Aquiflexum balticum gen. nov., sp. nov., a novel marine bacterium of the *Cytophaga–Flavobacterium–Bacteroides* group isolated from surface water of the central Baltic Sea

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A bacterial isolate from the Baltic Sea, BA160^T, was characterized for its physiological and biochemical features, fatty acid profile, G+C content and phylogenetic position based on 16S rRNA gene sequences. The strain was isolated from the surface water of the central Baltic Sea during the decay of a plankton bloom. Phylogenetic analyses of the 16S rRNA gene sequence revealed a clear affiliation with the family '*Flexibacteraceae*', and showed the closest phylogenetic relationship with the species *Belliella baltica* and *Cyclobacterium marinum*. The G+C content of the DNA was 38·4 mol%. The strain was red-coloured due to carotenoids, Gram-negative, rod-shaped, and catalase- and oxidase-positive. Growth was observed at salinities from 0 to 6 %, with an optimum around 1·5 %. Temperature for growth ranged from 4 to 40 °C, with an optimum around 30 °C. The fatty acids were dominated by branched-chain fatty acids (>87 %), with a high abundance of iso-$C_{15:0}$ (23 %) and anteiso-$C_{15:0}$ (19 %). According to its morphology, physiology, fatty acid composition, G+C content and 16S rRNA gene sequence, strain BA160^T is considered to represent a new genus of the family '*Flexibacteraceae*'. Due to its aquatic origin, the name *Aquiflexum balticum* gen. nov, sp. nov. is suggested for the type species (type strain, BA160^T=DSM 16537^T=LMG 22565^T=CIP 108445^T) of the new genus.

The *Cytophaga–Flavobacterium–Bacteroides* (CFB) group of bacteria is considered to be of special relevance for aquatic environments. In marine and freshwater environments, a high abundance of CFB bacteria can occur, and the CFB bacteria are considered to be of high relevance for the degradation of organic matter, such as complex polysaccharides (Cottrell & Kirchman, 2000; Höfle, 1982, 1992; Pinhassi *et al.*, 1999). An isolate, BA160^T, was obtained from surface water of the central Baltic Sea at the entrance of the Gulf of Finland during the decay of a phytoplankton bloom. CFB bacteria have been shown to be major degraders of organic matter derived from phytoplankton. The isolate can therefore be considered as a bacterium that contributes to the degradation of plankton blooms in an aquatic environment such as the Baltic Sea. Despite the relevance of the CFB group for aquatic environments, available information on the taxonomy of aquatic CFB bacteria and their biodegradation potential is still very limited.

A new genus, designated *Aquiflexum* gen. nov., is shown here to belong to the CFB group of bacteria, which is also referred to as the phylum '*Bacteroidetes*' (Ludwig & Klenk, 2001). The new genus can be assigned to the family '*Flexibacteraceae*', which includes 13 other genera and is one of three families belonging to the class '*Sphingobacteria*' (Ludwig & Klenk, 2001).

Strain BA160^T was isolated during a cruise on board the RV *Aranda* in September 1998 from surface water (5 m, 15 °C, 6 ‰ S, pH 8·2) from a site in the central Baltic Sea at the entrance of the Gulf of Finland (station LL12, 59·2900° N, 22·5398° E). All details on environmental conditions, sampling and isolation procedures are given elsewhere (Brettar *et al.*, 2002; Brettar & Rheinheimer, 1992; Brettar & Höfle, 1993; Höfle & Brettar, 1995). Medium for isolation was ZoBell agar (Oppenheimer & Zobell, 1952)

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Abbreviation: CFB, *Cytophaga–Flavobacterium–Bacteroides*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Aquiflexum balticum* BA160^T is AJ744861.

A phase-contrast micrograph of and a table giving details of API results for strain BA160^T are available as supplementary material in IJSEM Online.

with a reduced sea salt content of 1 %. The strain grew well on half-strength ZoBell agar and marine broth or agar (no.2216; Difco). Cultivation of the strain below 28 °C is not recommended because pronounced lag phases (>1 week) may occur.

