

Tenacibaculum skagerrakense sp. nov., a marine bacterium isolated from the pelagic zone in Skagerrak, Denmark

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A number of bacteria were isolated from sea water in Skagerrak, Denmark, at 30 m depth. Two of the isolates, strains D28 and D30^T, belonged to the *Flavobacteriaceae* within the *Cytophaga–Flavobacterium–Bacteroides* group. Sequencing of 16S rRNA genes of the two strains indicated strongly that they belonged to the genus *Tenacibaculum* and that they showed greatest similarity to the species *Tenacibaculum amylolyticum* and *Tenacibaculum mesophilum*. DNA–DNA hybridization values, DNA base composition and phenotypic characteristics separated the Skagerrak strains from the other species within *Tenacibaculum*. Thus, it is concluded that the strains belong to a novel species within the genus *Tenacibaculum*, for which the name *Tenacibaculum skagerrakense* sp. nov. is proposed, with strain D30^T (= ATCC BAA-458^T = DSM 14836^T) as the type strain.

Members of the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group are assumed to be important in the degradation of polymeric organic matter because of their ability to produce hydrolytic enzymes (Cottrell & Kirchman, 2000; Giovannoni & Rappé, 2000). Members of the CFB group are widespread in the marine environment and are often associated with surfaces where they may benefit from their hydrolytic activity (Cottrell & Kirchman, 2000; Giovannoni & Rappé, 2000; Johansen *et al.*, 1999; Suzuki *et al.*, 2001). In addition to attached forms, the CFB group also includes pelagic (free-living) marine species (Giovannoni & Rappé, 2000; Gosink *et al.*, 1998; Pinhassi *et al.*, 1997).

The taxonomy of the CFB group has had a turbulent history since the proposal of the genus *Cytophaga* (Winogradsky, 1929). In recent years, studies on phylogenetic characterization have led to several reclassifications and emended descriptions of species within this group. Bernardet *et al.* (1996) presented a new description of the family *Flavobacteriaceae* and rearranged the taxonomy of

the genus *Flavobacterium*. However, the generic relationship of members of the presumed [*Flexibacter*] *maritimus* rRNA branch was left open, which inspired a reclassification of several existing genera and descriptions of new genera, including *Psychroserpens* and *Gelidibacter* (Bowman *et al.*, 1997), *Psychroflexus* (Bowman *et al.*, 1998), *Polaribacter* (Gosink *et al.*, 1998), *Cellulophaga* (Johansen *et al.*, 1999; Bowman, 2000), *Salegentibacter* (McCammon & Bowman, 2000), *Zobellia* (Barbeyron *et al.*, 2001) and *Tenacibaculum*. The latter genus contains the former [*Flexibacter*] *maritimus*, which has been reclassified to *Tenacibaculum maritimum*, the type species of the genus (Suzuki *et al.*, 2001).

In the present study, two strains, D28 and D30^T, related to the CFB group were isolated from a sea-water sample taken in the pelagic zone at 30 m depth in Skagerrak (57° 96' N 10° 78' E), Denmark. Skagerrak is a water body between Denmark and Norway that is strongly influenced by the North Atlantic Ocean. The strains were isolated on 10%-strength ZoBell agar plates (ZoBell, 1946) from a 0.8 µm-filtered water sample (salinity 35 p.s.u., temperature 9 °C). Unless otherwise stated, full-strength ZoBell medium was used in all studies. The medium contained (l⁻¹): 5.0 g Bacto peptone, 1.0 g yeast extract, 0.015 g FePO₄·4H₂O and 35 g sea salts (Sigma-Aldrich) l⁻¹. For solidification of the medium, 1.5% (w/w) agar was added.

Colony size, shape and colour were determined after 5 days incubation at 20 °C on ZoBell plates. Following acridine orange staining (Hobbie *et al.*, 1977), photomicrographs of

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Abbreviations: AZCL, azurine-cross-linked; CFB, *Cytophaga–Flavobacterium–Bacteroides*.

The GenBank accession numbers for the 16S rDNA sequences of strains D30^T and D28 are respectively AF469612 and AF469613.

