

Xanthomarina gelatinilytica gen. nov., sp. nov., isolated from seawater

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A novel Gram-stain-negative, rod-shaped, yellow-pigmented, non-sporulating, non-motile bacterium, designated strain AK20^T, was isolated from seawater collected from Kochi city, Kerala state, India. Colonies on marine agar were circular, yellow, shiny, translucent, 2–3 mm in diameter, convex and with entire margin. Flexirubin-type pigment was present. The fatty acids were dominated by iso-branched units with a high abundance of iso-C_{15:0}, iso-C_{15:1} G, iso-C_{17:0} 3-OH, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and iso-C_{15:0} 3-OH. Polar lipids included phosphatidylethanolamine, two unidentified aminophospholipids, two unidentified phospholipids and four unidentified lipids. Menaquinone 6 (MK-6) was the predominant respiratory quinone. The DNA G + C content of strain AK20^T was 38.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain AK20^T was closely related to *Formosa spongicola* A2^T and *Bizionia paragorgiae* KMM 6029^T (pair-wise sequence similarities of 95.9 and 95.7 %, respectively), forming a distinct branch within the family *Flavobacteriaceae* and clustering with the clade comprising species of the genus *Bizionia*. Based on phenotypic and chemotaxonomic characteristics and phylogenetic analysis, strain AK20^T is different from the existing genera in the family *Flavobacteriaceae*, and is therefore considered to represent a novel species of a new genus, for which the name *Xanthomarina gelatinilytica* gen. nov., sp. nov. is proposed. The type strain of *Xanthomarina gelatinilytica* is AK20^T (=MTCC 11705^T=JCM 18821^T).

The family *Flavobacteriaceae* comprises, at the time of writing, 114 genera (<http://www.bacterio.net/flavobacteriaceae.html>), isolated from diverse ecological niches such as coastal hot spring (Arun *et al.*, 2009), tidal flat (Jeong *et al.*, 2013), mangrove sediment (Li *et al.*, 2013), green alga *Ulva fenestrata* (Nedashkovskaya *et al.*, 2009), soft coral *Paragorgia arborea* (Nedashkovskaya *et al.*, 2005), estuarine water (Surendra *et al.*, 2012), marine sediment (Xu *et al.*, 2013), blood culture of a patient with bacteraemia (Yassin *et al.*, 2012) and marine sponges (Haber *et al.*, 2013; Yoon *et al.*, 2013). Several members of the family are considered pathogenic to humans (Bernardet & Nakagawa, 2006). The family *Flavobacteriaceae* comprises Gram-negative, non-sporulating rods (Bernardet *et al.*, 2002).

Several representatives of the family are found in the marine environment, of which a few novel species (*Aestuariibaculum suncheonense*, *Sinomicrobium oceani*, *Muricauda antarctica*, *Arenitalea lutea* and *Hanstruepera neustonica*) have been described from tidal flat (Jeong *et al.*, 2013), marine sediment (Xu *et al.*, 2013), Antarctic seawater (Wu *et al.*, 2013), intertidal sand (Zhang *et al.*, 2013) and estuarine water (Hameed *et al.*, 2015). In the present study we report on the isolation and description of a novel member of the family *Flavobacteriaceae* (strain AK20^T) isolated from seawater collected from Kochi city, Kerala state, India. Based on 16S rRNA gene sequence analysis, fatty acid composition and phenotypic characteristics we propose that this strain represents a novel species of a new genus closely related to *Bizionia paragorgiae*.

Strain AK20^T was isolated from a seawater sample collected from Kochi city (9° 56' N 76° 15' E). For isolation, 1 ml of the water sample was serially diluted (up to 10-fold dilutions) in 2 % (w/v) NaCl solution; 100 µl of each dilution was plated on ZoBell's marine agar (ZMA; HiMedia) and incubated at 30 °C. A yellow colony was

All authors contributed equally to this work.

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One supplementary table and two supplementary figures are available with the online Supplementary Material.

isolated, subcultivated on ZMA at 30 °C and preserved at –80 °C in marine broth with 10 % glycerol. *B. paragorgiae* KCTC 12304^T was also cultivated under the same conditions as strain AK20^T.

