Belliella baltica 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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Two bacterial isolates from the Baltic Sea, BA1 and BA134^T, were characterized for their physiological and biochemical features, fatty acid profiles and phylogenetic position based on 16S rRNA gene sequences. The strains were isolated from surface water of the central Baltic Sea during the decay of a plankton bloom. Phylogenetic analysis of their 16S rRNA gene sequences revealed a clear affiliation to the family 'Flexibacteriaceae' and showed highest sequence similarity (91 %) to *Cyclobacterium marinum*. The G + C content of the DNA was 35.4 mol%. The strains were pink-coloured due to carotinoids, Gram-negative, rod-shaped and catalase- and oxidase-positive. Growth was observed at 0–6 % salinity, with good growth at 0–3 %. Temperature for growth was 4–37 °C, with an optimum around 25 °C. The fatty acid profiles were dominated by branched-chain fatty acids (70 %), with a high abundance of iso-C_{15:0} (29–33 %), iso-C_{17:1ω9c} (7–10 %) and C_{17:1ω6c} (5–10 %). According to their morphology, physiology, fatty acid composition, 16S rRNA gene sequences and DNA–DNA similarity, on one hand, the described bacteria are considered to be members of the same novel species; on the other hand, they are suggested as a novel genus of the family 'Flexibacteriaceae'. To honour the late aquatic microbiologist Russell T. Bell, the name *Belliella baltica* gen. nov., sp. nov. is suggested for the Baltic Sea isolates, for which the type strain is BA134^T (=DSM 15883^T =LMG 21964^T =CIP 108006^T).

The *Cytophaga–Flavobacterium–Bacteroides* (CFB) group is considered to be a bacterial group of special relevance for aquatic environments. In marine and freshwater environments, a high abundance of CFB organisms can occur; they are considered to be of high relevance for the degradation of organic matter, such as complex polysaccharides (Höfle, 1982, 1992; Pinhassi *et al.*, 1999; Cottrell & Kirchman, 2000). The isolates were obtained from surface water of the central Baltic Sea during the decay of a phytoplankton bloom. CFB organisms have been shown to be major degraders of

organic matter that is derived from phytoplankton; these organisms can therefore be considered to contribute to the degradation of plankton blooms in an aquatic environment such as the Baltic Sea. The view of CFB organisms as catalysts of the decay of phytoplankton blooms is supported by the results of earlier studies that were conducted during a calm summer period, when no decay of phytoplankton occurred. In this study period, no CFB isolates were obtained (Höfle & Brettar, 1996). Despite the relevance of the CFB group for aquatic environments, there is little information available on the taxonomy of aquatic CFB organisms or their physiological potential. The novel genus described here, of the CFB group or of the phylum 'Bacteroidetes', belongs to the class 'Sphingobacteria' according to the most recent issue of *Bergey's Manual of Systematic Bacteriology* (Ludwig & Klenk, 2001). This class comprises three families and a total of 15 genera. *Belliella baltica* gen. nov., sp. nov. was assigned to the largest family, 'Flexibacteriaceae', which currently comprises 10 genera.

Two strains, BA1 and BA134^T, were isolated during a cruise

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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BA134^T and BA1 are AJ564643 and AJ564642, respectively.

A phase-contrast micrograph of cells of strain BA134^T and tables showing the API test results and fatty acid profiles are available as supplementary material in IJSEM Online.

on board RV *Aranda* in September 1998, from surface water (5 m; 15 °C; salinity, 7‰; pH 8.4) of two stations of the central Baltic Sea [Gotland Deep (BY 15, 57.1920° N, 20.0302° E) and TEILI1 (59.2607° N, 21.3002° E)]. All details on environmental conditions, sampling and isolation procedures are given elsewhere (Brettar & Rheinheimer, 1992; Brettar & Höfle, 1993; Höfle & Brettar, 1995; Brettar *et al.*, 2002). The medium for isolation was ZoBell agar (Oppenheimer & ZoBell, 1952). The strains grew well on full- and half-strength ZoBell agar and on marine broth or agar 2216 (Difco).