Detailed Biolog test results for the novel strains are available as supplementary material in IJSEM Online.

cells in exponential phase (overnight cultures incubated at 20 °C) and stationary phase (3 days incubation at 20 °C) were recorded with an Axiovert 100 TV microscope (Zeiss) fitted with an AT200 CCD camera. To test for gliding motility, the hanging drop method (Perry, 1973; Suzuki *et al.*, 2001) was applied. Briefly, 20 µl drops of cell culture in full-strength and 1% ZoBell medium were placed on microscope cover slips and potential gliding motility was examined at ×1000 magnification. Lysis with 3% KOH (Gregersen, 1978) was used to determine whether the isolates were Gram-positive or negative.

To study growth at different temperatures, overnight cultures were washed once (8200 g, 12 min, 10 °C) in ZoBell medium followed by streaking of 1 µl cell suspensions on ZoBell agar plates to obtain single colonies. The plates were incubated at 5, 10, 15, 20, 25, 30, 37, 40 and 45 °C for 9 days. To test the salt tolerance of the strains, ZoBell plates were prepared containing 0, 5, 10, 15, 20, 25, 30, 35, 40, 50 or 60 g sea salts l⁻¹ or 35 g NaCl l⁻¹. NaCl-containing plates were used to test whether micronutrients were required for growth. Growth at different pH values was studied on ZoBell plates in which pH was adjusted with HCl or NaOH to pH 4, 5, 6, 7, 8 and 9. Temperature resistance was tested by heating exponentially growing cells to 40, 50, 60, 70, 80, 90 and 100 °C for a maximum of 30 min, followed by streaking of subsamples on ZoBell plates. The plates were subsequently incubated at 30 °C for 7 days before the presence of colonies was examined.

Catalase activity was detected by the presence of bubbles after addition of one drop of 3% H₂O₂ to colonies growing on solid ZoBell medium. Assays for cytochrome oxidase activity were considered positive when cells formed blue pigments after being streaked on filter paper wetted with 1% *N,N,N',N'*-tetramethyl-*p*-phenyldiamine dihydrochloride (Barrow & Feltham, 1993). Flexirubin pigment was detected by colour shift when bacteria growing on solid ZoBell medium were exposed to a 20% (w/v) KOH solution (Reichenbach, 1992). Growth on 0.4% (w/v) DL-aspartate, L-leucine and sucrose was determined in a basal medium containing (l⁻¹) 35 g sea salts (Sigma-Aldrich), 0.2 g NaNO₃, 0.2 g NH₄Cl and 0.05 g yeast extract according to Suzuki *et al.* (2001). Overnight cultures were washed three times in 1.5% (w/v) sea salt solution and cells were inoculated to an OD₅₉₀ of 0.1 in the growth medium. Bottles were incubated for 2 days at 20 °C and growth was followed spectrophotometrically. Growth in basal medium served as a control.

To test the ability of the strains to hydrolyse polymeric substrates, ZoBell plates were amended with 1% (v/v) CM-chitin-RBV (Loewe Biochemica) or 1% (w/v) of the following substrates: skimmed milk, starch and chromomeric azurine-cross-linked (AZCL) amylose, glucan, hydroxyethyl cellulose, curdlan, debranched arabinan, dextran, galactomannan, galactan, pullulan, xyloglucan, xylan, arabinoxytan, casein and collagen (all from Megazyme). Plates were incubated for 6 days at 30 °C and observed daily

for colour reactions (AZCL substrates) or clearing zones (chitin, skimmed milk and starch) around colonies. Clearing zones on starch-amended media were detected after dripping Lugol solution (Fluka) on the surface of the plates.

Degradation of crystalline cellulose was tested by spotting 20 µl cell culture on Whatman 1 filter paper and lens-cleaning tissues placed on full-strength or 1% ZoBell solid medium. Plates were incubated at 20 °C for 2 weeks and examined visually for dissolution of the cellulose by formation of translucent spots in the filter paper and lens-cleaning tissue. Growth on microcrystalline cellulose (Avicel; Merck) was tested in liquid basal medium containing 35 g sea salts and 0.67 g cellulose l⁻¹. Bottles were incubated for 2 weeks at 30 °C and growth was followed spectrophotometrically.

