Muricauda ruestringensis gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment

Alke Bruns,[1] Manfred Rohde[2] and Luise Berthe-Corti[3]

[1,3] Institut für Chemie und Biologie des Meeres[1] and Fachbereich Biologie, Geo- und Umweltwissenschaften[3], C. v. O. Universität Oldenburg, PO Box 2503, D-26111 Oldenburg, Germany

[2] Bereich Mikrobiologie, Abt. Mikrobielle Pathogenität und Impfstoffforschung, GBF – Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-38124 Braunschweig, Germany

Author for correspondence: Luise Berthe-Corti. Tel: +49 441 798 3290. Fax: +49 441 798 3250. e-mail: Luise.Berthe.Corti@uni-oldenburg.de

A Gram-negative, facultatively anaerobic bacterium with appendages was isolated from continuous cultures with a seawater-sediment suspension containing hexadecane as the sole carbon source. Although this organism was isolated from a hexadecane-degrading bacterial community, it was not able to degrade hexadecane. However, this bacterium was able to use different sugars and amino acids for growth, indicating that it probably profits from the lysis or from products like surfactants of other cells in the community. 16S rDNA analysis demonstrated that the isolated strain is phylogenetically related to the family *Flavobacteriaceae* of the phylum '*Cytophaga–Flavobacterium–Bacteroides*'. Evidence based on phenotypic characteristics and 16S rDNA analysis supports the conclusion that this bacterium is distinct from its nearest relative, *Zobellia uliginosa* (90·72% similarity in 16S rRNA gene sequence), and from the other genera of the *Flavobacteriaceae*. It is therefore proposed that the isolated marine bacterium represents a novel taxon, designated *Muricauda ruestringensis* gen. nov., sp. nov. The type strain is strain B1[T] (= DSM 13258[T] = LMG 19739[T]).

INTRODUCTION

Members of the order [*Cytophagales*] are ubiquitous and probably play a major role in the turnover of organic matter, since they are able to degrade natural polymers, e.g. starch, cellulose or gelatin (Reichenbach, 1992). Many marine species belonging to this order can use cell-wall components and exudates from macroalgae as nutrient sources (Johansen *et al.*, 1999). In addition to enhanced nutrient concentrations when living next to or as an epiphyte of phytoplankton, phytoplankton is available as a surface for colonization (Bowman *et al.*, 1997b). The ecological importance of the [*Cytophagales*] is confirmed by *in situ* hybridization data that show the great abundance (20–70% of the total bacterial biomass) of members of this order in the coastal pelagic zone of Antarctica (Glöckner *et al.*, 1999). Members of this group were found in all marine and freshwater

samples investigated by Glöckner *et al.* (1999) and they usually formed the largest bacterial group in marine water. In recent studies, these bacteria have also been detected in intertidal sediments, where they represent a large part of the natural microbial community (Llobet-Brossa *et al.*, 1998; Bruns & Berthe-Corti, 1998). Most of the recently described genera of the *Flavobacteriaceae*, e.g. *Psychroserpens* and *Gelidibacter* (Bowman *et al.*, 1997a), *Psychroflexus* (Bowman *et al.*, 1998), *Polaribacter* (Gosink *et al.*, 1998) and *Cellulophaga* (Johansen *et al.*, 1999), were isolated from marine environments, demonstrating that this seems to be an important habitat for members of the [*Cytophagales*].

Many studies within the last two decades have demonstrated the high degree of biological diversity of the order [*Cytophagales*] with respect to phenotypic characterization, fatty acid composition and phylogenetic analysis (Johansen *et al.*, 1999). Reichenbach (1989a) was also aware of the confusing nomenclature when stating that 'at the moment we do not yet know enough to clearly recognize the boundaries of the

order *Cytophagales* or to define other orders'. Today, the diversity of the [*Cytophagales*] is accepted and this has resulted in several reclassifications. Many members of the genus *Cytophaga* have been reclassified within the genus *Flavobacterium* (Bernardet *et al.*, 1996). In contrast to the former description of *Flavobacterium* as containing non-motile and non-gliding species, the emended genus represents predominantly gliding bacteria (Bernardet *et al.*, 1996). Nakagawa & Yamasato (1996) revealed by 16S rDNA analysis that the genera *Cytophaga*, *Flavobacterium* and *Flexibacter* are polyphyletic. These authors suggested that *Cytophaga hutchinsonii* and *Cytophaga aurantiaca* are the only genuine *Cytophaga* species that are able to decompose crystalline cellulose. The second edition of *Bergey's Manual of Systematic Bacteriology* (http://www.cme.msu.edu/Bergeys/index.html), publication of which has begun in 2001, also takes these results into account and suggests the new orders '*Flavobacteriales*' and '*Sphingobacteriales*', while the order [*Cytophagales*] will no longer exist in the new edition.

We isolated and characterized several strains from a series of marine enrichment cultures. One of these strains, isolate B1[T], originates from intertidal sediment taken from the German North Sea coast that was cultivated in a continuous culture containing only hexadecane as a source of carbon and energy. Despite the added carbon source, strain B1[T] does not degrade hexadecane and seems to play another role in the community of the continuous culture. 16S rDNA analysis revealed that strain B1[T] is affiliated with the *Flavobacteriaceae* and its closest relative is *Zobellia uliginosa*. In this study, we describe the new strain and analyse its relationship to members of the *Flavobacteriaceae*.

