

Robiginitalea biformata gen. nov., sp. nov., a novel marine bacterium in the family *Flavobacteriaceae* with a higher G + C content

Jang-Cheon Cho and Stephen J. Giovannoni

Department of Microbiology, Oregon State University, Corvallis, OR 97331, USA

Correspondence

Stephen J. Giovannoni
steve.giovannoni@
oregonstate.edu

Two Gram-negative, chemoheterotrophic, non-motile, rust-coloured, marine strains were isolated from the western Sargasso Sea by high-throughput culturing. Characterization of the two strains by polyphasic approaches indicated that they are members of the same species. Phylogenetic analyses based on 16S rRNA gene sequences using three treeing algorithms revealed that the strains formed a coherent and novel genus-level lineage within the family *Flavobacteriaceae*. The dominant fatty acids were branched or hydroxy acids, i15:0, i15:1 and 3-OH i17:0 being the most abundant. The higher DNA G + C content of the strains (55–56 mol%) clearly differentiated them from other genera of the family *Flavobacteriaceae* (27–44 mol%). It is proposed, from the polyphasic evidence, that the strains be placed into a novel genus and a novel species named *Robiginitalea biformata* gen. nov., sp. nov., with strain HTCC2501^T (=ATCC BAA-864^T=KCTC 12146^T) as the type strain.

The family *Flavobacteriaceae* (Reichenbach, 1989; Bernardet *et al.*, 2002) is one of the main phylogenetic lineages within the phylum 'Bacteroidetes' (Garrity & Holt, 2001), members of which generally comprise 6–30% of total bacterial communities in sea water, as determined by fluorescence *in-situ* hybridization (Glöckner *et al.*, 1999; Eilers *et al.*, 2000; Cottrell & Kirchman, 2000). According to the emended description of the family *Flavobacteriaceae* by Bernardet *et al.* (2002), members of the family are Gram-negative, non-spore-forming, short to moderately long rods that cannot degrade crystalline cellulose. Their major cellular fatty acids are branched or hydroxy fatty acids, with high levels of i15:0 and 3-OH i17:0, and the DNA G + C contents range from 27 to 44 mol%. In the last few years, polyphasic approaches (Vandamme *et al.*, 1996) powered by 16S rRNA gene sequence-based phylogeny have rapidly expanded the discovery and description of members of the family *Flavobacteriaceae*, including *Gelidibacter* and *Psychroserpens* (Bowman *et al.*, 1997), *Polaribacter* (Gosink *et al.*, 1998), *Psychroflexus* (Bowman *et al.*, 1998), *Salegentibacter* (McCammon & Bowman, 2000), *Cellulophaga* (Johansen *et al.*, 1999), *Arenibacter* (Ivanova *et al.*, 2001), *Muricauda* (Bruns *et al.*, 2001), *Aequorivita* (Bowman & Nichols, 2002), *Gillisia* (Van Trappen *et al.*, 2004), *Vitellibacter*, *Ulvibacter* and *Mesonina* (Nedashkovskaya *et al.*, 2003a, b, 2004) and *Croceibacter* (Cho & Giovannoni,

2003a), most of which were cultivated from marine environments. We have isolated and characterized several novel marine strains within the family *Flavobacteriaceae* from a series of high-throughput culturing (HTC) experiments. In this study, we describe two of these strains, HTCC2501^T and HTCC2514, as members of a new genus in the family *Flavobacteriaceae*. The two strains were isolated from the Sargasso Sea (Atlantic Ocean), using HTC, and were maintained on Marine agar 2216 (Difco) at 30 °C as described previously (Cho & Giovannoni, 2003a).