Oxidation of 113 different carbon sources was determined using duplicate Biolog GN and triplicate GP plates as recommended by the supplier. Prior to Biolog characterization, overnight cultures were washed three times in 1.5% (w/v) sea salts. Each well in the Biolog plates was inoculated with 150 µl cell suspension to an OD₄₅₀ of 0.5, after which the plates were incubated at 30 °C. Development of purple colour in the wells, caused by reduction of tetrazolium dye, was detected with a 96-well plate reader at 590 nm.

The DNA base composition (mol% G+C) was analysed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) by HPLC as described by Mesbah *et al.* (1989), Tamaoka & Komagata (1984) and Visuvanathan *et al.* (1989). Likewise, DNA-DNA hybridizations were performed by the DSMZ at 61 °C in 2 × SSC (1 × SSC is 0.15 M NaCl plus 15 mM sodium citrate) as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). Approximately 95% of the total 16S rDNA sequence of strain D30^T was determined by the DSMZ by direct sequencing of PCR-amplified 16S rDNA. Genomic DNA extraction, PCR amplification of the 16S rDNA and purification of PCR products were carried out as described by Rainey *et al.* (1996). A partial 16S rDNA sequence for strain D28 was obtained by extracting DNA by boiling. DNA encoding 16S rRNA was amplified by PCR using primers F1 and R13 (Dorsch & Stackebrandt, 1992) and purified using the QIAquick PCR purification kit (Qiagen). A partial sequence of the 16S rDNA was obtained by sequencing the PCR product in both directions using primer F1 and a primer (5'-CGTATTACCGCGGCTGCT) modified after Lane *et al.* (1985). Sequences were aligned in BIOEDIT (Hall, 1999) against sequences from the Ribosomal Database Project II (Maidak *et al.*, 2000). A phylogenetic tree was constructed by the neighbour-joining method (Jukes & Cantor, 1969) including *Flexibacter flexilis* as an outgroup. Ambiguous bases were excluded from the analysis. A tree and a bootstrap analysis with 500 sample replications were performed in TREECON for Windows (Van de Peer & De Wachter, 1994).

Strains D28 and D30^T were both Gram-negative and did

not produce flexirubin pigments. Colonies were convex, circular with spreading edges and bright yellow on ZoBell agar plates with 35 g sea salts l⁻¹. After 5 days of incubation at 20 °C, colonies were 5–20 mm in diameter, depending on colony closeness. Exponentially growing cells were long and thread-like whereas stationary phase cultures were dominated by rods and spherical forms (not shown). Gliding motility was not observed.

Growth was observed at temperatures between 10 and 40 °C, while optimal growth occurred between 25 and 37 °C. At 40 °C, relatively few but large colonies appeared. No growth was detected at 50 °C and above; however, cells that had been exposed to 50 °C remained viable and grew well when returned to 30 °C. The strains grew well at pH 6–8, whereas no growth was observed after 6 days at pH 5. Relatively few but large colonies appeared at pH 9. No growth occurred at sea salt concentrations below 10 g l⁻¹ (40 g sea salts l⁻¹ equals 100%-strength sea water). The strains grew well at sea-salt concentrations between 15 and 40 g l⁻¹, while growth was slow at 50 g l⁻¹ and very limited at 60 g l⁻¹. No growth was observed on plates containing 35 g NaCl l⁻¹, indicating that sea salts were required for growth.

Strains D28 and D30^T both showed catalase and oxidase activity and their Biolog carbon respiration profiles were almost identical (details results available as supplementary material in IJSEM Online). Both strains utilized all the amino acids, except D-alanine, L-histidine, L-phenylalanine and D-serine, and grew on most of the polymers. Approximately one-third of the carbohydrates and carboxylic acids were utilized; however, D30^T was capable of utilizing one more carbohydrate (D-melezitose), four more carboxylic acids (γ -aminobutyric acid, D-glucosaminic acid, malonic acid, quinic acid) and one more polymer (mannan) than D28. Almost none of the D-isomers, except for α -D-glucose (both strains) and D-melezitose and D-glucosaminic acid (strain D30^T), supported growth, and none of the amino sugars, amines, glucosides, brominated compounds, alcohols or carbohydrates with an alcoholic group was used by any of the two strains.