METHODS

Strain cultivation. Strain B1[T] was isolated from a continuous-flow culture with sediment originating from the German Wadden Sea (Jadebusen Bay), as described previously (Bruns & Berthe-Corti, 1999). Cultivation was performed routinely on solid modified nutrient agar containing 0·25% (w/v) Bacto Peptone (Difco), 0·15% (w/v) yeast extract, 0·2% (v/v) hexadecane, 1·5% (w/v) agar in artificial seawater with reduced salinity (2·25%). The artificial seawater contained (l[-1] distilled water): 23·6 g NaCl; 0·64 g KCl; 4·53 g $MgCl_2.6H_2O$; 5·94 g $MgSO_4.7H_2O$; 1·3 g $CaCl_2.2H_2O$ (Levring, 1946). In order to avoid precipitation, the $CaCl_2.H_2O$ was sterilized separately. The pH of the medium was adjusted with $NaHCO_3$ to 7·2 and incubation was carried out at 25 °C. For some phenotypic characterizations, the organism was cultivated on several other media, which are described below.

Phenotypic characterization. The bathochromic shift test with 20% (w/v) KOH was performed to detect flexirubin-type pigments (Fautz & Reichenbach, 1980). For this purpose, some biomass was deposited on a slide and covered with KOH. If the test is positive, the colour of the biomass changes from yellow to red, purple or red–brown when KOH is added and back to yellow again when the latter is replaced with 10% (v/v) HCl. The ability to grow with different

carbon sources was analysed in a seawater medium (SWM; Bruns & Berthe-Corti, 1999) supplemented with 0·1% (w/v) of the tested carbon source or with strips of filter paper for testing cellulolytic activity. Growth with peptone as the sole carbon source was performed on a solid medium containing 0·25% (w/v) peptone in artificial seawater of 2·25% salinity, pH 7·2. Agar degradation was studied on SW2 agar (Reichenbach, 1992). Acid production from fructose was studied as described by Smibert & Krieg (1994) using Hugh & Leifson's O/F medium (containing only NaCl and KH_2PO_4 as salts) and with Leifson's modified O/F medium, which contains artificial seawater. Analysis of gliding motility was performed on a low-concentration nutrient medium containing 0·052% (w/v) peptone, 0·03% (w/v) meat extract and 0·9% (w/v) agar in artificial seawater (2·25% salinity), pH 7·2 at 30 °C, under high moisture conditions, as recommended by Wolkin & Pate (1984). Starch and gelatin hydrolysis were analysed using PAC medium (see fatty acid analysis) supplemented with 0·2% (w/v) starch or 0·4% (w/v) gelatin, respectively. Indole production was tested as described by Smibert & Krieg (1994) using PAC medium supplemented with 0·01% (w/v) L-tryptophan. Production of H_2S was studied as described previously (Smibert & Krieg, 1994) using a medium containing 0·25% (w/v) peptone, 0·15% (w/v) meat extract, 0·015% (w/v) sodium thiosulfate, 0·027% (w/v) $FeSO_4.7H_2O$ and 1·5% (w/v) agar in artificial seawater of 2·25% salinity, described above. Tolerance of different NaCl concentrations was tested in the synthetic medium described by Gauthier *et al.* (1992), which contained 1·23% (w/v) Tris, 0·37% (w/v) $MgSO_4.7H_2O$, 0·15% (w/v) $CaCl_2$ and 0·075% (w/v) KCl. All of the inoculated media described above were incubated at 30 °C.

Further tests used to characterize the phenotype of the strain have been described in a previous paper (Bruns & Berthe-Corti, 1999).

In order to examine their denitrification ability, cells were grown anaerobically under a helium atmosphere in a modified liquid nutrient broth containing 0·3% acetate and 0·1% (w/v) KNO_3 (nitrate was labelled with the ^{15}N isotope). The formation of nitrite was tested after 4, 8, 13 and 17 d of incubation at 30 °C using MERCKOQUANT sticks (Merck). The production of gaseous ^{15}N nitrogen compounds was analysed on a type MAT 95 mass spectrophotometer (Finnigan MAT), applying the electron-ionization method (electron energy 70 eV, source temperature 220 °C; Budzikiewicz, 1998).

Electron microscopy. For negative staining, samples were absorbed onto carbon film, washed in TE buffer (0·02 M Tris/HCl, 0·001 M EDTA, pH 6·9), stained with 4% (w/v) aqueous uranyl acetate (pH 4·5) according to the method of Valentine *et al.* (1968) and picked up with 300-mesh copper grids. After air-drying, samples were examined in a Zeiss EM910 or EM109 transmission electron microscope (TEM) at an acceleration voltage of 80 kV and at calibrated magnifications.

For scanning electron microscopy (SEM), strain B1[T] was fixed on ice for 1 h in a fixation solution containing 3% (v/v) formaldehyde and 2% (v/v) glutaraldehyde. After three washing steps with PBS, samples were absorbed onto poly-L-lysine-coated cover slips and fixed with 3% (v/v) glutaraldehyde in TE buffer for 10 min at room temperature, washed with TE buffer, dehydrated in a graded series of acetone and subjected to critical-point drying with liquid CO_2. Samples were then covered with an approximately 10 nm thick gold film and examined with a field emission SEM (Zeiss DSM

982 Gemini) using the Everhart Thornley SE detector and the inlens detector at a ratio of 50:50.

For TEM, samples were fixed with the same fixation solution, pelleted and embedded into 1·5% (w/v) agar in cacodylate buffer (0·1 M sodium cacodylate, 0·09 M sucrose, 0·005 M CaCl$_2$, pH 6·9). Samples were fixed and contrasted with 1% (w/v) osmium tetroxide for 1 h at room temperature and 4% (w/v) uranyl acetate overnight. After dehydration with a graded series of ethanol, samples were embedded in LRWhite resin. After polymerization of the resin (50 °C, 1–2 d), ultrathin sections were cut with a diamond knife, counterstained with lead citrate and uranyl acetate and examined in a Zeiss EM910 microscope.