Phylogeny

16S rRNA gene fragments were generated by a PCR and directly sequenced as described by Cho & Giovannoni (2003a). Nearly full-length sequences of the 16S rRNA gene, 1434 and 1454 bp for strains HTCC2501^T and HTCC2514, respectively, were aligned using the ARB software package (Ludwig *et al.*, 1998) and 1169 unambiguously aligned nucleotide positions were used for phylogenetic analyses in PAUP* 4.0 beta 10. Phylogenetic trees were inferred by neighbour-joining with the Kimura two-parameter model, maximum parsimony and maximum likelihood (tree bisection-reconnection-branching; a transition/transversion ratio of 1.326). The tree topologies from neighbour-joining and maximum parsimony were evaluated by bootstrap analyses based on 1000 resamplings. The sequence similarity between strains HTCC2501^T and HTCC2514 was 99.99% (only 2 nucleotide differences), so they were considered to be the same species using this criterion as well as on the basis of their DNA relatedness (94.6 ± 3.9%; for methods, see Cho & Giovannoni, 2003b). None of the taxa with validly

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The GenBank accession numbers for the 16S rRNA gene sequences of strains HTCC2501^T and HTCC2514 are AY424899 and AY424900.

published names showed more than 90% 16S rRNA gene sequence similarity to the strains. The two HTCC isolates formed a distinct lineage within the family *Flavobacteriaceae* (Fig. 1), the most closely related genera being *Zobellia* (88.6–89.7%), *Arenibacter* (88.3–88.4%), *Aequorivita* (88.3–89.2%) and *Vitellibacter* (87.8–88.0%), and the lineage did not associate significantly with any of the described genera in the family. The branching orders and phylogenetic relationships between the strains and *Muricauda–Zobellia–Arenibacter–Aequorivita–Vitellibacter* were well conserved in all three phylogenetic trees (Fig. 1). The robustness of the phylogenetic relationships and the low sequence similarities between the strains and the other genera demonstrate that the two novel HTCC isolates represent a new genus in the family *Flavobacteriaceae*.

Phenotypic characteristics

Tests used for phenotypic characterizations were performed as described in previous studies (Cho & Giovannoni, 2003a, b), except that the incubation temperature used was 30 °C. Biochemical tests were carried out using API 20NE strips (bioMérieux). Custom-made 48-well microplates containing 47 different carbon compounds at a final concentration of 0.2% (w/v or v/v) were used to test sole-carbon-source utilization. Cell morphology varied clearly with the growth stage of the cells. Cells in early exponential phase (1–2 days

incubation) were predominantly straight rods, 1.6–5.6 µm long and 0.3–0.7 µm wide, while those in stationary phase (5–8 days incubation) tended to transform into coccoid cells that were 0.6–1.2 µm in diameter (Fig. 2). This pleomorphic property has been reported for other genera of the family *Flavobacteriaceae*, including *Gelidibacter* and *Psychroserpens* (Bowman *et al.*, 1997). Colonies on Marine agar 2216 were 1.0–2.0 mm in diameter, rusty orange-coloured, uniformly circular, pulvinate, opaque and possessed a smooth surface. Gliding motility and flagella were not observed. They did not grow under either strictly anaerobic conditions or microaerobic conditions. The temperature range for growth was 10–44 °C, with optimum growth at 30 °C. No growth was observed at 4 or 48 °C. The pH range for growth was pH 6.0–9.0 (optimum 8.0–8.5). The isolates were moderately halophilic, showing good growth at NaCl concentrations of 0.25–10% (w/v; optimum 2.5%). Both strains produced carotenoid pigments with wavelength absorbance spectral peaks at 339 and 457 nm, with a major peak at 457 nm. The bathochromic shift test results indicated that flexirubin pigments were absent. Other phenotypic results for biochemical characteristics, sole-carbon-source utilization, antibiotic susceptibility and degradation of macromolecules are given in Table 1 and in the species description.