Strain AK20^T was characterized simultaneously with *B. paragorgiae* KCTC 12304^T and *Formosa algae* KCTC 12364^T. Colony morphology of strain AK20^T was observed after 48 h of growth on ZMA at 30 °C. Cell morphology was studied by light microscopy (Zeiss, at 1000×) and transmission electron microscopy (JEM2100; JEOL). Gram staining was performed using a Gram staining kit (HiMedia) according to the manufacturer's instructions and gliding motility was assessed based on protocols described by Bernardet *et al.* (2002). Spore formation was determined by the malachite green staining method. The presence of flexirubin-type pigments was studied as described by Bernardet *et al.* (2002). For determination of optimum salt concentration and NaCl tolerance, the isolate was grown in nutrient broth containing 0–8 % (w/v) NaCl (in increments of 0.5 %). For calculating optimum pH value for growth and pH tolerance level, the isolate was grown in ZMA with suitable buffers to maintain desired pH values (4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 12): acetate or citrate buffer (pH 4–6), 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7–8), 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–10) or 100 mM Na₂CO₃/NaOH buffer (pH 11–12) (Nakajima *et al.*, 2005). For determination of the temperature range for growth, the isolate was grown at 5–50 °C (in increments of 5 °C) on ZMA.

Hydrolysis of aesculin, casein, gelatin, starch and Tweens, Voges–Proskauer, methyl red, oxidation–fermentation tests, catalase and oxidase activity, oxidation of tetramethyl-*p*-phenylenediamine dihydrochloride, production of H₂S and reduction of nitrate were determined as described by Lányi (1987) and Cowan & Steel (1965). Sensitivity of the isolate to different antibiotics (µg per disc), lincomycin (2), chloramphenicol (30), cefprozil (30), ampicillin (25), amoxicillin (30), cephadroxil (30), novobiocin (30), ciprofloxacin (10), kanamycin (30), gentamicin (10), tetracycline (30), bacitracin (10), vancomycin (30), neomycin (30), ceftazidime (30), chlortetracyclin (30) and polymyxin B (300), was determined using commercially available antibiotic discs (HiMedia). The sensitivity, resistance and intermediate sensitivity to different antibiotics was determined based on the worksheet provided by the manufacturer (HiMedia). Acid production from different carbohydrate sources was determined using bromocresol purple as an acid–base indicator (Gordon *et al.*, 1974). Activity of β-galactosidase was determined using ONPG discs (HiMedia). Biochemical and enzyme characterization of strain AK20^T was also performed using the Vitek 2 GN system (bioMérieux) according to the manufacturer's protocol. For this the inoculum was prepared using sterile 2.0 % (w/v) NaCl and incubated at 30 °C.

Analysis of cellular fatty acid methyl esters followed the method described by Anil Kumar *et al.* (2012) and

components were analysed by the Sherlock Microbial Identification System (MIDI; Agilent Technologies) according to the manufacturer's protocol. Polar lipids and quinones were analysed from freeze-dried cells. Isoprenoid quinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1997). Cells were extracted for polar lipid analysis (Bligh & Dyer, 1959) and polar lipids were analysed by two-dimensional TLC followed by spraying with appropriate detection reagents (5 % ethanolic molybdato-phosphoric acid, molybdenum blue, ninhydrin and molisch reagents) (Komagata & Suzuki, 1987). The G + C content of strain AK20^T was determined from the melting point (*T*_m) curves (Sly *et al.*, 1986) obtained using a Lambda 2 UV-Vis spectrophotometer equipped with the Templab 2.0 software package (Perkin Elmer). *Escherichia coli* DH5α DNA was used as a standard in determining the G + C content of the DNA.