For the determination of anionic sites on the bacterial surface and appendages, fixed samples were treated with TE buffer, pH 2·5, for 10 min and then incubated with cationic gold (gold particle size of 15 nm) for 15 min at room temperature, washed with the TE buffer and absorbed onto carbon film. The carbon film was picked up with 300-mesh copper grids and samples were stained with 4% (w/v) aqueous uranyl acetate before examination in the TEM.

Fatty acid analysis. To obtain biomass for fatty acid analysis, strain B1^T was grown in a liquid PAC medium containing: 0·1% (w/v) CH$_3$COCOONa, 0·416% (w/v) CH$_3$COONa, 0·1% Casamino acids, 3 ml vitamin solution (Schlegel, 1992), 1 ml mineral salt solution (DSMZ medium no. 124), 0·146% (w/v) NaNO$_3$ and 0·14 g Na$_2$HPO$_4$ in artificial seawater of 2·25% salinity, pH 7·2. The incubation was performed at 30 °C. To prepare the esters for GC analysis, harvested cells were saponified, methylated, extracted and washed with 0·24 M NaOH according to the procedure of the SHERLOCK microbial identification system (MIDI). The extracted fatty acid methyl esters were chromatographed on an apolar capillary column (HP-Ultra2, 25 m, 0·22 mm; Hewlett Packard) with helium as the carrier gas. The analysis was carried out with an HP 5890 series II gas chromatograph (split-less injection, temperature between 170 and 310 °C, flame-ionization detector) with peak integration by the HP3365 ChemStation software. Esters were identified using known internal standards.

DNA base composition. Genomic DNA was extracted from strain B1^T and then analysed using the HPLC method as described by Mesbah *et al.* (1989).

16S rDNA sequencing and analysis. The 16S rDNA gene sequence was determined by Sequence Laboratories Göttingen (Seqlab, Germany). The genomic DNA was extracted as described by Sambrook *et al.* (1989). For amplification of the 16S rDNA, primers M13Fgm3F (5′-TGTAAAACGACGGCCAGTTAGAGTTTGATCM-TGGC-3′) and M13Rgm4R (5′-CAGGAAACAGCTAT-GACCTTACCTTGTTACGACTT-3′) were used, which were coupled to the 16S rDNA-specific primers GM3F (positions 8–24 according to *Escherichia coli* numbering) and GM4R (positions 1492–1507) (Muyzer *et al.*, 1995). PCR amplification was performed with 35 cycles of 96 °C for 20 s, 60 °C for 60 s and 72 °C for 90 s. The sequencing reaction was performed using the ABI Dye Terminator technology according to the manufacturer (Applied Biosystems). An Applied Biosystems model 377 DNA sequencer was used for electrophoresis of the sequence reaction mixtures.

The 16S rDNA sequence was compared to sequences from GenBank using the program BLASTN 2.0 (Altschul *et al.*,

1997) available through the National Center for Biotechnology Information (NCBI) internet site. Sequences of other members of the *Flavobacteriaceae* were downloaded and aligned with the 16S rDNA sequence of strain B1^T using CLUSTAL W (Thompson *et al.*, 1994).

Evolutionary distances were calculated by the method of Jukes & Cantor (1969) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the program MEGA (Kumar *et al.*, 1994).

RESULTS

Cell and colony morphology

Strain B1^T is a Gram-negative, non-motile organism. Cells grown in PAC medium were rod-shaped with rounded ends, 0·3–0·6 μm wide and 1·1–2·7 μm long (Fig. 1a). Ultrathin sections showed the typical ultrastructure of a Gram-negative bacterium (Fig. 1g). One main characteristic, however, was the formation of holes in the outer membrane in older cultures (Fig. 1h). Cells of older cultures (3–4 d) were characterized by long and relatively thick (20–35 nm), mostly polar-located appendages (Fig. 1a–c; Fig. 2). However, not all of the cells produced such appendages. Appendages were often characterized by vesicle-like structures at the end, which exhibited fibrillar-like structures on the surface (Fig. 1c). Ultrathin sections revealed that the appendages are formed as a continuum of the outer membrane (Fig. 1i) and are covered by a membrane (Fig. 1f). We assume that these appendages are used to connect cells with each other (Fig. 1a), since we observed that cells grew mostly in small flocks in older cultures. Another explanation might be that these structures are used for colonization of a given substratum, because strain B1^T was able to colonize glass beads easily. The use of cationic gold particles allowed us to determine whether or not anionic components of the outer membrane are involved in the formation of appendages. During the early formation of appendages (Fig. 1d), we could demonstrate that more anionic sites were exposed in this process compared with anionic sites on the remaining cell surface. Furthermore, the entire appendage and the vesicle-like structure at its end was covered with numerous anionic sites (Fig. 1e).