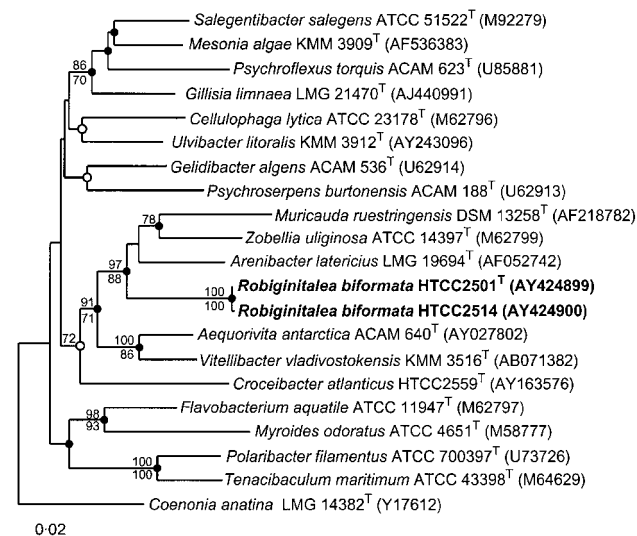


Fig. 1. Neighbour-joining 16S rRNA-based phylogenetic tree showing relationships between the marine HTCC isolates and representatives of the family *Flavobacteriaceae*. Bootstrap proportions above 70% from both neighbour-joining (above nodes) and maximum parsimony (below nodes) are shown. Closed and open circles respectively indicate nodes recovered reproducibly by all treeing methods and by two treeing methods. *Coenonia anatina* LMG 14382^T (Y17612) was used as an outgroup to define the root of the tree. Bar, 0.02 substitutions per nucleotide position.

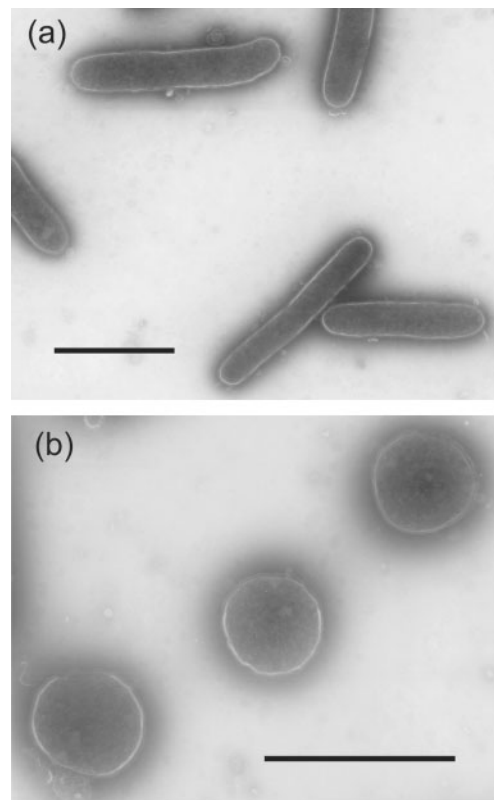


Fig. 2. Electron micrographs of negatively stained cells of strain HTCC2501^T in exponential phase (a) and stationary phase (b). Bars, 2.0 µm.

Table 1. Phenotypic characteristics of *Robiginitalea biformata* gen. nov., sp. nov. HTCC2501^T and HTCC2514

Where two results are given, they refer to strains HTCC2501^T/HTCC2514. Otherwise, the two strains show the same response.

Characteristic	<i>R. biformata</i> (n=2)
Biochemical properties (API 20NE)	
Catalase, oxidase, aesculin hydrolysis, β -galactosidase	+
Denitrification, indole production, glucose acidification, arginine dihydrolase, urease, gelatin hydrolysis	–
Sole-carbon-source utilization	
D-Ribose, D-xylose, D-glucose, D-fructose, L-sorbose, D-mannose, sucrose, β -lactose, D-trehalose D-cellobiose, D-maltose, D-melibiose, D-raffinose, N-acetyl D-glucosamine, succinic acid, propionic acid, lactic acid, L-glutamate, L-ornithine, L-proline, L-serine	+
DL-Glyceraldehyde, D-arabinose, D-galactose, L-rhamnose, D-sorbitol, adonitol, methanol, ethanol, glycerol, pyruvic acid, itaconic acid, citric acid, gluconic acid, D-malic acid, malonic acid, formic acid, L-alanine, L-lysine, D-glucosamine, L-alanine, L-leucine, L-isoleucine, glycine, L-arginine	–
D-Melezitose	+/-
D-Mannitol, m-inositol	-/+
Degradation of macromolecules	
Starch	+
Aesculin, dextran, cellulose, alginate, chitin, carrageenan, casein, elastin, gelatin, DNA	–
Susceptibility to antibiotics	
Nalidixic acid, tetracycline, erythromycin, rifampicin, benzylpenicillin	+
Chloramphenicol, kanamycin, carbenicillin, streptomycin, ampicillin, puromycin, vancomycin, gentamicin, cycloheximide	–
Fatty acid composition (%)*	
15:0	5.5
16:0	1.3
i15:0	26.0
a15:0	3.7
i15:1	17.2
i17:0	0.9
2-OH 17:0	1.2
3-OH i15:0	4.3
3-OH i17:0	25.8
2-OH i15:0 + 16:1 ω 7c	3.5