For 16S rRNA gene sequencing, DNA was prepared using a microbial DNA isolation kit (Mo Bio Laboratories) and sequenced as described by Lane (1991). The resultant sequence of the 16S rRNA gene (1461 bp) was compared via the EzBioCloud (Kim *et al.*, 2012) to identify the nearest taxa. To reveal the exact phylogenetic position of the strain in the domain *Bacteria*, the 16S rRNA gene sequence was aligned against an ARB dataset using the ARB program package (Ludwig *et al.*, 2004). The 16S rRNA gene sequences of 15 closely related type strains of species of genera in the family *Flavobacteriaceae* were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL W program within the MEGA5 package (Tamura *et al.*, 2011). Phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood tree-making algorithms, using the MEGA5 package (Tamura *et al.*, 2011). There were in total 16 sequences and 1470 characters

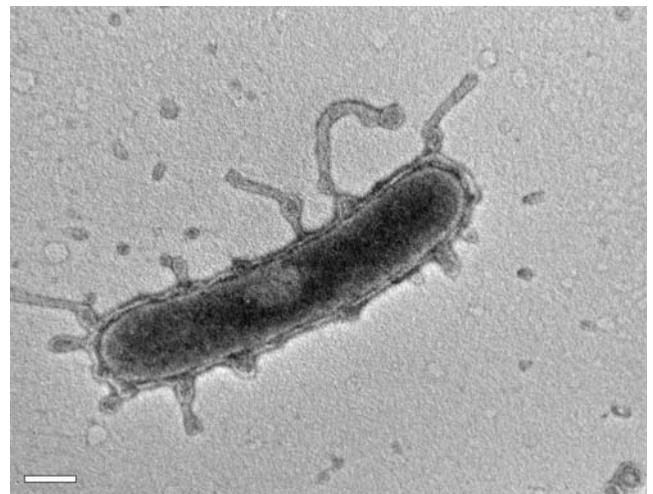


Fig. 1. Scanning electron micrograph of a cell of strain AK20^T. Bar, 0.2 µm.

Table 1. Differential characteristics between strain AK20^T and the type strains of closely related members of the family *Flavobacteriaceae*

Strains: 1, AK20^T; 2, *B. paragorgiae* KCTC 12304^T; 3, *F. algae* KCTC 12364^T. All strains were positive for catalase, gelatinase, Ala-Phe-Pro arylamidase and phosphatase activities, and ONPG hydrolysis. All strains were susceptible to lincomycin, chloramphenicol, cefprozil and ampicillin, but resistant to bacitracin and neomycin. All strains were negative for nitrate reduction, methyl red and Voges-Proskauer reactions, H₂S and indole production, aesculin, starch, Tween 20 and Tween 60 hydrolysis, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, β -galactosidase, *N*-acetyl- β -glucosaminidase, γ -glutamyltransferase, β -xylosidase, β -alanine arylamidase, α -glucosidase, *N*-acetyl- β -galactosaminidase, α -galactosidase and β -glucuronidase activities, acid production from adonitol, arabitol, cellobiose, glucose, fructose, L-arabinose, mannose, salicin, sucrose, trehalose, dulcitol, erythritol, galactose, glycerol, inositol, inulin, lactose, maltose, melezitose, melibiose, raffinose, rhamnose, sodium gluconate, sorbitol, xylitol, xylose, methyl α -D-glucoside and methyl α -D-mannoside, utilization of adonitol, L-arabitol, cellobiose, citrate, malonate, D-glucose, maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, sucrose, D-tagatose, trehalose, courmarate, 5-keto-D-gluconate, L-lactate, L-histidine and L-malate, L-lactate and succinate alkalization, fermentation of glucose, 0/129 resistance and Ellman reaction. +, Positive; -, negative; (+), weakly positive; I, intermediate; S, sensitive; R, resistant; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PL, unidentified phospholipid. All data are from the present study.