This organism produced colonies (diameter 0·5–1·0 mm) on modified nutrient agar that were yellow in colour, circular and convex in shape with regular edges. Strain B1^T has no flexirubin-type pigment, as determined by the bathochromic shift test. Colonies with shapes other than those with a regular edge but with a halo of fine hairy roots were observed only after 3 weeks of incubation on low-concentration nutrient medium under high-moisture conditions. This halo contains cells. However, the colonies do not show the typical spread shape of the edges, as demonstrated for gliding cells of [*Flexibacter*] *filiformis* and *Flavobacterium columnare* (formerly [*Cytophaga columnaris*]) (Reichenbach, 1992). Cells were non-motile in hanging-drop preparations; they showed
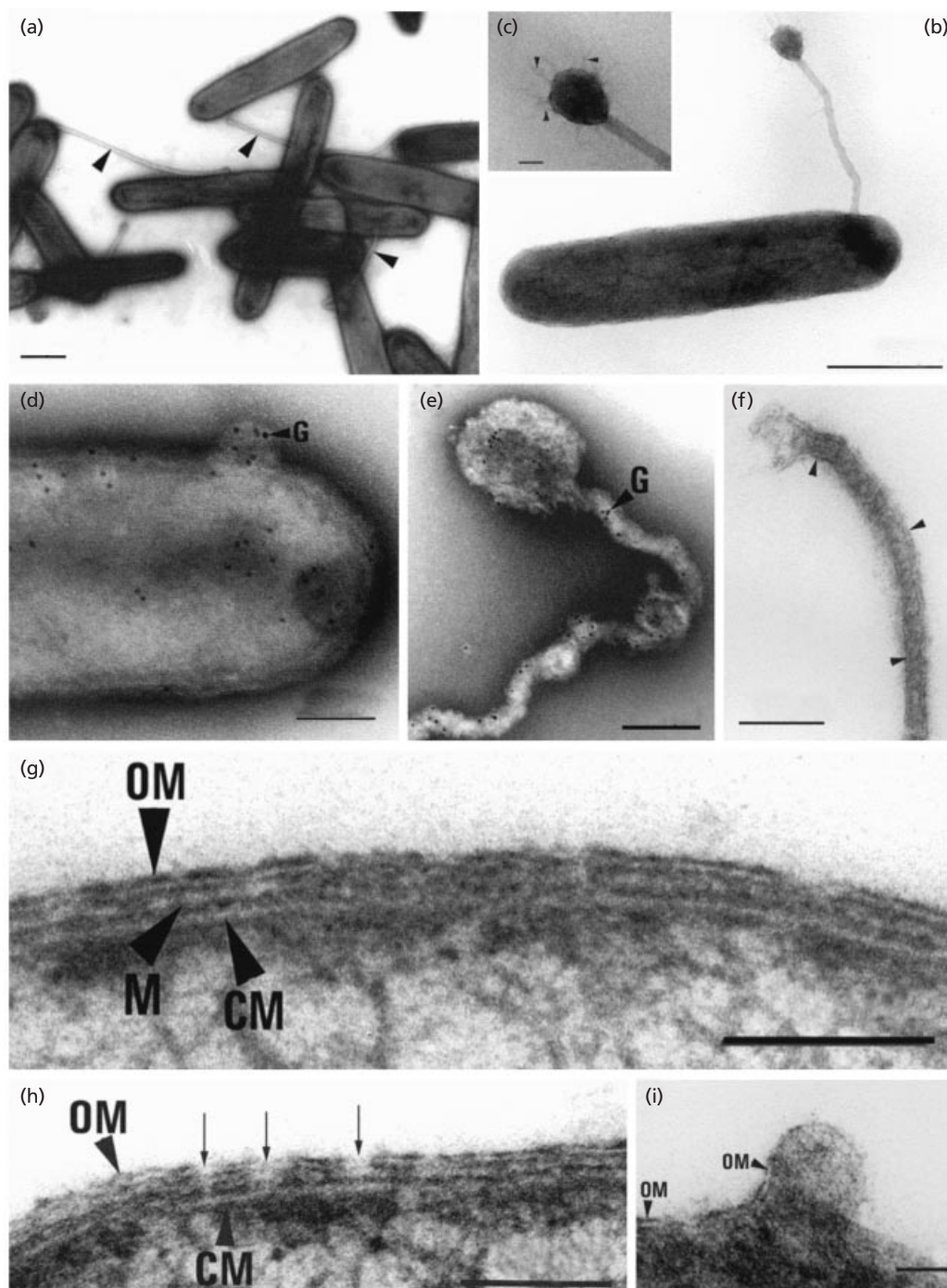
Fig. 1. Transmission electron micrographs showing the cell morphology and ultrastructure of *M. ruestringensis* strain B1[T]. (a)–(e) Negative staining of cells. Arrowheads mark appendages, which can be seen in small flocks of the bacteria (a). (b) and (c) show an appendage on a single