The test of Suzuki *et al.* (2001) for the genus *Tenacibaculum* demonstrated growth of both strains on aspartate and sucrose in basal medium with 0.05 g yeast extract l⁻¹, while weak growth was found on leucine (Fig. 1). The hydrolytic profiles of the strains were also identical, as they hydrolysed the same proteins (skimmed milk, AZCL casein and AZCL collagen) and showed positive reactions for glucose polymer α -1,4-glucoside bonds (starch, AZCL α -amylose and AZCL pullulan) and β -1,4-glucoside bonds (AZCL hydroxyethyl cellulose, AZCL barley β -glucan). None of the other tested substrates (CM-chitin-RBV, AZCL debranched arabinose, AZCL arabinoxylan, AZCL curdlan, AZCL dextran, AZCL galactan, AZCL galactomannan; AZCL xylan, AZCL xyloglucan) was hydrolysed by either strain. No degradation or growth on crystalline cellulose by D30^T was observed.

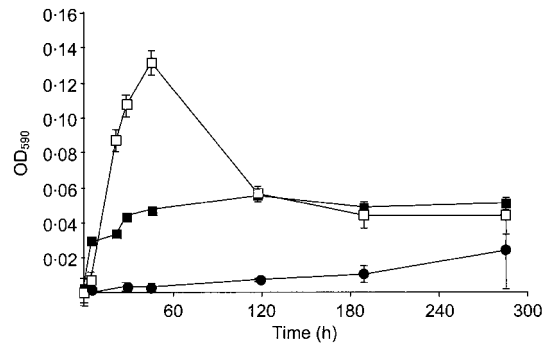


Fig. 1. Growth of strain D30^T on sucrose (□), aspartate (■) and leucine (●). Values were corrected for basal medium OD. Error bars represent ± 1 SD ($n=3$). Strain D28 showed similar growth patterns.

Sequence similarity of 100% was observed between the overlapping part of the 16S rDNA sequences of strains D28 (449 bp) and D30^T (1465 bp). The 16S rDNA sequence similarity between D30^T and the type strains of the two most distantly related *Tenacibaculum* species, *Tenacibaculum ovolyticum* IAM 41318^T and *T. maritimum* NCIMB 2154^T, was respectively 95.4 and 94.0%, i.e. below the 97% limit suggested for species identity (Schloter *et al.*, 2000; Stackebrandt & Goebel, 1994). The similarity values between D30^T and *Tenacibaculum mesophilum* MBIC 1140^T and *Tenacibaculum amylolyticum* MBIC 4355^T, on the other hand, were respectively 98.1 and 97.7%. Thus, a DNA–DNA hybridization test was necessary to determine the relationship between D30^T and these species (Schloter *et al.*, 2000; Stackebrandt & Goebel, 1994). This showed that the DNA–DNA relatedness between D30^T and *T. mesophilum* MBIC 1140^T was 35.9%, while that for *T. amylolyticum* MBIC 4355^T was 34.3%. Since the DNA relatedness values were well below the suggested 70% limit for species identity (Wayne *et al.*, 1987), we propose that D30^T represents a novel species within the genus *Tenacibaculum*. The position of D30^T among other members of the family *Flavobacteriaceae* is shown in Fig. 2.

The association of D30^T and D28 with the genus *Tenacibaculum* was further supported by shared colony morphologies and phenotypic characteristics (Table 1). Some differences were observed, however. The G+C content of strain D30^T (35.2 mol%) was higher than those reported for any of the other *Tenacibaculum* species (30.3–32.5 mol%). In addition, unique characters of D28 and D30^T were endocellulase activity, growth on sucrose, high salinity tolerance, formation of spherical cells in stationary phase, lack of Tween 80 metabolism and the formation of bright yellow colonies (Table 1).

The genus name *Tenacibaculum* means ‘rod-shaped bacterium that adheres to surfaces’, and all hitherto-reported species within the genus *Tenacibaculum* have been isolated from surfaces of marine organisms (Suzuki *et al.*, 2001).