The isolate was tested for a number of key characteristics using standard procedures (Gerhardt *et al.*, 1994), such as KOH string test (Gram stain), cell size and morphology (phase-contrast microscopy), cytochrome oxidase and catalase (3 % H_2O_2). Furthermore, production of indole, growth on 0·5 % yeast extract, nitrate reduction, and hydrolysis of aesculin, casein, tyrosine, starch, gelatin and DNA were tested. Chitinase, cellulase and pectinase activities were tested as described by Atlas (1993). The strain was also characterized by using the whole test spectrum of the identification systems API 50CHE, API 20NE and API ZYM (bioMérieux) and Biolog GN2 at 28 °C. Growth was assessed at 4, 10, 20, 25, 30, 37, 40 and 45 °C. Different salinities were tested: 0, 0·8, 1·5, 3, 6 and 10 % NaCl. Growth at different pH values was also tested (pH 7, 9 and 10). For these tests, we used half-strength marine broth (no. 2216; Difco), except for the salinity test for which half-strength salt-free ZoBell medium was supplemented with the respective amount of NaCl.

Ethanol extracts of cells grown at 30 °C on half-strength marine agar were examined by spectroscopy to check for pigments and flexirubin, as outlined by Gosink *et al.* (1998). Briefly, cells were extracted in 95 % ethanol, and spectral analysis was performed from 250 to 700 nm before and after alkalinization with 0·1 vol. of 0·1 M NaOH.

Genomic DNA was prepared from individual colonies as described by Moore *et al.* (1996). 16S rRNA genes were amplified by PCR (Mullis & Faloona, 1987) and the PCR products were sequenced directly as described previously (Moore *et al.*, 1999).

For phylogenetic analyses, related sequences were selected from a database of 92 000 pre-aligned bacterial 16S rRNA gene sequences and according to a BLAST against the latest release from the EBI (European Bioinformatics Institute). In a first analysis, 105 sequences were selected according to the results of the BLAST query (100 sequences) and to previous analyses. The new sequences were automatically and then manually aligned against these already aligned sequences. Among these sequences, 40 that were from cultured bacteria (type species and type strains when available) were selected for the final analysis. Phylogenetic trees were then constructed using these different datasets of sequences and three different methods (BIONJ, maximum-likelihood and maximum-parsimony). For the neighbour-joining analysis, distance matrices were calculated using Kimura's two-parameter correction. BIONJ was run according to Gascuel (1997), and maximum-likelihood and maximum-parsimony were run from PHYLIP (Felsenstein, 1995); for more details, see http://bioinfo.unice.fr (section 'publication', document: 'Phylogeny_How'). Analyses made with or without domains with potential homoplasies yielded the same results. The phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996). The topology shown (Fig. 1) is that of the bootstrap neighbour-joining analysis. Bootstrap values (expressed as percentages of 1000 replications) are shown only for those branches that were retrieved by all three methods. There is no scale bar with the tree because it is a bootstrap tree.

The DNA G+C content (mol%) of the strain was determined using HPLC analysis of hydrolysed DNA according to Tamaoka & Komagata (1984) and Mesbah *et al.* (1989).

For fatty acid analysis, the strains were grown on half-strength marine agar (no. 2216; Difco) for 24 h at 28 °C. The fatty acid methyl esters were obtained from washed cells by saponification, methylation and extraction. Analysis by gas chromatography was controlled by MIS software (Microbial ID, Newark, DE, USA) and peaks were automatically integrated and identified by the Microbial Identification software package (Sasser, 1990).