cell and the characteristic vesicle-like structure at the end with fibrillar-like structures. Arrowheads in (d) and (e) show localization of anionic structures with cationic gold particles (G) on the bacterial surface and the appendages. (f)–(i) Ultrathin sections showing ultrastructural features. (g) Typical Gram-negative arrangement of the cell periphery of a young bacterial cell with a cytoplasmic membrane (CM), murein (M) and outer membrane (OM). Older cultures were characterized by several holes in the outer membrane (arrows in h). Appendages are formed at first as a continuum of the outer membrane (i) and exhibit a typical membrane structure when fully grown (f). Bars, 0·5 (a, b), 0·2 (d, e), 0·1 (f–h) and 0·05 (c, i) µm.
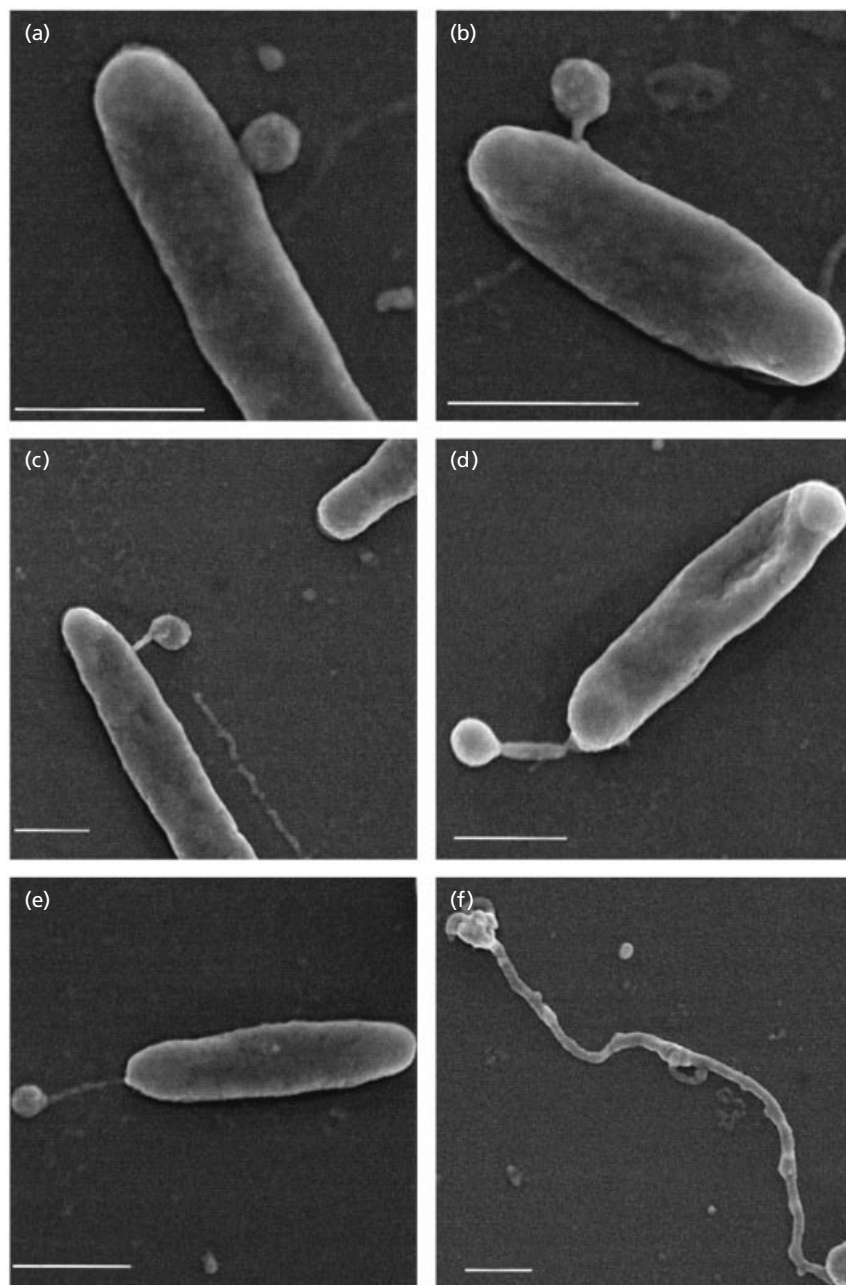
Fig. 2. Scanning electron micrographs of appendage formation in *M. ruestringensis* strain B1[T]. Appendage formation probably starts with the building of vesicle-like structures (blebs) on the bacterial surface (a), which then grow behind the vesicle-like structures up to a length of approximately 5 μm (b–f). Bars, 0·5 μm.