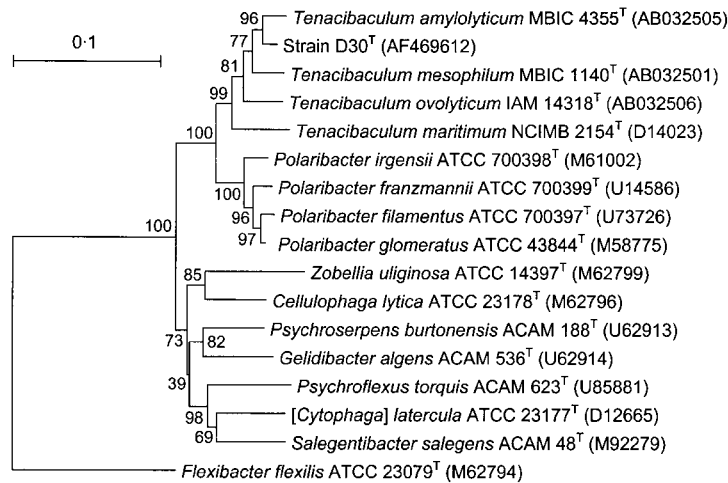


Fig. 2. Phylogenetic tree showing the relationship of strain D30^T to other members of the family *Flavobacteriaceae*. The topology of the tree is based on the neighbour-joining method. Bootstrap percentages of 500 replicates are indicated at the branches. *F. flexilis* ATCC 23079^T was used as outgroup. Sequence accession numbers are shown in parentheses.

T. mesophilum MBIC 1140^T was isolated from a Japanese sponge homogenate, *T. amylolyticum* MBIC 4355^T was isolated from Japanese macroalgae (Suzuki *et al.*, 2001), *T. maritimum* NCIMB 2154^T was isolated from a diseased red sea bream fingerling in Japan (Wakabayashi *et al.*, 1986)

and *T. ovolyticum* IAM 41318^T was isolated from halibut eggs in Norway (Hansen *et al.*, 1992). In contrast, strains D28 and D30^T appeared to be free-living, as they were isolated from filtered (0.8 µm) sea water. However, they were capable of hydrolysing several polymeric compounds,

Table 1. Differential characteristics between strains D28 and D30^T and the other species within the genus *Tenacibaculum*

Species: 1, *T. skagerrakense* sp. nov. D28 and D30^T; 2, *T. amylolyticum* ($n=1$); 3, *T. mesophilum* ($n=4$); 4, *T. ovolyticum* ($n=3$); 5, *T. maritimum* ($n=2$). Data for reference species were taken from Suzuki *et al.* (2001). ND, No data; W, weak growth.

Characteristic	1	2	3	4	5
Origin	Pelagic (Denmark)	Macroalgae (Japan)	Sponge and macroalgae (Japan)	Halibut egg (Norway)	Diseased red sea bream fingerling (Japan)
Cell size (µm)	0.5 × 2–15	0.4 × 2–4	0.5 × 1.5–10	0.5 × 2–20	0.5 × 2–30
Colony morphology:					
Shape	Circular, spreading edge	Circular, spreading edge	Irregular, spreading edge	Regular edge	Uneven edge
Diameter at 5 days (mm)	5–20	23–27	30–60	ND	<5
Colour	Bright yellow	Yellow	Yellow	Pale yellow	Pale yellow
Spherical cells	Frequent	Very rare	Very rare	ND	ND
Salinity range: (%)					
NaCl	No growth	3 (W)	1–7	No growth	No growth
Sea salts*	25–150	50–100	10–100	70–100	30–100
pH range	6–9	5.3–8.3	5.3–9	5.9–8.6	5.9–8.6
Temperature range	10–40	20–35	15–40	4–25	15–34
Growth on:					
L-Leucine	W	–	–	–	–
Sucrose	+	–	–	–	–
DL-Aspartate	+	–	+	–	–
Degradation of:					
Chitin	–	–	–	+	–
Starch	+	+	–	–	–
Tween 80	–	+	+	+	+
Endocellulase activity	+	ND	ND	ND	ND
Nitrate reduction	+	W	–	+	+
DNA G+C content (mol%)	35.2	30.9	31.6–32.0	30.3–32.0	31.3–32.5

*Values are percentages of sea-water strength.

as would be expected for surface-associated bacteria (Kirchman & Mitchell, 1982; Smith *et al.*, 1992). Substantial release of bacteria from sinking particles to the surrounding water in pelagic marine environments has recently been demonstrated (Azam & Long, 2001; Riemann & Winding, 2001). Hence, prior to isolation, D28 and D30^T could have been attached to particles.