Strain BA160^T was Gram-negative and formed slender, long rods of 0·3–0·6 μm in width and 1·1–4·8 μm in length (Fig. S1, available as supplementary material in

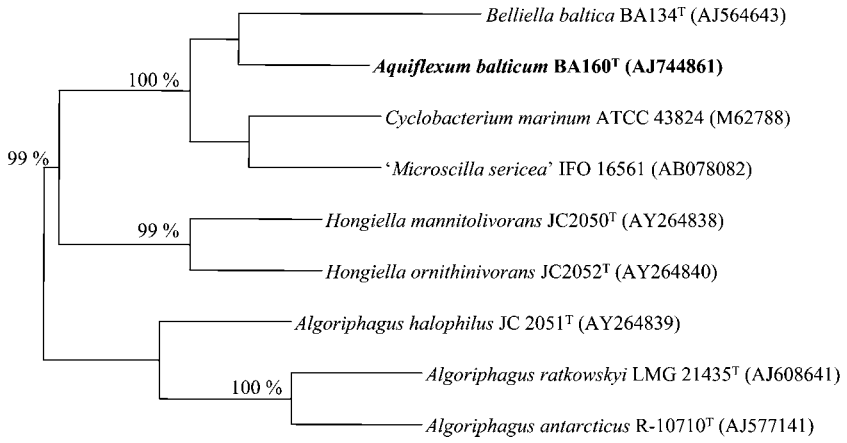

Fig. 1. Unrooted phylogenetic tree resulting from the analysis of nearly complete 16S rRNA gene sequences. The species shown were chosen according to the results of a larger analysis. The topology shown was obtained using a neighbour-joining algorithm and 1000 bootstrap replications. Bootstrap values (expressed as percentages) are indicated only for branches found also by parsimony and maximum-likelihood ($P<0·01$), and therefore define robust clusters.

IJSEM Online). Occasionally, chains of up to five cells in length were observed. Gliding motility could not be observed by phase-contrast microscopy. Colonies were circular, smooth, transparent and bright red in colour on half-strength marine agar. With ongoing incubation, colonies became opaque. The strain was catalase-, cytochromoxidase- and aminopeptidase-positive. Growth was observed from 4 to 40 °C, with good growth between 10 and 35 °C, and optimum growth between 30 and 35 °C. Growth was observed at salinities from 0 to 6 %, with an optimum at 1·5 %. Growth occurred from pH 7 to 9, with an optimum around neutral conditions.

Strain BA160^T did not show a change in colour after alkalinization in the KOH string test (used as an indicator of Gram stain) nor after alkalinization of the ethanol extract of cells. The spectrum of the ethanol extract showed a broad peak with a maximum around 475 nm and two shoulder peaks at 450 and 505 nm – peaks that are typical for carotenoids. Alkalinization did not show a bathychromatic shift of the peaks. Therefore, we assume that strain BA160^T contained carotenoids but no flexirubins, as often observed for marine CFB bacteria (Reichenbach *et al.*, 1981).

Strain BA160^T was able to reduce nitrate to nitrite and hydrolyse aesculin and starch. It did not produce indole, and did not degrade tyrosine or cellulose. It did not grow on agar supplemented with DNA, casein, chitin, pectin or 0·5 % yeast extract.

In the API 50CHE test system, strain BA160^T showed acid production from 28 substrates (see species description). API 20NE test results indicated that six substrates were assimilated. The strain showed 12 enzymic activities. As a general rule, phenotypic features were rated positive when a signal was obtained, either weak or more pronounced (for details see Table S1, available as supplementary material in IJSEM Online).

In terms of its phenotypic features, strain BA160^T could be differentiated from the closely related species *Cyclobacterium marinum* (Raj & Maloy, 1990) by cell morphology, salt dependence, salt tolerance, nitrate reduction, hydrolysis of starch and gelatin, and the ability to produce acid from three carbohydrates, to assimilate malate and to use eight carbonic acids (Table 1). It could be distinguished from *Belliella baltica* by the temperature range and optimum for growth, hydrolysis of gelatin, acid production from 13 substrates, assimilation of three substrates and utilization of seven substrates (Table 1). Compared to *B. baltica*, BA160^T showed a higher versatility concerning the use of organic substrates except for amino acids.

Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain BA160^T formed a very robust clade (all methods, 100 % of bootstrap replications) with *Belliella*, *Cyclobacterium* and *Hongiella* species while *Algoriphagus* species formed a closely related outgroup (Fig. 1). Furthermore, strain BA160^T formed a robust clade with *C. marinum* and *B. baltica*, but could not be grouped robustly with any currently recognized species, suggesting that strain BA160^T should be recognized as representing a different genus. More extensive analyses including related sequences of described and undescribed bacteria demonstrated that strain BA160^T is located among a large set of sequences of undescribed bacteria.

The 16S rRNA gene sequence of strain BA160^T was most similar to those of *B. baltica* (GenBank/EMBL/DDBJ accession no. AJ564643, 92·4 % similar, 110 differences; Brettar *et al.*, 2004), *Hongiella ornithinivorans* (AY264840, 92·3 % similar, 114 differences; Yi & Chun, 2004), *Hongiella mannitolivorans* (AY264838, 91·4 % similar, 118 differences; Yi & Chun, 2004), *Algoriphagus halophilus* (AY264839, 90·6 % similar, 134 differences; Yi & Chun, 2004; Nedashkovskaya *et al.*, 2004), *Algoriphagus ratkowskyi* (AJ608641, 89·8 % similar, 147 differences; Bowman *et al.*, 2003), *Algoriphagus antarcticus* (AJ577141, 90·2 % similar, 142 differences; Van Trappen *et al.*, 2004) and *C. marinum* (M62788, 89·4 % similar, 155 differences; Raj & Maloy 1990; Woese *et al.*, 1990). The similarity of the 16S rRNA gene sequence of strain BA160^T with that of *C. marinum* might be higher than the calculated value of 89·4 %, due to a number of non-determined nucleotides of the sequence of the latter.

The G+C content of strain BA160^T was 38·4 mol% (Table 1). The G+C contents of *C. marinum* range from 34 to 38 mol% (Raj & Maloy, 1990; Gosink *et al.*, 1998), while those of *B. baltica* range from 35·3 to 35·5 %.

The cellular fatty acid composition of strain BA160^T showed a restricted spectrum of 17 fatty acids with a pronounced dominance of branched-chain C_{15} fatty acids. Abundant fatty acids were iso-$C_{15:0}$ (22·6 %), anteiso-$C_{15:0}$ (18·6 %), iso-$C_{15:0}$ G (9·4 %) and iso-$C_{16:1}$ H (9·5 %) (Table 2). The branched-chain fatty acids formed a fraction of at least 87 %, with iso-branching fatty acids forming the major fraction of 62 %. This calculation is based on the assumption that the detected double peak (ecl 15·19–15·48) represents $C_{16:1}\omega7c$, which cannot be differentiated by the MIDI system from iso-$C_{15:0}$ 2OH. Otherwise, the fraction of iso-branching fatty acids would be even higher. When compared with *B. baltica*, the number of fatty acid compounds detected in strain BA160^T is lower and the composition differs considerably. The major difference is the high abundance of anteiso-$C_{15:0}$ in strain BA160^T (*B. baltica*, 4·5 %). A comparison of strain BA160^T with *C. marinum* Raj^T (Urakami & Komagata, 1986) showed for both a high fraction of iso- and anteiso-$C_{15:0}$ fatty acids, but contrasts with respect to the overall fatty acid composition (*C. marinum*: iso-$C_{15:0}$ 44·6 %; anteiso-$C_{15:0}$ 31·5 %).

Of the currently recognized species, the Baltic Sea strain BA160^T is phylogenetically most closely related to *C. marinum* and *B. baltica* (Fig. 1). Due to the low level of 16S rRNA gene similarity and the lack of strong clustering

Table 1. Features useful for distinguishing strain BA160^T from closely related species

Species: 1, *Aquiflexum balticum* (strain BA160^T); 2, *Belliella baltica* [data from Brettar *et al.* (2004), comprising strains BA1 and BA134^T]; 3, *Cyclobacterium marinum* [data from Raj & Maloy (1990), comprising strains Raj^T (ATCC 25205^T), WH-A (ATCC 43824) and WH-B (43825)]. Responses to substrates: +, good; W, weak; V, variable; −, no response; ND, no data available.