The isolates were tested for a number of key characteristics by using standard procedures (Gerhardt *et al.*, 1994), such as the KOH string test, cell size and morphology (phase-contrast microscopy) and activities of cytochrome oxidase and catalase (3% H₂O₂). 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). Strains were additionally characterized by using the whole test-spectrum of the API 50CH, API 20NE, API ZYM (bioMérieux) and Biolog GN2 identification systems at 28 °C. Growth was assessed at 4, 10, 20, 25, 30, 33 and 37 °C, at pH 6, 7, 9 and 10, and at 0, 0.8, 1.5, 3, 6 and 10% NaCl. For these tests, half-strength marine broth 2216 (Difco) was used except for the salinity test, where half-strength, salt-free ZoBell medium was supplemented with the respective amount of sea salt.

Ethanol extracts of cells grown at 21 °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, then spectral analysis was performed at 250–700 nm, before and after alkalization with 0.1 vol. 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 products were sequenced directly, as described by Moore *et al.* (1999).

For phylogenetic analyses, related sequences were selected according to previous phylogenetic analyses of a database of 62 000 pre-aligned bacterial 16S rRNA gene sequences and BLAST searches against the latest release of EBI (European Bioinformatics Institute). In a preliminary analysis, 150 sequences were selected according to the results of a BLAST query (100 sequences) and previous analyses (50 sequences). The new sequences were aligned automatically (Abdeddaim, 1997) and then manually [by using SeaView (Galtier *et al.*, 1996)] against these already aligned sequences. The first set of phylogenetic trees allowed us to select 43 sequences of related cultured bacteria (type species, when available) and uncultured clones that were very closely related to the strains studied. When several sequences were available for a type species, the sequence with the least ambiguities was

selected. Phylogenetic trees were then constructed by using three different methods: neighbour-joining (BIONJ), maximum-likelihood and maximum-parsimony. For neighbour-joining analysis, distance matrices were calculated by using Kimura's two-parameter correction. BIONJ was performed according to Gascuel (1997) and maximum-likelihood and maximum-parsimony were from PHYLIP (Phylogeny Inference Package, version 3.573c; distributed by J. Felsenstein, Department of Genetics, UW, Seattle, WA, USA). Because of the loose relationships between the 43 selected sequences, homoplasies and aligning difficulties were detected and domains that corresponded to positions 68–150, 190–430, 445–803, 813–988 and 1002–1407 of strain BA134^T were selected for phylogenetic analysis. Only these domains were used for the analysis shown in Fig. 1. The phylogenetic trees were drawn by using NJPLOT (Perrière & Gouy, 1996).

DNA was isolated by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Escara & Hutton (1980) and Huss *et al.* (1983), using a Gilford system model 2600 spectrometer. Renaturation rates were calculated with the TRANSFER.BAS program (Jahnke, 1992). The DNA G + C content (mol%) of the strains was determined by using HPLC analysis of hydrolysed DNA, according to Tamaoka & Komagata (1984) and Mesbah *et al.* (1989).

For cellular fatty acid analysis, strains were grown on half-strength marine agar 2216 (Difco) for 24 h at 28 °C. Fatty acid methyl esters were obtained from washed cells by saponification, methylation and extraction. Analysis by GC was controlled by MIS software (Microbial ID) and peaks were integrated and identified automatically by the Microbial Identification software package (Sasser, 1990).

The strains were Gram-negative, tender, straight rods of 0.3–0.5 µm in width and 0.9–3.0 µm in length (Supplementary Fig. A, IJSEM Online). Occasionally, chains of up to six cells were observed. Gliding motility was not observed by phase-contrast microscopy. Colonies were circular, smooth, transparent and pink on half-strength marine agar. With ongoing incubation, colonies became opaque and their colour intensified and changed to orange. In terms of physiological features, the two strains analysed showed comparable responses. The strains were catalase-, cytochrome oxidase- and aminopeptidase-positive. Growth was observed at 4–33 °C for strain BA1 and at 4–37 °C for strain BA134^T. Both strains showed good growth between 10 and 30 °C, with optimum growth between 20 and 25 °C for strain BA1 and between 25 and 30 °C for strain BA134^T. Growth was observed at salinities from 0 to 6%, with good growth between 0 and 3%. Growth occurred at pH 6–10, with an optimum around neutral conditions. Both strains were able to reduce nitrate to nitrite. They hydrolysed aesculin, starch and DNA. They did not produce indole and did not degrade tyrosine, gelatin or cellulose. No growth occurred on media that contained chitin, pectin or casein.

