Lacinutrix algicola sp. nov. and *Lacinutrix mariniflava* sp. nov., two novel marine alga-associated bacteria and emended description of the genus *Lacinutrix*

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Two heterotrophic, aerobic, yellow-pigmented, Gram-negative, non-gliding bacteria, designated AKS293^T and AKS432^T, isolated from a red alga, were analysed using a polyphasic taxonomic approach. 16S rRNA gene sequence analysis revealed that the novel strains were affiliated to the genus *Lacinutrix*, a member of the family *Flavobacteriaceae*, showing sequence similarities of 96.1–96.4 % with respect to the type strain of *Lacinutrix copepodicola*. The two novel isolates shared 99.5 % 16S rRNA gene sequence similarity and 55.0 % DNA–DNA relatedness. They grew optimally at 17.5 °C and pH 6.5. The main cellular fatty acids of strain AKS293^T were iso-C_{15:0}, iso-C_{15:0} 3-OH and iso-C_{16:0} 3-OH, while those of strain AKS432^T were anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{15:1} and iso-C_{15:0} 3-OH. In both cases, the major isoprenoid quinone was MK-6. The DNA G + C contents were 34.7 and 37.0 mol% for strains AKS293^T and AKS432^T, respectively. The phylogenetic evidence, phenotypic data and DNA–DNA hybridization results support the differentiation of strains AKS293^T and AKS432^T from each other and from their closest relative, *L. copepodicola* DJ3^T. Therefore, strains AKS293^T and AKS432^T represent two novel species, for which the names *Lacinutrix algicola* sp. nov. and *Lacinutrix mariniflava* sp. nov. are proposed, respectively. The type strain of *L. algicola* sp. nov. is AKS293^T (=KCCM 42313^T=JCM 13825^T) and the type strain of *L. mariniflava* sp. nov. is AKS432^T (=KCCM 42306^T=JCM 13824^T). An emended description of the genus *Lacinutrix* is also proposed.

The genus *Lacinutrix* was created by Bowman & Nichols (2005), within the family *Flavobacteriaceae* (Bernardet *et al.*, 2002), to accommodate Gram-negative, heterotrophic, aerobic, non-gliding, yellow-pigmented bacteria. A single strain, *Lacinutrix copepodicola* DJ3^T was isolated from a calanoid copepod, *Paralabidocera antarctica*, collected from a meromictic Antarctic lake (Bowman & Nichols, 2005). The genera *Olleya* (Nichols *et al.*, 2005) and *Mesoflavibacter* (Asker *et al.*, 2007) are the closest phylogenetic neighbours of the genus *Lacinutrix* (Fig. 1).

During a survey of the microbial diversity of Antarctic marine environments, two novel bacterial strains, designated

AKS293^T and AKS432^T, were isolated from alga and studied using a polyphasic taxonomic approach. On the basis of the results of this study, two novel species of the genus *Lacinutrix* are proposed.

Strains AKS293^T and AKS432^T were isolated from a red alga belonging to the family *Gigartinales*, growing in the subtidal zone of Marian Cove, King George Island, Antarctica. For strain isolation, a small piece of algal frond was ground, diluted with sterile seawater and spread on marine agar 2216 (Difco). Inoculated plates were cultivated at 4 °C (AKS432^T) or at 25 °C (AKS293^T) for 1 week. After primary isolation and purification, strains were cultivated at 15 °C on the same medium and stored at –80 °C in marine broth (Difco) supplemented with 20 % (v/v) glycerol.

The 16S rRNA gene was amplified by means of direct PCR and then sequenced using an ABI 3100 automatic DNA

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains AKS293^T and AKS432^T are DQ167238 and DQ167239, respectively.

Fig. 1. Rooted neighbour-joining phylogenetic tree, based on almost-complete 16S rRNA gene sequences (1294 unambiguously aligned bp) showing the relationships between strains AKS293^T and AKS432^T and closely related members of the family *Flavobacteriaceae*. The 16S rRNA gene sequences of *Bacteroides fragilis* ATCC 25285^T (NC003228) and *Sphingobacterium spiritivorum* DSM 2582^T (AJ459411) served as outgroups (not shown). Bootstrap values greater than 50 % (based on 1000 replicates) are shown at nodes. Nodes with bootstrap values greater than 50 % (open circles) or greater than 70 % (filled circles) recovered in a maximum-parsimony tree based on distances calculated by the maximum-likelihood method are indicated. Bar, 0.01 nucleotide substitutions per nucleotide position.

sequencer (ABI) according to the manufacturer's instructions. Comparisons of the 16S rRNA gene sequence with sequences retrieved from GenBank and phylogenetic analyses were conducted according to the procedure described by Kwon *et al.* (2005).