neither swimming nor gliding motility as defined by Henrichsen (1972).

Growth properties

Strain B1[T] is a facultatively anaerobic, seawater-requiring chemoheterotroph. The organism is able to grow aerobically and grows weakly under anaerobic conditions. It is slightly halophilic, showing good growth in media containing 0·5–9·0 % (w/v) NaCl, with optimal growth at 3 % (w/v) NaCl. The pH tolerance ranges between pH 6·0 and 8·0; the optimum for growth is pH 6·5–7·5. The strain has a wide temperature range for growth, showing growth between 8 and 40 °C, with optimal growth between 20 and 30 °C.

Physiology and metabolism

The physiological characteristics of strain B1[T] are summarized in Tables 1 and 2. The organism was isolated from a continuous-flow culture, containing

Table 1. Growth of strain B1^T with different carbon sources

Growth is scored as: +, turbidity; −, no turbidity. The amino acid mixtures contained the following amino acids (each at 200 µM): 1, alanine, arginine, asparagine and cysteine; 2, glutamine, glutamic acid, glycine and histidine; 3: isoleucine, leucine, lysine and methionine; 4, phenylalanine, proline and serine; 5, tryptophan, tyrosine and valine; 6, all 19 amino acids contained in mixtures 1–5.

Substrate	Growth
Raffinose	+
Cellobiose	+
Sucrose	+
Lactose	+
Glucose	−
Fructose	+
Mannose	+
Acetate	−
Pyruvate	−
Alanine	+
Arginine	+
Serine	−
Ethanol	−
Mannitol	−
Hexadecane	−
Amino acid mixtures:	
1	+
2	+
3	+
4	+
5	+
6	+

mud-flat sediment and hexadecane as the sole source of carbon at a low oxygen concentration (0·4 % DOT, equivalent to 0·8 µmol O$_2$ l^{-1}). However, it showed no growth with hexadecane as the sole source of carbon. Hexadecane was also not used when it was offered in combination with other carbon sources such as lactose, fructose or a mixture of the 20 amino acids. Furthermore, strain B1^T was unable to use pyruvate or acetate when these compounds were offered as the sole carbon source. The other substrates that strain B1^T used for growth are listed in Table 1. It was able to grow with a wide spectrum of different amino acids as sole carbon and energy sources. In contrast to related halophilic organisms of the *Flavobacteriaceae*, strain B1^T was not able to degrade high-molecular-mass carbohydrates like starch or agar (Table 2). Moreover, it showed no proteolytic properties in hydrolysing gelatin, but it was able to grow with peptone as the sole carbon and nitrogen source.

Under strictly anoxic or suboxic conditions, strain B1^T showed no production of the gaseous nitrogen compounds N$_2$, N$_2$O or NO (analysed by mass spec-

trometry) when incubated in liquid nutrient broth. Nitrate was not consumed and not reduced to nitrite. Nevertheless, strain B1^T grew in liquid nutrient broth under anoxic conditions. Aerobic growth was tested in Hugh & Leifson's O/F medium and Leifson's modified O/F medium (Smibert & Krieg, 1994). Strain B1^T only showed growth and weak acid production with the modified O/F medium, which, in contrast to Hugh & Leifson's medium, contains artificial seawater (Smibert & Krieg, 1994). This indicates that this bacterium is capable of metabolizing fructose with acid production and that it requires the salts of artificial seawater.

Fatty acid profile

The composition of the fatty acid profile of strain B1^T was as follows: 13:1 (1·50 %), i15:1 (16·29 %), a15:1 (0·55 %), i15:0 (15·52 %), a15:0 (2·40 %), 15:0 (1·08 %), 2-OH i15:0 and 16:1ω7c (2·54 %), 16:0 (1·10 %), 3-OH i15:0 (4·88 %), 2-OH 15:0 (0·46 %), i17:1ω9c (1·01 %), 3-OH 15:0 (0·62 %), 3-OH i16:0 (2·73 %), 3-OH 16:0 (2·91 %), 3-OH i17:0 (28·70 %), 2-OH 17:0 (2·83 %) and unidentified fatty acids (14·86 %). The fatty acid profile of strain B1^T is characterized by high proportions of the branched fatty acids 13-methyl tetradecenoic acid, 13-methyl tetradecanoic acid and 3-hydroxy-15-methyl hexadecanoic acid, which represented 60·51 % of the total fatty acids. It is important to note that 72·1 % of the whole-cell fatty acids were of the branched type.

DNA base composition

The G+C content of the DNA is 41·4 mol %, as determined by the HPLC method.
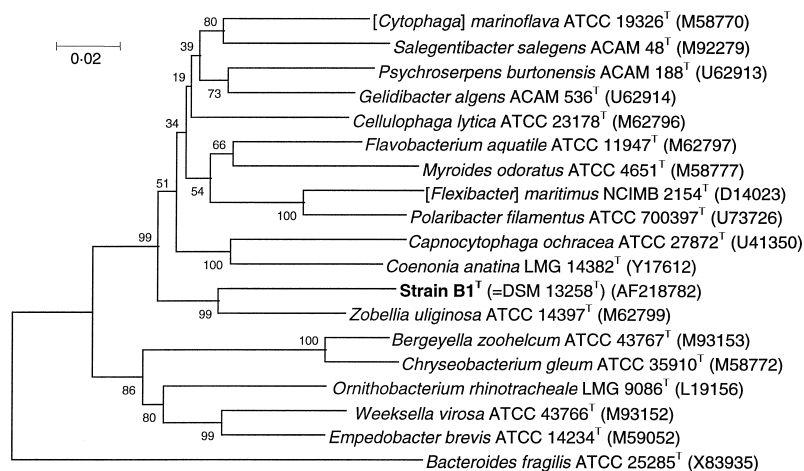
Phylogeny

The phylogenetic relationships of the strain were examined using the almost complete 16S rDNA sequence (1426 bp) determined by the Sequence Laboratories Göttingen. A comparison of the sequence with sequences from GenBank demonstrated that strain B1^T belongs to the family *Flavobacteriaceae* within the phylum 'Cytophaga–Flavobacterium–Bacteroides'. This was confirmed by whole-cell hybridization with the 16S rRNA-directed oligonucleotide probe CF319a (target position 319–336, 5′-TGGTCCGTGTCTCAGTAC-3′) developed by Manz et al. (1996).