The closest phylogenetic relative of strains D28 and D30^T is *T. amylolyticum*, characterized by starch hydrolysis. Strains D28 and D30^T also hydrolysed starch but, in addition, they tested positive for endocellulase activity and growth on sucrose. Hence, they may be separated easily from the other *Tenacibaculum* species by these characters. The bright-yellow colour of the colonies may be used as a secondary unique characteristic.

Description of *Tenacibaculum skagerrakense* sp. nov.

Tenacibaculum skagerrakense (ska.ger.rak.en'se. N.L. neut. adj. *skagerrakense* of Skagerrak, Denmark, referring to the place of isolation).

Gram-negative, oxidase- and catalase-positive. Cells are rods (0.5 × 2–15 µm) during exponential growth. Spherical cells occur in stationary phase. Colonies are bright yellow and flexirubin-type pigment is absent. Requires at least 1/4-strength sea water for growth and grows in up to 150 % strength sea water. Mesophilic: growth occurs at 10–40 °C, optimal growth around 25–37 °C and temperature resistance up to 50 °C. Grows at pH 6–9. Casein, collagen, hydroxyethyl cellulose, starch, barley β-glucan and pullulan are hydrolysed. Growth occurs on sucrose and aspartate. Nitrate is reduced. G + C content of the DNA is 35.2 mol%.

The type strain is D30^T (=ATCC BAA-458^T=DSM 14836^T). Isolated from the <0.8 µm fraction of a sea-water sample taken from Skagerrak, Denmark, at a depth of 30 m.

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References

Azam, F. & Long, R. A. (2001). Sea snow microcosms. *Nature* **414**, 495–498.

Barbeyron, T., L'Haridon, S., Corre, E., Kloareg, B. & Potin, P. (2001). *Zobellia galactanovorans* gen. nov., sp. nov., a marine species of *Flavobacteriaceae* isolated from a red alga, and classification of [*Cytophaga*] *uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 985–997.

Barrow, G. I. & Feltham, R. K. A. (1993). *Cowan and Steel's Manual for Identification of Medical Bacteria*, 3rd edn. Cambridge: Cambridge University Press.

Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.

Bowman, J. P. (2000). Description of *Cellulophaga algicola* sp. nov., isolated from the surfaces of Antarctic algae, and reclassification of *Cytophaga uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Cellulophaga uliginosa* comb. nov. *Int J Syst Evol Microbiol* **50**, 1861–1868.

Bowman, J. P., McCammon, S. A., Brown, J. L., Nichols, P. D. & McMeekin, T. A. (1997). *Psychroserpens burtonensis* gen. nov., sp. nov., and *Gelidibacter algens* gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. *Int J Syst Bacteriol* **47**, 670–677.

Bowman, J. P., McCammon, S. A., Lewis, T., Skerratt, J. H., Brown, J. L., Nichols, D. S. & McMeekin, T. A. (1998). *Psychroflexus torquis* gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of *Flavobacterium gondwanense* (Dobson *et al.* 1993) as *Psychroflexus gondwanense* gen. nov., comb. nov. *Microbiology* **144**, 1601–1609.

Cottrell, M. T. & Kirchman, D. L. (2000). Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Appl Environ Microbiol* **66**, 5116–5122.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Dorsch, M. & Stackebrandt, E. (1992). Some modifications in the procedure of direct sequencing of PCR amplified 16S rDNA. *J Microbiol Methods* **16**, 271–279.

Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.