Characteristic	1	2	3
Cell shape	Slightly curved rods	Regular rods	Regular rods
Cell size (μm)	1.6 \times 0.3–0.4	1.9–2.3 \times 0.4–0.5	0.8–2.0 \times 0.4–0.9
Gliding motility	+	–	+
Temperature growth range ($^{\circ}\text{C}$)	10–40	4–35	10–30
Optimum temperature ($^{\circ}\text{C}$)	30–35	20–25	25
pH growth range (optimum)	6–11 (7)	7–11 (7)	6–9 (8)
Salinity range (% NaCl, w/v) (optimum)	0.5–7.5 (2–3)	0.5–7.5 (2–4)	1–3.5 (2)
Arginine dihydrolase activity	+	+	–
Hydrolysis of:			
Casein	–	+	–
Tween 40	–	+	+
Tween 80	+	+	–
Oxidase	+	+	–
Flexirubin-type pigment production	+	–	–
Enzyme activity of:			
L-Pyrrolidonyl arylamidase	–	–	+
Glutamyl arylamidase	+	+	–
β -Glucosidase	–	–	+
L-Proline arylamidase	+	–	+
Glycine arylamidase	+	–	–
Lipase	+	+	–
Tyrosine arylamidase	+	+	–
Urease	–	+	–
Glu-Gly-Arg arylamidase	+	–	–
Susceptibility to:			
Amoxicillin	I	S	S
Ceftazidime	R	R	S
Cephadroxil	I	S	S
Chlortetracycline	R	S	S
Ciprofloxacin	R	S	S
Gentamicin	R	S	S
Kanamycin	R	S	S
Novobiocin	I	I	S
Tetracycline	R	R	S
Cefaclor	R	S	S
Vancomycin	R	S	S
Polymyxin B	R	S	S
Polar lipids	PE, APL1–2, PL1–2, L1–4	PE, APL3–4, PL1–5, L1–2	PE, APL5–7, L2, L4–7
DNA G + C content (mol%)	38.8	37.6	34.0

in the final dataset. The robustness of the trees was inferred using bootstrap analysis of 1000 replicates. Evolutionary distances were calculated using the Jukes–Cantor method (Jukes & Cantor, 1969).

Cells of strain AK20^T were Gram-stain-negative, rod-shaped, yellow-pigmented, non-sporulating and non-motile. Cells were 1.6 µm in length and 0.3–0.4 µm in width (Fig. 1). Colonies on ZMA were circular, yellow, shiny, translucent, 2–3 mm in diameter, convex and with entire margin after 48 h at 30 °C. The cellular morphology of strain AK20^T differed slightly from that of *B. paragorgiae* KCTC 12304^T. Cells of strain AK20^T were relatively longer. A list of distinguishing features between strain AK20^T and *B. paragorgiae* KCTC 12304^T and *F. algae* KCTC 12364^T is provided in Table 1 and other related halophilic genera in the family *Flavobacteriaceae* in Table S1 (available in the online Supplementary Material).

The cellular fatty acids of strain AK20^T were dominated by iso-branched units with a high abundance of iso-C_{15:0}, iso-C_{15:1} G, iso-C_{17:0} 3-OH, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and iso-C_{15:0} 3-OH (Table 2). The fatty acid profile of strain AK20^T differed considerably from those of *B. paragorgiae* KCTC 12304^T and *F. algae* KMM 3553^T (Table 2). The polar lipid composition included phosphatidylethanolamine (PE), two unidentified aminophospholipids (APL), two unidentified phospholipids (PL) and four unidentified lipids (L) (Fig. S1a). The polar lipid profile of *B. paragorgiae* KCTC 12304^T was similar (PE, APL, PL and L) but it differed in the absence of APL1 and 2 and L3 and 4 and the presence of APL3 and 4 and PL3–5 (Fig. S1b). A similar composition was found for *F. algae* KCTC 12364^T (PE, APL and L) but it differed from strain AK20^T in the absence of APL1–4, PL1–5 and L1, 3 and 4 and the presence of APL5–7 and L5–7 (Fig. S1c). *B. paragorgiae* KMM 6029^T, *Psychroserpens burtonensis* ACAM 188^T and *F. algae* KMM 3553^T contained phosphatidylethanolamine as the major polar lipid (Ivanova *et al.*, 2004; Lee *et al.*, 2013; present study). Menaquinone 6 (MK-6) was the predominant respiratory quinone in strain AK20^T and the reference type strains. The DNA G+C content of strain AK20^T was 38.8 mol%.