Fig. 1. Unrooted phylogenetic tree resulting from analysis of nearly complete 16S rRNA gene sequences. The topology shown was obtained by using the neighbour-joining algorithm. Bootstrap values (%) are only indicated for branches that were also retrieved by parsimony (a single, most parsimonious tree) and maximum-likelihood [$P < 0.01$; 661 trees examined; $\ln(\text{likelihood})$, -814.63698] and therefore define robust clusters. Strains BA134^T and BA1 formed a robust cluster with the alkaliphilic strains Bacterium ikaite c9 and Soda lake bacterium Z3.

In the flexirubin test, the strains did not show a change in colour after alkalinization, either with the KOH string test or after alkalinization of ethanol extract of cells. The spectrum of the ethanol extract of both strains showed a broad peak with a maximum around 475 nm and two shoulder peaks at 450 and 505 nm, peaks that are typical for carotinoids. Alkalinization did not show a bathochromic shift of the peaks. We therefore assume that the strains contained carotinoids but no flexirubins, as often observed for marine CFB organisms (Reichenbach *et al.*, 1981).

Results of the API tests are given in detail in Supplementary Table A in IJSEM Online. In the API 50CH test system, the strains produced acid from a total of 20 substrates, with use of 19 substrates by strain BA1 and 16 substrates by strain BA134^T. By the API 20NE test, the same six positive reactions were observed for both strains. By the API ZYM test, 11 enzymic activities were detected, with one lacking for strain BA1. In the Biolog GN2 test system, the substrate spectra of the strains differed: strain BA1 used 14 substrates and strain BA134^T used 22 substrates, with 12 commonly used substrates. As a general rule, phenotypic features were rated as positive when a signal was obtained, either weak or more pronounced.

In terms of phenotypic features, the strains could be differentiated from the most closely related species with a validly published name, *Cyclobacterium marinum* (Raj & Maloy, 1990), by cell morphology, salt dependence and tolerance, pH tolerance, nitrate reduction, hydrolysis of starch and abilities to produce acid from four carbohydrates and to use five carbonic acids (Table 1). Only those

phenotypic features that were expressed for both strains were used as differential features, i.e. variable features were not included.

Phylogenetic analysis based on 16S rRNA gene sequences revealed that the two *B. baltica* strains, BA1 and BA134^T, formed a very robust clade (all methods, 100 % of bootstrap replications; Fig. 1). The strains also formed a robust clade with *C. marinum* that was separate from any other described species. The Baltic Sea strains could therefore be recognized as members of the genus *Cyclobacterium*. On the other hand, the *B. baltica* strains were very distinct from *C. marinum*, with a low 16S rRNA gene sequence similarity of 90.8 % (133 sequence differences). Additionally, bacteria that originated from alkaline environments (e.g. bacterium ikaite c9, Soda lake bacterium Z3) are more closely related to the *B. baltica* strains than *C. marinum*, and form a robust clade.

In terms of 16S rRNA gene sequence similarity, strain BA134^T showed a very high similarity of 99.93 % to strain BA1. Sequence similarities with submitted sequences of either strains or clones always showed similarities of < 94 %. Highest similarity was found with the CFB group bacterium ikaite c9 (GenBank accession no. AJ431335; 93.6 % 16S rRNA similarity), which was isolated from alkaline ikaite tufa columns in Greenland (Stougaard *et al.*, 2002), and an unidentified bacterium from Hailaer soda lake in China (AF275712; 92.3 % 16S rRNA similarity). In terms of species with validly published names, highest similarity was found with *C. marinum* (M62788; 90.8 % 16S rRNA similarity) (Raj & Maloy, 1990; Woese *et al.*, 1990). All other species of

Table 1. Differential features of *Belliella baltica* strains and *Cyclobacterium marinum*

Taxa: 1, BA1; 2, BA134^T; 3, *C. marinum* [data from Raj & Maloy (1990), comprising strains *C. marinum* Raj^T (ATCC 25205^T), WH-A (ATCC 43824) and WH-B (ATCC 43825)]. Response: +, good response; w, weak response; –, no response.