Phylogenetic analysis of the almost-complete 16S rRNA gene sequences of strains AKS293^T and AKS432^T (1294 nt in each case) revealed that the strains formed a distinct lineage in the vicinity of *L. copepodicola* DJ3^T, sharing 96.1–96.4 % sequence similarity with this strain and 99.5 % sequence similarity with each other (Fig. 1). DNA was isolated according to the procedure described by Marmur (1961) and the DNA relatedness between the two isolates was measured fluorometrically by using the microplate hybridization method described by Ezaki *et al.* (1989). The strains shared 55.0 % DNA relatedness, a value that is well below the threshold accepted for species delineation (Wayne *et al.*, 1987). Thus, strains AKS293^T and AKS432^T represent two different species.

DNA G+C contents were analysed by using HPLC with a symmetry reversed-phase C18 column (Stackebrandt & Liesack, 1993); the values for strains AKS293^T and AKS432^T were 37.0 and 34.7 mol%, respectively.

Analysis of fatty acid methyl esters was carried out by using the MIDI/Hewlett Packard Microbial Identification System (MIS; Sasser, 1990), according to the manufacturer's

instructions, on cells grown on marine agar 2216 for 3 days at 25 °C. The predominant fatty acids of strain AKS293^T were C_{15:0} (6.7 %), iso-C_{15:0} (12.5 %), iso-C_{15:1} (7.2 %), iso-C_{16:1} (7.2 %), iso-C_{15:0} 3-OH (10.0 %), iso-C_{16:0} 3-OH (14.4 %) and summed feature 3 (9.2 %) and those of strain AKS432^T were iso-C_{15:0} (10.6 %), anteiso-C_{15:0} (12.5 %), iso-C_{15:1} (11.6 %), anteiso-C_{15:1} (7.5 %), iso-C_{15:0} 3-OH (12.2 %), iso-C_{16:0} 3-OH (8.9 %), iso-C_{17:0} 3-OH (7.6 %) and summed feature 3 (5.8 %) (Table 1). Despite the existence of differences in culture conditions and analytical methods, the two novel isolates and *L. copepodicola* DJ3^T contained large amounts of iso-branched C₁₅ fatty acids. However, significant differences in fatty acid composition and in the proportion of some components were identified between the two isolates (Table 1). Isoprenoid quinones were extracted from lyophilized cells and analysed according to the procedure described by Collins (1985). The major respiratory quinone was MK-6 in both isolates. The chemotaxonomic data obtained in this study were in accordance with the characteristics of members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002).

Unless otherwise stated, phenotypic analysis of strains AKS293^T and AKS432^T was performed according to the minimal standards for describing new taxa in the family *Flavobacteriaceae* proposed by Bernardet *et al.* (2002) and by using previously described methods (Bae *et al.*, 2005,

Table 1. Fatty acid profiles (%) of strain AKS293^T, strain AKS432^T and *L. copepodicola* DJ3^T

Strains: 1, AKS293^T; 2, AKS432^T; 3, *L. copepodicola* DJ3^T. Data are from Bowman & Nichols (2005) and this study. Fatty acids amounting to <0.5% of the total fatty acids in all strains were omitted. tr, Trace (<1%); Br, branched fatty acid but branching position is unclear. The culture conditions and analytical procedures used for isolates AKS293^T and AKS432^T differed from those used for *L. copepodicola* DJ3^T.