For phylogenetic assessment of strain AK20^T, 1461 nt of the 16S rRNA gene sequence was compared with the database of type strains of recognized prokaryotes (<http://eztaxon-e.ezbiocloud.net/>; Kim *et al.*, 2012), which revealed its closeness to the genera *Formosa* and *Bizionia* of the family *Flavobacteriaceae*. Based on pair-wise sequence similarity, strain AK20^T was related most closely to *Formosa spongicola* A2^T (95.9 %) and *B. paragorgiae* KMM 6029^T (95.7 %). In the neighbour-joining tree, strain AK20^T formed a distinct branch within the family *Flavobacteriaceae* and clustered with species of the genera *Bizionia* and *Formosa* (Fig. 2). In the maximum-likelihood phylogenetic tree, strain AK40^T was located between the genera *Formosa*, *Bizionia*, *Gelidibacter*, *Subsaxibacter* and *Subsaximicrobium* (Fig. S2).

Based on the phylogenetic analysis, a comparison was made between the characteristics of strain AK20^T and *B. paragorgiae* KCTC 12304^T and *F. algae* KCTC 12364^T, of the family *Flavobacteriaceae* (Table 1), and a number of differences were observed. Strain AK20^T differed with respect to cell shape, size, gliding motility, growth range and optimum for temperature, pH and salinity, biochemical characteristics, antibiotic sensitivity, polar lipid composition, fatty acid composition and DNA G+C content (Tables 1 and 2). Strain AK20^T could also be differentiated from other phylogenetically related halophilic genera in the family *Flavobacteriaceae* with respect to characteristics such as gliding motility, presence of flexirubin pigment, requirement of sodium ions for growth, amino acid requirement, carbohydrate utilization, starch hydrolysis and DNA G+C content (Table S1). Based on the observed phenotypic and chemotaxonomic characteristics and phylogenetic analysis, strain AK20^T is sufficiently different from existing genera in the family *Flavobacteriaceae* to warrant placement in a novel species of a new genus, for which the name *Xanthomarina gelatinilytica* gen. nov., sp. nov. is proposed.

Table 2. Fatty acid composition of strain AK20^T and the type strains of closely related members of the family *Flavobacteriaceae*

Strains: 1, AK20^T; 2, *B. paragorgiae* KCTC 12304^T; 3, *F. algae* KCTC 12364^T. Results are presented as a percentage of the total fatty acids. Fatty acids amounting to 5 % or more of the total are in bold type. All data are from the present study. The strains were grown on MA plates at 30 °C for 2–3 days. ND, Not detected. Fatty acids comprising less than 1 % for all strains are not shown.

Fatty acid	1	2	3
iso-C _{14:0}	ND	1.9	1.0
C _{15:0} 2-OH	ND	ND	2.5
C _{15:0} 3-OH	ND	ND	2.3
iso-C _{15:0}	39.3	33.2	22.2
iso-C _{15:0} 3-OH	6.1	6.4	8.6
anteiso-C _{15:0}	ND	6.9	9.0
iso-C _{15:1} G	29.5	13.8	10.2
anteiso-C _{15:1} A	ND	ND	1.8
C _{15:1} ω6c	ND	4.0	6.0
iso-C _{16:0}	ND	4.5	1.5
iso-C _{16:0} 3-OH	ND	5.6	8.4
iso-C _{16:1} H	ND	2.8	2.3
C _{17:0} 2-OH	ND	ND	2.0
iso-C _{17:0} 3-OH	13.4	7.5	7.4
C _{17:1} ω6c	ND	2.7	1.1
iso-C _{17:1} ω9c	3.8	3.3	1.4
Summed feature 3*	7.9	7.5	6.9

*Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

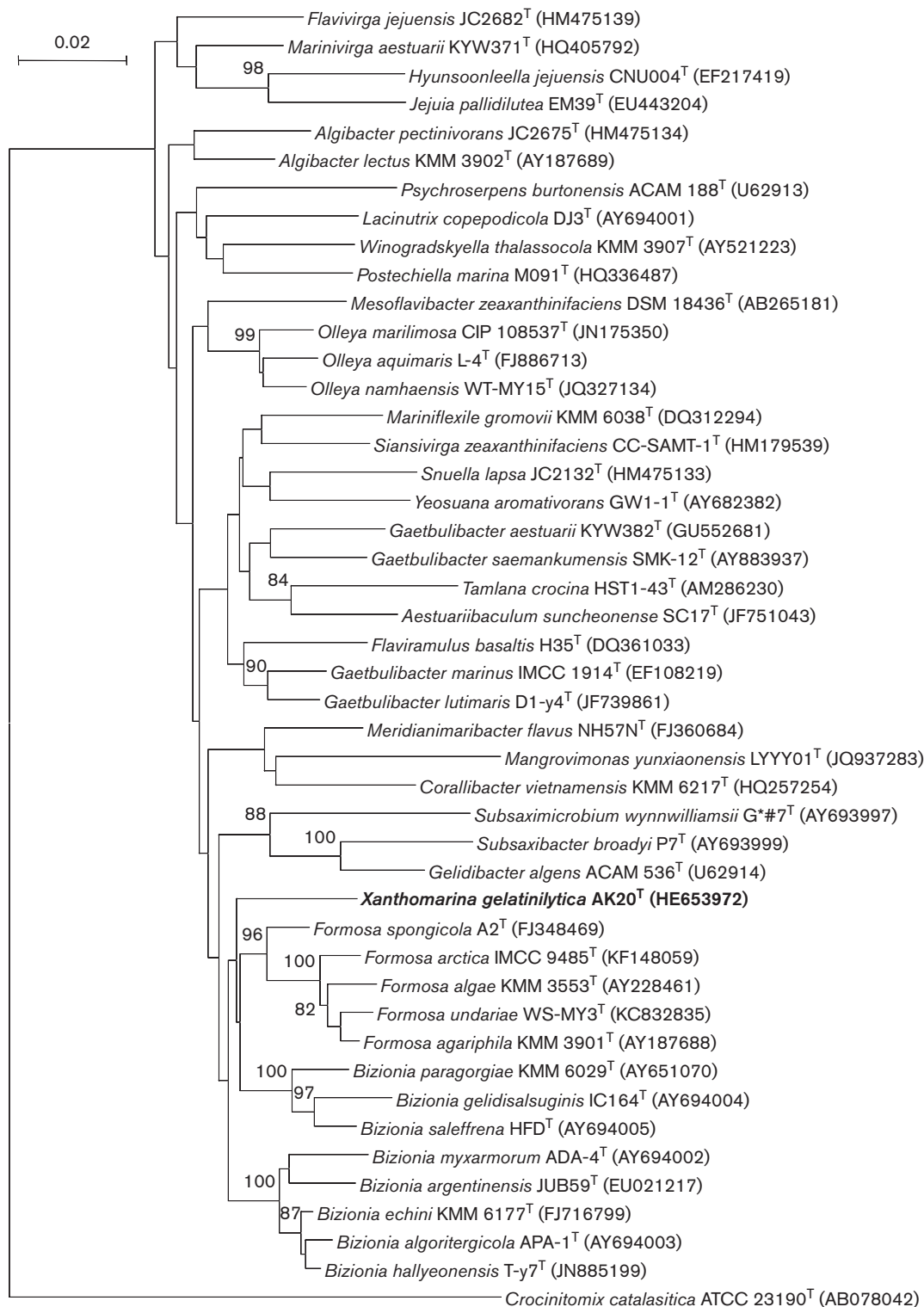


Fig. 2. Neighbour-joining tree showing the phylogenetic relationships of strain AK20^T with closely related members of the family *Flavobacteriaceae* based on 16S rRNA gene sequences. Bootstrap support values of more than 70 % are shown at nodes. *Crocinitomix catalasitica* ATCC 23190^T was used as an outgroup taxon. Bar, 0.02 substitutions per nucleotide position.

Description of *Xanthomarina* gen. nov.

