8 µm. Cells are Gram-negative and non-motile and have aerobic metabolism, do not grow under (facultative) anaerobic conditions. Colonies on TSA + 20‰ sea salts at 20 °C are round, yellow, show

gliding motility and with a pronounced metallic tinge at temperatures between 18 and 26 °C. Growth occurs at pH 7–8 and 2–30 °C (optimally at 26–30 °C), no growth occurs over 32 °C. Colony morphology changes significantly depending on the temperature, substrate and salinity. Members of this species are weakly halophilic and grow on substrates containing 10–50‰ sea salts (optimally around 20–30‰). Elevated sea salt concentration is necessary for good growth. No bathochromic shift is observed on the addition of 20% KOH to colonies. The DNA G+C content is 32.4 mol% (type strain). This species is catalase-positive and oxidase-negative. The potential to metabolize different carbon sources is presented in Tables 2 and 3. Source and habitat: isolated from the surface of the brown alga *Fucus serratus* L. on the island of Hirsholm, offshore from the town of Frederikshavn, Jutland, Denmark. The type strain is NN015860^T and has been deposited in the BCCM/LMG Bacteria Collection as LMG 18536^T.

Description of *Cellulophaga lytica* (Lewin, 1969) gen. nov., comb. nov.

The description is identical to that given for [*Cytophaga*] *lytica* by Lewin (1969) and additional data by Reichenbach (1989). Type strain is ATCC 23178^T, isolated from marine beach mud, Limon, Costa Rica.

ACKNOWLEDGEMENTS

We thank Henrik Jespersen and Steen Kroghsbøll for help with collection of macroalgae in Hullehavn, Bornholm, and Kaare Johnsen and Anita Jørgensen, Geological Survey of Denmark and Greenland, for introduction to and performance of REP-PCR. We are thankful to Dr Jørgen Kristiansen, Dept of Mycology and Phycology, University of Copenhagen, for assistance with nomenclature. Finally, we would like to thank Dr Fiona Duffner, Novo Nordisk A/S, and Lone Madsen for proofreading the manuscript.

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