*Fatty acids comprising more than 1 % of the total content are shown. The following fatty acids were rarely present in the strains: 14:0, i13:0, a15:1, i16:0, i16:1, a17:0, i17:1 ω 9c, 15:1 ω 6c, 16:1 ω 5c, 17:1 ω 8c, 17:1 ω 6c, 18:1 ω 9c, 3-OH i14:0, 2-OH 15:0, 3-OH 15:0, 3-OH 16:0, 3-OH i16:0 and 3-OH 17:0.

Chemotaxonomy

The DNA G + C content was analysed by HPLC according to Mesbah *et al.* (1989), using a Platinum EPS reverse-phase C18 column (Alltech Associates), and phage λ DNA was used as a standard throughout the analyses. The DNA G + C contents of strains HTCC2501^T and HTCC2514 were respectively 56.4 ± 0.2 and 54.7 ± 0.4 mol%. The G + C content of the strains was at least 10 mol% higher than that of other members of the family *Flavobacteriaceae* (Table 2). Cellular fatty acid methyl esters were prepared and analysed using GC according to the instructions of the Microbial Identification System (MIDI). The predominant fatty acids in the strains were i15:0 (24–28%), i15:1 (14–21%) and 3-OH i17:0 (25–27%) (Table 1). The major fatty acid types were branched acids and hydroxy acids, which comprised 75.7–77.9% of total fatty acids. The fatty acid profiles of the

two strains were almost identical to each other. Although the fatty acid profile of the HTCC isolates was similar to that of *Muricauda ruestringensis*, it differed significantly from those of the other phylogenetically related genera in the family *Flavobacteriaceae* (Table 2).

Taxonomic conclusions

The two strains showed almost the same phenotypic characteristics except for differences in the utilization pattern for three carbon compounds, and shared very similar genotypic characteristics, such as >99% 16S rDNA sequence similarity and >90% DNA–DNA hybridization, so they were regarded as members of the same species (Wayne *et al.*, 1987). Polyphasic taxonomy based on 16S rRNA gene sequence analyses (Fig. 1) and phenotypic characteristics together with chemotaxonomic data (Tables 1 and

Table 2. Differential characteristics of *R. biformata* from phylogenetically related species in the family *Flavobacteriaceae*

Species: 1, *Robiginitalea biformata* gen. nov., sp. nov. (this study); 2, *Muricauda ruestringensis* (Bruns *et al.*, 2001); 3, *Zobellia uliginosa* (Barbeyron *et al.*, 2001; Reichenbach, 1989); 4, *Arenibacter latericius* (Ivanova *et al.*, 2001); 5, *Aequorivita antarctica* (Bowman & Nichols, 2002); 6, *Vitellibacter vladivostokensis* (Nedashkovskaya *et al.*, 2003a); 7, *Croceibacter atlanticus* (Cho & Giovannoni, 2003a). Symbols: +, positive; -, negative; V, variable; -, not detected; ND, not determined.

Characteristic	1	2	3†	4	5	6	7
Flexirubin	-	-	+	-	-	+	-
Gliding motility	-	-	+	-	-	-	-
Reaction to oxygen*	A	F	A	A	A	A	A
Growth at 42 °C	+	-	+	-	-	+	-
Nitrate reduction	-	-	+	+	-	-	-
Oxidase/catalase production	+ / +	+ / -	+ / +	+ / +	ND / +	+ / +	- / +
β -Galactosidase	-	ND	+	V	-	-	-
Glucose acidification	-	-	+	ND	-	-	-
Degradation of:							
Starch	+	-	V	-	+	-	+
Aesculin	+	ND	+	-	+	ND	-
Gelatin	-	-	+	-	+	+	+
DNA	-	ND	+	-	-	+	+
Cellulose	-	-	-	-	-	-	-
Urea	-	ND	-	+	V	-	+
Arginine	-	+	-	-	-	ND	+
Major fatty acids (%):							
15:0	5-6	1.1	10.4	13.3	0.6	3.7	3.9
i15:0	24-28	15.5	38.2	17.3	7.6	68.8	13.3
a15:0	3-4	2.4	-	6.6	15.7	8.4	1.6
i15:1	14-21	16.3	8.7	19.3	9.5	2.4	9.1
a15:1	0.3-0.6	0.6	-	1.7	16.0	-	-
16:1 ω 7c	-	-	9.8‡	11.0	7.8	0.1	-
3-OH i15:0	4.3	4.9	-	-	5.4	1.6	4.3
3-OH i16:0	0.9	2.8	-	-	9.2	-	4.4
3-OH i17:0	25-27	28.7	16.1	-	2.0	0.8	28
G+C content (mol%)	55-56	41.4	42	37-38	38-39	41.3	34.8