Characteristic	1	2	3
Cell morphology	Long rods, straight	Rods, straight	Vibrioid, ring-like, spiral, coils
Cell size (μm)	0·3–0·6 × 1·1–4·8	0·3–0·5 × 0·9–3·0	0·3–0·7 × 0·8–1·5
Colour	Red	Pink/orange	Pink
G+C content (mol%)	38·4	35·4	34–38
Salinity growth range (%)	0–6	0–6	1·5–15
Salinity optimum (%)	1–2	0–3·4	1·5–5
Temperature growth range (°C)	4–40	4–37	4–40
Temperature optimum (°C)	30–35	20–30	25
Growth at pH 10	−	W	−
Nitrate reduction to nitrite	+	+	−
Hydrolysis of gelatin	+	−	−
Hydrolysis of starch	+	+	−
Acid production from (API 50CHE):			
L-Arabinose	−	+	+
D-Xylose	−	+	+
D-Mannose	W	−	+
Rhamnose	+	−	+
Methyl α-D-mannoside	W	−	ND
Methyl α-D-glucoside	W	−	+
N-Acetylglucosamine	W	−	ND
Amygdalin	W	−	ND
Cellobiose	+	+	−
Melezitose	W	−	ND
Xylitol	W	−	ND
D-Turanose	W	−	ND
L-Fucose	W	−	ND
5-Ketogluconate	W	−	ND
Assimilation of (API 20NE):			
Mannose	+	−	ND
N-Acetylglucosamine	+	−	ND
Gluconate	+	−	+
Malate	−	−	+
Utilization of (Biolog GN2):			
D-Mannose	W	−	ND
Methyl β-D-glucoside	W	−	ND
D-Psicose	W	−	ND
D-Sorbitol	W	−	ND
Monomethyl succinate	W	−	ND
Citric acid	−	−	+
D-Gluconic acid	−	−	+
α-Ketobutyric acid	−	+	ND
α-Ketoglutaric acid	W	W	−
α-Ketovaleric acid	−	+	ND
DL-Lactic acid	+	V	−
Malonic acid	−	−	+
Propionic acid	W	V	−
Succinic acid	−	−	+
L-Alanine	−	V	ND
L-Alanyl glycine	−	V	ND
L-Glutamic acid	−	+	+
L-Proline	−	V	ND
L-Serine	−	V	−
L-Threonine	−	V	ND

Table 2. Fatty acid composition of strain BA160^T in comparison to *B. baltica* and *C. marinum*

Species: 1, *A. balticum* BA160^T; 2, *B. baltica* [data from Brettar *et al.* (2004), comprising strains BA1 and BA134^T]; 3, *C. marinum* (data from Urakami & Komagata, 1986). Results are presented as a percentage of the total fatty acids; only those fatty acids contributing more than 1 % are given. Fatty acids representing more than 5 % are in bold. ND, Not detected.

Fatty acid	1	2	3
$C_{13:1}$ AT 12–13	ND	<1·44	
iso-$C_{14:0}$	4·77	1·98	
$C_{15:0}$	ND	2·94	0·2
iso-$C_{15:0}$	**22·59**	**20·70**	**44·6**
anteiso-$C_{15:0}$	**18·53**	4·53	**31·5**
iso-$C_{15:0}$ 3OH	1·59	2·18	
iso-$C_{15:1}$ G	**9·40**	**10·20**	
$C_{15:1}\omega6c$	ND	2·07	
iso-$C_{16:0}$	4·23	2·64	
$C_{16:0}$ 3OH	ND	<1·10	
$C_{16:1}$ straight chain			**14·2**
iso-$C_{16:1}$ H	**9·45**	3·49	
iso-$C_{16:1}$ 3OH	2·00	1·94	
$C_{16:1}\omega5c$	1·98	3·30	
$C_{16:1}\omega7c$/iso-$C_{15:0}$ 2OH*	**6·10**	**9·25**	
iso-$C_{17:0}$ 3OH	1·44	3·17	
anteiso-$C_{17:1}$ B	2·55	3·69	
$C_{17:1}\omega6c$	2·99	**7·28**	
$C_{17:1}\omega8c$	ND	1·19	
iso-$C_{17:1}\omega9c$	**5·22**	**8·38**	
anteiso-$C_{17:1}\omega9c$	1·10	ND	
$C_{18:1}$ straight chain			**8·1**
iso-$C_{19:1}$ I	1·46	ND	
Not identified	4·60	7·70	