The dendrogram shown in Fig. 3 was constructed by the neighbour-joining method (Saitou & Nei, 1987). It shows that the branch containing *Zobellia uliginosa* and strain B1^T is related to the other members of the *Flavobacteriaceae*. The similarity between the 16S rDNA sequences of *Zobellia uliginosa* and strain B1^T was 90·72 %. The other members of the *Flavobacteriaceae* are even more distantly related to strain

Table 2. Phenotypic characteristics that differentiate strain B1^T from other halophilic members of the *Flavobacteriaceae*

Taxa are identified as: 1, *Muricauda ruestringensis* sp. nov. B1^T; 2, *Zobellia uliginosa*; 3, *Cellulophaga lytica*; 4, [*Flexibacter*] *maritimus*; 5, *Salegentibacter salegens*. Characters are scored as: +, positive reaction; −, negative reaction; NA, no data available. Data were taken from this study (strain B1^T), Reichenbach (1989b) (*Zobellia uliginosa*, *Cellulophaga lytica* and [*Flexibacter*] *maritimus*) and Dobson *et al.* (1993) and McCammon & Bowman (2000) (*Salegentibacter salegens*). All taxa are oxidase-positive.

Character	1	2	3	4	5
Cell length (μm)	1·1–2·7	1·2–4·0	1·5–3·5	2·0–5·0	1·2–11·5
Cell width (μm)	0·3–0·6	0·4	0·4	0·4	0·5–0·8
Pigment colour	Yellow	Golden yellow	Bright yellow	Yellow-orange	Yellow
Flexirubin reaction	−	+	−	NA	−
Gliding motility	+	+	+	+	NA
Growth on peptone alone	+	+	+	+	NA
Hydrolysis of:					
Gelatin	−	+	+	+	+
Starch	−	+	+	−	+
Cellulose (filter paper)	−	−	−	−	NA
Agar	−	+	+	NA	−
Production of:					
Indole	−	−	NA	−	NA
H$_2$S	−	−	+	−	−
Nitrate reduced	−	+	−	+	+
Strict aerobe	−	+	+	+	+
Facultative anaerobe	+	−	−	−	−
Catalase	−	+	+	+	+
Optimum temperature for growth (°C)	20–30	20–30	22–30	30	NA
Highest NaCl concentration tolerated (%)	9	NA	6	NA	20
DNA G+C content (mol%)	41	42	33	31	41

Fig. 3. Phylogenetic dendrogram showing the relationships of *Muricauda ruestringensis* sp. nov. to members of the family *Flavobacteriaceae* and other related organisms. GenBank accession numbers and strain culture accession numbers are shown in parentheses. Bootstrap percentages indicated at the branches were calculated from 1000 trees. *Bacteroides fragilis* served as the outgroup. Bar, sequence dissimilarity value of 2%.

B1^T, with less than 86% sequence similarity (the second closest relative is *Gelidibacter algens*, 86·05%).

DISCUSSION

The most striking results of the phenotypic analysis are the appendages of strain B1^T (Figs 1 and 2), which are formed as a continuum of the outer membrane. These appendages are morphologically similar to those described for *Flavobacterium aquatile* strain ATCC 11947^T (Thomson *et al.*, 1981) and for *Flexibacter* strain BH3 (Humphrey *et al.*, 1979). Humphrey *et al.* (1979) revealed that the vesicular-tubular material was derived from the lipopolysaccharide membrane and the authors speculated as to whether the extruded material had any function in gliding, adhesion or slime

excretion. The only member of the *Flavobacteriaceae* that possesses flagella is *Polaribacter irgensii*, but motility has never been observed in wet mounts (Gosink *et al.*, 1998). In hanging-drop preparations or in wet mounts, strain B1^T also did not show motility. We observed neither a swimming motility nor a gliding motility as defined by Henrichsen (1972). Thus, the function of the appendages still has to be investigated in detail. We assume the connection of cells with each other as one possible function of the appendages (Fig. 1a). This may explain why the strain forms flocks when growing in PAC medium. When cells were provided with surfaces for growth, such as glass beads, they seemed to prefer colonizing these surfaces to living freely in the medium. Adhesion to surfaces and flock formation may be a selective advantage for cells growing in intertidal habitats, which are characterized by considerable water currents. Marshall *et al.* (1971) suggested that extracellular material may play a role in the initial contact with a surface by providing a means of polymer bridging between the cell and the surface. This may support our assumption.

The phylogenetic tree (Fig. 3) reveals a distant relationship between *Zobellia uliginosa* (formerly [*Cellulophaga*] *uliginosa*, Barbeyron *et al.*, 2001; [*Cytophaga*] *uliginosa*, Bowman, 2000) and strain B1^T. The stability of this group was supported by the high bootstrap value of 98 %. However, the rather low sequence similarity of 90·7 % argues in favour of classifying these two organisms into separate genera. In addition, these strains are morphologically and physiologically different. As shown in Table 2, strain B1^T is a facultatively anaerobic organism, while *Zobellia uliginosa* has a strictly aerobic respiratory metabolism.

Strain B1^T is well adapted to the environment from which it was isolated. The intertidal mud-flat zone of the North Sea is characterized by a sharp drop from oxic to anoxic conditions in the sediment within a few centimetres. Thus, the ability to switch between an aerobic and an anaerobic metabolism is very useful. In addition, its wide temperature range for growth indicates a good adaptation of strain B1^T to the environmental conditions of the mud-flat zone. The temperature changes in summer occur cyclically with the tides and lie between about 17 °C when the flats are covered with water and 60 °C when they dry out and are exposed to the sun.

In contrast to *Zobellia uliginosa*, strain B1^T is not able to reduce nitrate, has no pigment of the flexirubin type and is not able to degrade high-molecular-mass carbohydrates such as starch or agar. In common with all members of the emended family *Flavobacteriaceae* (Bernardet *et al.*, 1996), it is not able to degrade crystalline cellulose (filter paper).