Feature	1	2	3
Cell morphology	Rods, straight	Rods, straight	Vibroid, ringlike, spiral, coils
Cell size (µm)	0.4–0.5 × 0.9–2.2	0.3–0.4 × 1.5–3.0	0.3–0.7 × 0.8–1.5
Colony colour	Pink/orange	Pink/orange	Pink
G + C content (mol%)	35.5	35.3	34–38
Salinity (%):			
Growth range	0–6	0–6	1.5–15
Optimum	0–3.4	0–3.4	1.5–5
Temperature (°C):			
Growth range	4–33	4–37	4–40
Optimum	20–25	25–30	25
pH growth range	6–10	6–10	6–9
Nitrate reduction to nitrite	+	+	–
Hydrolysis of starch	+	+	–
Acid production from:			
D-Mannose	–	–	+
Rhamnose	–	–	+
Methyl α-D-glucoside	–	–	+
Cellobiose	w	+	–
Utilization of:			
Malate	–	–	+
Citric acid	–	–	+
D-Gluconic acid	–	–	+
Malonic acid	–	–	+
Succinic acid	–	–	+

the ‘*Flexibacteriaceae*’ with validly published names were related more distantly and showed 16S rRNA gene sequence similarities of < 89 %.

DNA–DNA hybridization between strains BA134^T and BA1 ranged from 72.9 to 76.0 %. According to Wayne *et al.* (1987), the DNA relatedness of the strains is thus still > 70 %, which is considered to be the lower limit for members of the same species. Based on DNA binding data and the high 16S rRNA gene sequence similarity of 99.93 %, we suggest that the two strains are members of the same species.

The DNA G + C contents of strains BA134^T and BA1 were 35.3 and 35.5 mol%, respectively (Table 1). The G + C contents of the related species *C. marinum* ranged from 34 to 38 mol% (Raj & Maloy, 1990; Gosink *et al.*, 1998) and are therefore within the same range as the strains of *B. baltica* described here.

Predominant fatty acids of the *B. baltica* strains were iso-C_{15:0} (18.9–22.5 %), iso-C_{15:0} G (10.1–10.3 %), iso-C_{17:1}ω9c (6.6–10.2 %), C_{16:1}ω7c (7.1–11.45 %) and C_{17:1}ω6c (4.8–9.8 %) (for details see Supplementary Table B, IJSEM Online). Fatty acids of minor contribution (1–5 % of total fatty acids) were C_{13:1} (at 12–13), iso-C_{14:0},

C_{15:0}, anteiso-C_{15:0}, iso-C_{15:0} 3-OH, C_{15:1}ω6c, iso-C_{16:0}, iso-C_{16:0} 3-OH, iso-C_{16:1} H, C_{16:1}ω5c, iso-C_{17:0} 3-OH, anteiso-C_{17:1} B, C_{17:1}ω6c and C_{17:1}ω8c. The cellular fatty acid composition of the *B. baltica* strains was dominated by branched-chain fatty acids, which formed 70 % of the total. This calculation is based on the assumption that the detected double peak (equivalent chain length, 15.19–15.48) represents C_{16:1}ω7c, which cannot be differentiated from iso-C_{15:0} 2-OH by the MIDI system. Otherwise, the fraction of iso-branching fatty acids would be even higher. Comparison of the *B. baltica* strains with *C. marinum* Raj^T (Urakami & Komagata, 1986) showed, for both species, a high fraction of iso-branched C_{15:0} fatty acids, but contrasted with respect to the high content of anteiso-C_{15:0} for *C. marinum* (*C. marinum*, 31.5 %; *B. baltica*, 4.5 %).