*Not differentiated by the MIDI system.

by phylogeny, strain BA160^T was regarded as belonging to a new genus. The phylogenetic conclusions are supported by the fatty acid profile, cell morphology and physiological features of strain BA160^T. Physiological features that differentiate strain BA160^T from *C. marinum* are the NaCl dependence for growth, the salinity range for growth, the reduction of nitrate and the spectrum of substrates utilized for growth and acid production; those that differentiate strain BA160^T from *B. baltica* are the temperature growth range and optimum, the pH range for growth and a rather distinct spectrum of substrates used for acid production and growth, showing a higher metabolic versatility for strain BA160^T. Strain BA160^T was very distinct in terms of its cellular fatty acid composition. Based on this polyphasic study and its results for the novel Baltic Sea isolate, we propose a new genus, *Aquiflexum* gen. nov. Strain BA160^T is proposed as the type strain of the novel species *Aquiflexum balticum* sp. nov.

Description of *Aquiflexum* gen. nov.

Aquiflexum (A.qui.flex′um. L. fem. n. *aqua* water; L. part. adj. *flexus* bent, winding; N.L. neut. n. *Aquiflexum* to indicate the bacterium's aquatic origin and its long flexible rods).

Cells are Gram-negative, rod-shaped and oxidase- and catalase-positive. Growth is heterotrophic and aerobic. Dominant fatty acids are of iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{16:1}$ H. Cells contain carotenoids but no flexirubin. NaCl is not needed for growth, but growth is improved by its presence. The genus belongs to the phylum '*Bacteroidetes*'.

The type species is *Aquiflexum balticum.*

Description of *Aquiflexum balticum* sp. nov.

Aquiflexum balticum (bal′ti.cum. N.L. neut. adj. *balticum* from the Baltic Sea, referring to the source of the type strain).

Colonies are circular, smooth, convex and entire. Red and transparent when young, but turn opaque with ongoing incubation (>1 week, 30 °C, on half-strength marine agar). Cells are Gram-negative, rod-shaped (width, 0·3–0·6 μm; length, 1·1–4·8 μm), and oxidase- and catalase-positive. Reduces nitrate to nitrite. Temperature range for growth is 4–40 °C, with an optimum around 30 °C. NaCl is not needed for growth, but growth occurs in the presence of 0 to 6 % NaCl, with an optimum around 1·5 %. Grows from pH 7 to 9, with an optimum around neutral pH. Does not grow on 0·5 % yeast extract. Hydrolyses aesculin, starch and gelatin. Produces acid from galactose, D-glucose, D-fructose, D-mannose, rhamnose, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, *β*-gentobiose, D-turanose, L-fucose and 5-ketogluconate. Shows α- and *β*-glucosidase, *β*-galactosidase, acid and alkaline phosphatase, leucine, valine and cystine arylamidase, trypsin, chymotrypsin, naphthol-phosphohydrolase and *N*-acetyl-*β*-glucosaminase activities. Assimilates glucose, arabinose, mannose, *N*-acetylglucosamine, maltose and gluconate. Utilizes L-arabinose, cellobiose, L-fructose, D-galactose, gentobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannose, methyl *β*-D-glucoside, D-psicose, D-sorbitol, sucrose, D-trehalose, turanose, monomethyl succinate, acetic acid, α-ketoglutaric acid, lactic acid and propionic acid as substrates. No use of amino acids is detected, but aminopeptidase is produced. Of marine or estuarine origin. The G+C content is 38·4 mol%.

The type strain is BA160^T (=DSM 16537^T=LMG 22565^T=CIP 108445^T).

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