Xanthomarina (Xan.tho.ma.ri'na. Gr. adj. *xanthos* yellow; L. masc. adj. *marinus* from the sea; N.L. fem. n. *Xanthomarina* yellow organism from seawater).

Cells are Gram-stain-negative, rod-shaped, oxidase- and catalase-positive, yellow-pigmented, non-motile and aerobic. Flexirubin-type pigment is present. The major fatty acids are iso-C_{15:0}, iso-C_{15:1} G, iso-C_{17:0} 3-OH, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and iso-C_{15:0} 3-OH. The predominant polar lipids are phosphatidylethanolamine, two unidentified aminophospholipids, two unidentified phospholipids and four unidentified lipids. MK-6 is the predominant respiratory quinone. Based on 16S rRNA gene sequence analysis, the genus is a member of the family *Flavobacteriaceae* of the class *Flavobacteriia*. The type species is *Xanthomarina gelatinilytica*.

Description of *Xanthomarina gelatinilytica* sp. nov.

Xanthomarina gelatinilytica [ge.la.ti.ni.ly'ti.ca. N.L. neut. n. *gelatinum* gelatin; N.L. fem. adj. *lytica* (from Gr. fem. adj. *lutikê*) able to loosen, able to dissolve; N.L. fem. adj. *gelatinilytica* gelatin-dissolving, referring to the property of being able to hydrolyse gelatin].

Exhibits the following properties in addition to those given in the genus description. Grows at 10–40 °C (optimum, 30–35 °C) and at pH 6–11 (optimum, 7.0). Optimum growth occurs with 2–3 % (w/v) NaCl and tolerates up to 7.5 % (w/v) NaCl; there is no growth in the absence of salt (grows with 0.5 % NaCl). β-Galactosidase activity is present. In the Hi25 *Enterobacteriaceae* identification kit, lysine decarboxylase, ornithine decarboxylase and phenylalanine deaminase activities are negative. Nitrate is not reduced; H₂S and indole are not produced. The methyl red and Voges-Proskauer reactions are negative. Gelatin and Tween 80 are hydrolysed but aesculin, casein, starch and Tweens 20, 40 and 60 are not. In the Vitek GN ID card, positive for Ala-Phe-Pro arylamidase, glutamyl arylamidase, L-proline arylamidase, lipase, tyrosine arylamidase, urease, phosphatase, glycine arylamidase and Glu-Gly-Arg arylamidase activities but negative for L-pyrrolidonyl arylamidase, β-galactosidase, N-acetyl-β-glucosaminidase, γ-glutamyltransferase, β-xylosidase, β-glucosidase, α-galactosidase, β-alanine arylamidase, N-acetyl-β-galactosaminidase and β-glucuronidase. Does not utilize L-arabitol, D-mannitol, D-mannose, palatinose, D-glucose, maltose, D-sorbitol, D-tagatose, 5-keto-D-glucuronate, L-histidine, courmarate, L-malate or L-lactate. Negative for L-lactate and succinate alkalization, fermentation of glucose, and Ellman reaction and O/129 resistance (Vitek GN card system). Does not produce acid from cellobiose, glucose, fructose, L-arabinose, mannose, salicin, sucrose, trehalose, adonitol, arabitol, dulcitol, erythritol, galactose, glycerol, inositol, inulin, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, sodium gluconate, sorbitol, xylitol, xylose, methyl α-D-glucoside or methyl α-D-mannoside in the Hi25 *Enterobacteriaceae* identification kit, and is unable to ferment the same sugars, as well as citrate, using the

HiCarbohydrate kit. Susceptible to lincomycin, chloramphenicol, cefprozil and ampicillin, moderately susceptible to amoxicillin, cephadroxil and novobiocin, but resistant to ciprofloxacin, kanamycin, gentamicin, tetracycline, bacitracin, vancomycin, neomycin, ceftazidime, chlortetracycline and polymyxin B. The cellular fatty acid composition is given in Table 2.

The type strain, AK20^T (=MTCC 11705^T=JCM 18821^T), was isolated from seawater sampled from Kochi, Kerala, India. The G+C content of the genomic DNA of the type strain is 38.8 mol%.

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