When supplied with a mixture of amino acids (Casamino acids as given in PAC medium) or a mixture of 19 amino acids (Table 1), strain B1^T showed good growth. However, with mixtures of only four amino acids, with alanine or arginine as substrate, growth was considerably weaker. This organism presumably prefers to use some specific amino acids for growth; this remains to be tested in further studies. The bacterium is able to grow anaerobically when supplied with peptone and yeast extract. It would be quite interesting to determine whether strain B1^T ferments amino acids by the Stickland reaction (Buckel, 1991), which would explain why it showed good growth with a mixture of amino acids but weak growth with single amino acids. The absence of acid fermentation when strain B1^T was growing anaerobically with peptone and yeast extract (data not presented) may support this assumption, since the Stickland reaction is characterized by the production of NH_4^+ and an acid, resulting in a neutral pH. This has to be investigated further.

The fact that strain B1^T is able to use sugars and amino acids for growth but not hexadecane, even though the strain was isolated from a culture with hexadecane as the sole source of carbon, demonstrates that it probably profits from the lysis or from secondary products like surfactants of other cells in the hexadecane-degrading community.

Kaneda (1991) reported the existence of two different fatty acid families, a straight-chain fatty acid family and a branched-chain fatty acid family. Bacteria with the straight-chain membrane system usually require unsaturated fatty acids for growth, while this is not the case for bacteria with the branched-chain membrane system. Strain B1^T has the latter membrane system. It possesses extremely large proportions of branched-chain fatty acids (72·1 % of the whole-cell fatty acids). This has also been reported for several phylogenetic relatives of strain B1^T, e.g. [*Cytophaga*] *latercula*, with 88·4 % branched-chain fatty acids, *Gelidibacter algens* (71·4 %), *Cellulophaga lytica* (formerly [*Cytophaga*] *lytica*) (70·9 %; Bowman *et al.*, 1998) and *Capnocytophaga ochracea* (83·0 %; Vandamme *et al.*, 1996). Comparison of the fatty acid profile of strain B1^T with those of other members of the *Flavobacteriaceae* (Bowman *et al.*, 1998; Bowman, 2000) reveals that the nearest relative is *Cellulophaga lytica*, which has almost the same major fatty acid profile. This clearly confirms that strain B1^T belongs to the family *Flavobacteriaceae*. Moreover, according to the fatty acid profile, this strain seems to be closely related to the genus *Cellulophaga*, members of which, however, have quite different physiological traits (Table 2). Furthermore, the phylogenetic data do not support such a classification.

To sum up, strain B1^T cannot be assigned phylogenetically to an existing genus. Its nearest relative, *Zobellia uliginosa*, and members of the other genera of the *Flavobacteriaceae* exhibit distinct characteristics that are not shared completely by the novel strain. Therefore, we propose that the novel strain B1^T belongs to a new genus and species within the family *Flavobacteriaceae*, for which we propose the name *Muricauda ruestringensis* gen. nov., sp. nov.

Description of *Muricauda* gen. nov.

Muricauda (Mu′ri.cau.da. L. masc. gen. n. *muris* of the mouse; L. fem. n. *cauda* the tail; N.L. fem. n. *Muricauda* tail of the mouse, referring to the cellular appendages observed on some cells).

Gram-negative, rod-shaped cells. Facultatively anaerobic. Cells can grow optimally over a mesophilic temperature range between 20 and 30 °C. Seawater is required for growth. The fatty acid profile is characterized by large amounts of branched fatty acids. According to 16S rDNA analysis and binding of the rRNA to the oligonucleotide CF319a, *Muricauda* is a member of the family *Flavobacteriaceae* within the phylum 'Cytophaga–Flavobacterium–Bacteroides'.

Description of *Muricauda ruestringensis* sp. nov.

Muricauda ruestringensis (rue.strin′gen.sis. N.L. adj. *ruestringensis* of Rüstringen, referring to the former village of Rüstringen, which was destroyed by a tidal wave in 1362, near the site of which the type strain was isolated).

The cells are Gram-negative rods, 0·3–0·6 µm wide and 1·1–2·7 µm long. Cells of older cultures (3–4 d) are characterized by long and relatively thick (20–30 nm) appendages, which seem to be used to connect cells to each other. Cells are non-motile in hanging-drop preparations. Colonies are circular, 0·5–1·0 mm in diameter and yellow-pigmented. The strain has no flexirubin-type pigment. The temperature for growth ranges from 8 to 40 °C, with optimal growth at 20–30 °C. Strain B1^T requires the salt components of artificial seawater and grows at NaCl concentrations of 0·5–9·0 % (w/v), with an optimum at 3 % (w/v). *M. ruestringensis* is a facultatively anaerobic organism, being able to grow anaerobically with complex carbon sources. No ability to break down high-molecular-mass carbohydrates like starch, agar or crystalline cellulose is observed. The strain shows no denitrifying properties and no proteolytic properties in hydrolysing gelatin, but does show good growth with peptone as the sole carbon and nitrogen source. It is catalase-negative but oxidase-positive. Major cellular fatty acids are i15:1, i15:0 and 3-OH i17:0. The G+C content of the DNA is 41 mol%.

The type strain is strain B1^T (= DSM 13258^T = LMG 19739^T).

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