Fatty acid	1	2	3
Straight-chain			
C _{14:0}	tr	tr	tr
C _{15:0}	6.7	1.6	3.7
C _{16:0}	1.0	tr	2.9
Branched			
iso-C _{14:0}	4.1	tr	1.4
iso-C _{15:0}	12.5	10.6	19.9
anteiso-C _{15:0}	4.6	12.5	18.2
iso-C _{16:0}	3.4	2.2	7.1
Unsaturated			
C _{15:1} ω6c	2.7	1.4	1.2
C _{17:1} ω6c	1.8	tr	
C _{17:1} ω8c	tr		
C _{18:1} ω5c		tr	
Br-C _{14:1}			tr
iso-C _{15:1}	7.2	11.6	12.0
anteiso-C _{15:1}	1.2	7.5	14.2
Br-C _{16:1}	7.2	3.1	4.6
iso-C _{17:1} ω9c	1.1	1.3	–
anteiso-C _{17:1} ω9c		1.6	
Hydroxy			
C _{15:0} 2-OH	1.9	2.5	
C _{15:0} 3-OH	1.1		tr
C _{16:0} 3-OH	tr	tr	
C _{17:0} 2-OH	tr	3.9	
iso-C _{14:0} 3-OH	1.0		
iso-C _{15:0} 3-OH	10.0	12.2	3.1
anteiso-C _{15:0} 3-OH			2.1
iso-C _{16:0} 3-OH	14.4	8.9	2.7
iso-C _{17:0} 3-OH	3.6	7.6	2.3
anteiso-C _{17:0} 3-OH			1.7
Summed feature 3*	9.2	5.8	2.8

*Summed features represent fatty acids that could not be separated by GLC with the MIDI system; summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

2007; Sohn *et al.*, 2004). Scanning electron micrographs were taken using a JSM-2000EXII (JEOL) electron microscope after bacterial cells had been dehydrated using a graded series of ethanol dilutions. The bacterial suspensions used to inoculate API 20E, API 20NE (bioMérieux) and Microlog GN2 (Biolog) systems were prepared in 2% sea salts (Sigma) solution. The tolerance range for NaCl was tested in marine broth prepared with distilled water and supplemented with 0–7% NaCl (w/v).

The physiological, biochemical and morphological characteristics of the two strains are given in the species descriptions and those that serve to differentiate the two isolates from each other and from *L. copepodicola* DJ3^T are listed in Table 2.

The results of the 16S rRNA-based phylogenetic analysis taken together with the phenotypic findings (Table 2) allow the affiliation of strains AKS293^T and AKS432^T to the genus *Lacinutrix* and their classification as two separate species, for which the names *Lacinutrix algicola* sp. nov. and *Lacinutrix mariniflava* sp. nov. are proposed, respectively. Additional data are included in an emended description of the genus *Lacinutrix*.

Emended description of the genus *Lacinutrix* Bowman and Nichols 2005

The description of the genus *Lacinutrix* is as given by Bowman & Nichols (2005), with the following amendments. Cells are approximately 0.4–0.8 µm wide and 0.7–2.5 µm long. The DNA G+C content range is 35–37 mol%.

Description of *Lacinutrix algicola* sp. nov.

Lacinutrix algicola [al.gi.co'la. L. fem. n. *alga* a seaweed; L. suffix *-cola* (from L. masc. or fem. n. *incola*) a dweller; N.L. fem. n. *algicola* an alga dweller].

Cells are Gram-negative, non-gliding, straight or slightly curved rods 0.5–0.6 µm wide and 0.7–1.7 µm long. On marine agar, colonies are circular, 1–3 mm in diameter, convex, shiny, butyrous, have entire edges and are yellow-pigmented. Flexirubin-type pigments are not formed. Does not form resting cells or spores. Does not require Na⁺ ions for growth. Growth occurs in the presence of up to 2.5% NaCl, at 0–25 °C and at pH 5.5–8.5. Optimal growth is observed with 0.5% NaCl, at 17.5 °C and at pH 6.5. Oxidase, catalase, alkaline phosphatase and β-galactosidase activities are present, but arginine dihydrolase activity is absent. Gelatin and casein are hydrolysed, but aesculin, agar, Tweens 40 and 80 and urea are not hydrolysed. Acid is not produced from D-glucose. In the API 20NE system, arabinose, glucose, mannose, maltose, N-acetylglucosamine, gluconate, caprate, adipate, malate, citrate and phenyl acetate are not utilized. In the Biolog GN2 microplate, the following substrates are utilized: D-arabitol, D-mannitol, D-psicose, methyl pyruvate, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketoglutaric acid, L-glutamic acid and glycyl L-glutamic acid. In the API ZYM system, esterase (C4), esterase lipase (C8), leucine-, valine- and cystine arylamidases, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but lipase (C14), α-galactosidase, β-glucuronidase, α- and β-glucosidases, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. Nitrate is not reduced. Indole is not produced. The major fatty acids

Table 2. Phenotypic properties of *Lacinutrix* species

All strains gave positive results in tests for the following properties: respiratory metabolism; catalase, alkaline phosphatase and leucine arylamidase activities and hydrolysis of gelatin. All strains gave negative results in tests for the following properties: gliding motility; arginine dihydrolase, tryptophan deaminase, α -galactosidase, β -glucuronidase, α - and β -glucosidases, α -fucosidase and nitrate reductase activities; flexirubin-type pigments; hydrolysis of agar and urea; acid production from glucose; utilization of arabinose, sucrose, L-alanine, L-histidine, citrate, D-gluconate, adipate, caprate, malate and phenylacetate. Data are from Bowman & Nichols (2005) and this study.