Giovannoni, S. & Rappé, M. (2000). Evolution, diversity, and molecular ecology of marine prokaryotes. In *Microbial Ecology of the Oceans*, pp. 47–84. Edited by D. L. Kirchman. Chichester: Wiley-Liss.

Gosink, J. J., Woese, C. R. & Staley, J. T. (1998). *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga*–*Flavobacterium*–*Bacteroides* group and reclassification of '*Flectobacillus glomeratus*' as *Polaribacter glomeratus* comb. nov. *Int J Syst Bacteriol* **48**, 223–235.

Gregersen, T. (1978). Rapid method for distinction of gram-negative from gram-positive bacteria. *Eur J Appl Microbiol* **5**, 123–127.

Hall, T. A. (1999). Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

Hansen, G. H., Bergh, Ø., Michaelsen, J. & Knappskog, D. (1992). *Flexibacter ovolyticus* sp. nov., a pathogen of eggs and larvae of Atlantic halibut, *Hippoglossus hippoglossus* L. *Int J Syst Bacteriol* **42**, 451–458.

Hobbie, J. E., Daley, R. J. & Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* **33**, 1225–1228.

Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

- Johansen, J. E., Nielsen, P. & Sjøholm, C. (1999).** Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp. nov. and reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 1231–1240.
- Jukes, T. H. & Cantor, C. R. (1969).** Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kirchman, D. & Mitchell, R. (1982).** Contribution of particle-bound bacteria to total microheterotrophic activity in five ponds and two marches. *Appl Environ Microbiol* **43**, 200–209.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985).** Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A* **82**, 6955–6959.
- Maidak, B. L., Cole, J. R., Lilburn, T. G. & 9 other authors (2000).** The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.
- McCannon, S. A. & Bowman, J. P. (2000).** Taxonomy of Antarctic *Flavobacterium* species: description of *Flavobacterium gillisiae* sp. nov., *Flavobacterium tegetincola* sp. nov. and *Flavobacterium xanthum* sp. nov., nom. rev. and reclassification of [*Flavobacterium*] *salegens* as *Salegentibacter salegens* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 1055–1063.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Perry, L. B. (1973).** Gliding motility in some non-spreading flexibacteria. *J Appl Bacteriol* **36**, 227–232.
- Pinhassi, J., Zweifel, U. L. & Hagström, Å. (1997).** Dominant marine bacterioplankton species found among colony-forming bacteria. *Appl Environ Microbiol* **63**, 3359–3366.
- Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996).** The genus *Norcardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Norcardiopsaceae* fam. nov. *Int J Syst Bacteriol* **46**, 1088–1092.
- Reichenbach, H. (1992).** The order Cytophagales. In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, pp. 3631–3675. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.
- Riemann, L. & Winding, A. (2001).** Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microb Ecol* **42**, 274–285.
- Schlöter, M., Lebuhn, M., Heulin, T. & Hartmann, A. (2000).** Ecology and evolution of bacterial microdiversity. *FEMS Microbiol Rev* **24**, 647–660.
- Smith, D. C., Simon, M., Alldredge, A. L. & Azam, F. (1992).** Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. *Nature* **359**, 139–142.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Suzuki, M., Nakagawa, Y., Harayama, S. & Yamamoto, S. (2001).** Phylogenetic analysis and taxonomic study of marine *Cytophaga*-like bacteria: proposal for *Tenacibaculum* gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov. *Int J Syst Evol Microbiol* **51**, 1639–1652.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Van de Peer, Y. & De Wachter, R. (1994).** TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.
- Visuvanathan, S., Moss, M. T., Stanford, J. L., Hermon-Taylor, J. & McFadden, J. J. (1989).** Simple enzymatic method for isolation of DNA from diverse bacteria. *J Microbiol Methods* **10**, 59–64.
- Wakabayashi, H., Hikida, M. & Masumura, K. (1986).** *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. *Int J Syst Bacteriol* **36**, 396–398.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Winogradsky, S. (1929).** Études sur la microbiologie du sol. *Ann Inst Pasteur* **43**, 549–633.
- ZoBell, C. (1946).** *Marine Microbiology; a Monograph on Hydrobacteriology*. Waltham, MA: Chronica Botanica.