Of species with validly published names, strains BA1 and BA134^T are most closely related phylogenetically to *C. marinum* (Fig. 1). Phylogenetic analyses based on 16S rRNA gene sequences revealed, furthermore, that some alkaliphilic strains are affiliated more closely to the *B. baltica* strains than *C. marinum*. As for ikaite and soda lakes, the surface water of the Baltic Sea with its phytoplankton bloom can be regarded as an occasionally alkaline environment (pH 8.4, with higher pH values in the vicinity of algal cells)

due to alkalinization caused by HCO_3^- and/or NO_3^- uptake by algae (Giraldez *et al.*, 1998; Ullrich *et al.*, 1998). Due to the phylogenetic distance, the Baltic Sea strains were regarded as members of a different genus from *C. marinum*. These phylogenetic conclusions are furthermore supported by the fatty acid profiles, cell morphology and physiological features. Physiological features that differentiate the Baltic Sea strains from *C. marinum* are NaCl dependence for growth, salinity and pH ranges for growth, reduction of nitrate and spectrum of substrates utilized for growth and acid production. A major difference of the cellular fatty acids was the high content of anteiso- $\text{C}_{15:0}$, which was almost an order of magnitude higher for *C. marinum* than for *B. baltica*. Based on the results of this polyphasic approach for the novel Baltic Sea isolates, we propose a novel genus and species, *Belliella baltica* gen. nov., sp. nov. Strain BA134^T is proposed as the type strain of this novel species. The two strains described are considered to belong to the novel species *B. baltica* based on DNA–DNA similarity, 16S rRNA gene sequence comparison, fatty acid composition and phenotypic traits.

Description of *Belliella* gen. nov.

Belliella [Bel.li.el'la. N.L. fem. n. *Belliella* after the late aquatic microbiologist Russell Bell, University of Uppsala, in recognition of his work on aquatic bacteria (Bell *et al.*, 1983)].

Cells are Gram-negative, rod-shaped and oxidase- and catalase-positive. Growth is heterotrophic, aerobic and chemoheterotrophic. Predominant fatty acids are iso- $\text{C}_{15:0}$, iso- $\text{C}_{17:1\omega9c}$ and $\text{C}_{17:1\omega6c}$. Cells contain carotenoids, but no flexirubin. NaCl is not needed for growth, but good growth occurs at up to 3% NaCl. The genus *Belliella* belongs to the class 'Sphingobacteria' and the family 'Flexibacteriaceae'. The type species is *Belliella baltica*.

Description of *Belliella baltica* sp. nov.

Belliella baltica (bal'ti.ca. M.L. fem. adj. *baltica* from the Baltic Sea, referring to the source of the type strain).

Colonies are circular, smooth, convex and entire; they are pink and transparent when young, but become orange and opaque with ongoing incubation (>2 weeks at 20 °C on half-strength marine agar). Cells are Gram-negative, rod-shaped (width, 0.3–0.5 µm; length, 0.9–3.0 µm) and oxidase- and catalase-positive. Nitrate is reduced to nitrite. Temperature for growth is 4–37 °C, with an optimum around 25 °C. NaCl is not needed for growth; strains are tolerant of salinities up to 6%, with optimal growth between 0 and 3%. Strains grow at pH 6–10, with an optimum around neutral pH. Growth occurs on 0.5% yeast extract; aesculin, starch and DNA are hydrolysed. Acid is produced from L-arabinose, D-xylose, galactose, D-glucose, D-fructose, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, D-raffinose and starch. The following enzymic activities are present: α - and β -glucosidases,

β -galactosidase, acidic and alkaline phosphatases, lipase (C8), leucine, valine and cystine arylamidases, trypsin, chymotrypsin and naphthol phosphohydrolase. Glucose, arabinose and maltose are assimilated. D-Galactose, gentobiose, α -D-glucose, α -D-lactose, lactulose, maltose, D-trehalose, acetic acid, α -ketobutyric/glutaric/valeric acids and L-glutamic acid are utilized as substrates. DNA G+C content of the type strain is 35.4 mol%.

The type strain is BA134^T (=DSM 15883^T=LMG 21964^T=CIP 108006^T). Of marine or estuarine origin.

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