Characteristic	Strain AKS293 ^T	Strain AKS432 ^T	<i>L. copepodicola</i> DJ3 ^T
β -Galactosidase	+	+	–
<i>N</i> -Acetyl- β -D-glucosaminidase	–	–	+
Salinity range (% NaCl)	0–2.5	0.5–4.5	0.5–10
Temperature range (°C)	0–25	0–22	–2–25
pH value for growth	5.5–8.5	6.0–8.5	ND
Hydrolysis of:			
Aesculin	–	+	–
Casein	+	+	–
Tween 40	–	+	–
Tween 80	–	–	+
Utilization of:			
D-Glucose, glycogen, maltose	–	+	+
D-Mannose	–	–	+
Mannitol, L-serine	+	+	–
Dextrin, D-galactose, L-aspartic acid	–	+	ND
D-Arabitol, D-psicose, methylpyruvate, D-glucosaminic acid, D-glucuronic acid, α - and β -hydroxybutyric acids, α -ketoglutaric acid, glycyl L-glutamic acid	+	–	ND
DNA G + C content (mol%)	37.0	34.7	37.0

(>5 %) are C_{15:0}, iso-C_{15:0}, iso-C_{15:1}, branched C_{16:1}, iso-C_{15:0} 3-OH, iso-C_{16:0} 3-OH and summed feature 3. The complete fatty acid composition is given in Table 1.

The type strain, AKS293^T (=KCCM 42313^T=JCM 13825^T), was isolated from a marine red alga collected at Marian Cove, King George Island, South Shetland Islands, Antarctica. The DNA G + C content of the type strain is 37.0 mol%.

Description of *Lacinutrix mariniflava* sp. nov.

Lacinutrix mariniflava (ma.ri.ni fla'va. L. adj. *marinus* marine; L. adj. *flavus* golden yellow; N.L. fem. adj. *mariniflava* marine and yellow-pigmented).

Cells are Gram-negative, non-gliding, straight or slightly curved rods 0.6–0.8 µm wide and 1.1–2.5 µm long. On marine agar, colonies are circular, 1–3 mm in diameter, convex, shiny, butyrous, have entire edges and are yellow-pigmented. Flexirubin-type pigments are not formed. Does not form resting cells or spores. Requires Na⁺ ions for growth. Growth occurs in the presence of 0.5–4.5 % NaCl, at 0–22 °C and at pH 6.0–8.5. Optimal growth is observed with 2.5 % NaCl, at 17.5 °C and at pH 6.5. Oxidase, catalase, alkaline phosphatase and β -galactosidase activities are present, but arginine dihydrolase activity is absent. Gelatin, aesculin, casein and Tween 40 are hydrolysed, but agar, Tween 80 and urea are not hydrolysed. Acid is not produced from D-glucose. In the API 20NE system,

arabinose, glucose, mannose, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate and phenylacetate are not utilized. In the Biolog GN2 microplate, the following substrates are utilized: dextrin, glycogen, D-galactose, α -D-glucose, maltose, D-mannitol, L-aspartic acid and L-glutamic acid. In the API ZYM system, esterase (C4), esterase lipase (C8), leucine-, valine- and cystine arylamidases, trypsin, α -chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but lipase (C14), α -galactosidase, β -glucuronidase, α - and β -glucosidases, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. Nitrate is not reduced. Indole is not produced. The major fatty acids (>5 %) are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{15:1}, anteiso-C_{15:1}, iso-C_{15:0} 3-OH, iso-C_{16:0} 3-OH, iso-C_{17:0} 3-OH and summed feature 3. The complete fatty acid composition is given in Table 1.

The type strain, AKS432^T (=KCCM 42306^T=JCM 13824^T), was isolated from a marine red alga collected at Marian Cove, King George Island, South Shetland Islands, Antarctica. The DNA G + C content of the type strain is 34.7 mol%.

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