*A, Obligate aerobe; F, facultative anaerobe.

†Grown on Marine agar 2216 for fatty acid analysis.

‡16:1 ω 7c+2-OH i15:0.

2) demonstrated that the two HTCC strains represent a coherent and novel genus within the family *Flavobacteriaceae*. The phenotypic and genotypic characteristics of the strains generally met the minimal standards for the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Furthermore, comparisons of phenotypic and chemotaxonomic characteristics between the phylogenetically associated genera indicated clearly that the isolates could not be placed in any of the previously described genera (Table 2). The property of the strains that best differentiated them from other members of the family *Flavobacteriaceae* was the DNA G + C content, which was more than 10 mol% higher than those of the other genera within the family. In view of all the data presented in this study, the novel strains belong to a new genus and species within the family *Flavobacteriaceae*; therefore, we propose for them the name *Robiginitalea biformata* gen. nov., sp. nov.

Description of *Robiginitalea* gen. nov.

Robiginitalea (Ro.bi.gi.ni.tal'e.a. L. gen. n. *robiginis* of rust; L. fem. n. *talea* a rod; N.L. fem. n. *Robiginitalea* a rust-coloured rod).

Gram-negative. Morphology varies from straight rods, in exponential phase, to coccoid cells, in stationary phase. Cells do not exhibit motility or gliding motility. Oxidase- and catalase-positive. Carotenoid pigments are produced, but flexirubin pigments are not produced. Obligately aerobic and chemoheterotrophic. NaCl is required for growth. Starch and aesculin are degraded. The major fatty acid types are branched acids and hydroxy acids. The predominant fatty acids are i15:0 (24-28%), i15:1 (14-21%) and 3-OH i17:0 (25-27%). DNA G + C content is 55-56 mol%. Phylogenetically, a novel member of the family *Flavobacteriaceae*. The type and only species of the genus is *Robiginitalea biformata*.

Description of *Robiginitalea biformata* sp. nov.

Robiginitalea biformata (bi.for.ma'ta. L. fem. adj. *biformata* double-formed, two-shaped, pertaining to the different cell morphology in different growth phases).

Description is the same as that for the genus with the following additional properties. Cells in exponential phase are straight rods, 1.6–5.6 µm long and 0.3–0.7 µm wide. Cells in stationary phase are coccoid, 0.6–1.2 µm in diameter. Endospores and poly-β-hydroxybutyrate granules are not formed. Colonies are rusty-orange-coloured, circular, pulvinate and opaque. Growth occurs at 10–44 °C, but not at 4 or 48 °C. The pH and salinity ranges for growth are 6.0–9.0 and 0.25–10% (w/v), respectively. Moderately halophilic. Maximum absorption spectral peak occurs at 457 nm. No denitrification activity detected. A variety of carbon compounds are used as sole carbon sources, including pentoses, hexoses, oligosaccharides, sugar acids and amino acids. Cellular fatty acid composition, biochemical characteristics, sole-carbon-source utilization, antibiotic susceptibility and degradation of macromolecules are detailed in Table 1. The DNA G+C content of the type strain, HTCC2501^T, is 56.4 ± 0.2 mol% (HPLC method). Strains have been isolated from the western Sargasso Sea, Atlantic Ocean.

The type strain is HTCC2501^T (=ATCC BAA-864^T = KCTC 12146^T).

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