Algibacter lectus gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from green algae

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Three strains of the marine, gliding, pigmented, facultatively anaerobic, heterotrophic, Gram-negative bacteria were isolated from the green algae *Acrosiphonia sonderi* (Kütz) Kornm and *Ulva fenestrata* Ruprecht inhabiting the Sea of Japan. 16S rDNA sequence analysis indicated that the strains were members of the family *Flavobacteriaceae*, in which they occupied separate lineages. The predominant cellular fatty acids were i15:0, a15:0, i15:1, 15:0, 15:1 ω 6c, i15:0 3-OH and i17:0 3-OH. The DNA base compositions were 31–33 mol% G+C. Based on the phenotypic, genotypic, chemotaxonomic and phylogenetic analyses, the novel bacteria should be placed in a novel taxon as *Algibacter lectus* gen. nov., sp. nov. with type strain KMM 3902^T (=KCTC 12103^T=DSM 15365^T).

The family *Flavobacteriaceae* accommodates chemo-organotrophic, Gram-negative bacteria that can be non-pigmented, or pigmented by carotenoid or flexirubin pigments or both. They can move by gliding or be non-motile and are rod-shaped bacteria with menaquinone 6 as their main respiratory quinone (Bernardet *et al.*, 1996, 2002). The majority of the members of this family are aerobic bacteria belonging to genera such as *Aequorivita*, *Arenibacter*, *Bergeyella*, *Cellulophaga*, *Chryseobacterium*, *Empedobacter*, *Formosa*, *Gelidibacter*, *Mesonina*, *Myroides*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter*, *Weeksella* and *Zobellia* (Bernardet *et al.*, 2002; Bowman & Nichols, 2002;

Ivanova *et al.*, 2001, 2004; Nedashkovskaya *et al.*, 2003b, c, 2004). Representatives of the genera *Capnocytophaga*, *Coenonia*, *Ornithobacterium* and *Riemerella* are characterized by microaerobic and anaerobic growth (Bernardet *et al.*, 2002). Recently, a novel marine bacterium *Muricauda ruestringensis*, with a facultatively anaerobic type of metabolism and appendages, has been described (Bruns *et al.*, 2001).

In this work we report the isolation and identification of three Gram-negative, facultatively anaerobic, gliding, agarolytic marine bacteria isolated from the surface of green algae. Based on the polyphasic study of these bacteria including phylogenetic, genotypic, chemotaxonomic and phenotypic methods, the description of a novel genus *Algibacter* is proposed.

Agarolytic strains KMM 3902^T and KMM 3914 were isolated from samples of the green alga *Ulva fenestrata* Ruprecht and strain KMM 3970 was isolated from the green alga *Acrosiphonia sonderi* (Kütz) Kornm. They were

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The GenBank accession numbers for the 16S rDNA sequences of *Algibacter lectus* KMM 3902^T and KMM 3914 are AY187689 and AY187690, respectively.

collected in Troitsa Bay, Gulf of Peter the Great, Sea of Japan, during June 2000. For strain isolation, 0.1 ml homogenates of algal fronds were transferred onto plates of marine agar 2216 (Difco). After primary isolation and purification, strains were cultivated at 28 °C on the same medium and stored at -80 °C in marine broth (Difco) supplemented with 20 % (v/v) glycerol.

Phylogenetic analysis

Genomic DNA extraction, PCR and sequencing of 16S rDNA followed previous procedures (Kim *et al.*, 1998). Sequence data obtained were aligned with those of representative members of selected genera belonging to the family *Flavobacteriaceae* using PHYDIT version 3.2 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic trees were inferred using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated using the Kimura two-parameter model (Kimura, 1980) and trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1993) algorithms. Bootstrap analysis was performed with 1000 resampled datasets using the SEQBOOT and CONSENSE programs of the PHYLIP package.

Phylogenetic analysis of almost-complete 16S rDNA sequences of strains KMM 3902^T and KMM 3914 (1431 nucleotides) revealed that the strains formed a distinct lineage within the family *Flavobacteriaceae* (Fig. 1). The close relatives of the strains studied, *Psychroserpens burtonensis* and *Gelidibacter algens*, had 93.6 and 93.3 % 16S rDNA sequence identity, respectively, to strain KMM 3902^T, and 93.5 and 93.8 % 16S rDNA sequence identity, respectively, to strain KMM 3914.

Genotypic methods

DNA was isolated following the method of Marmur (1961) and the G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). DNA-DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970).

The DNA G+C contents of KMM 3902^T, KMM 3914 and KMM 3970 were 31.9, 32.5 and 32.9 mol%, respectively. The level of DNA-DNA relatedness between strains KMM 3902^T, KMM 3914 and KMM 3970 was 91–93 %.

Chemotaxonomic methods

Lipids were extracted according to the method of Bligh & Dyer (1959). Two-dimensional micro-thin layer chromatography of polar lipids was carried out using the method of Svetashev & Vaskovsky (1972), using chloroform/methanol/benzene/28 % NH₄OH (65:30:10:6, by vol.) for the first direction and chloroform/methanol/benzene/acetone/acetic acid/water (70:30:10:5:4:1, by vol.) for the second direction (Vaskovsky & Terekhova, 1979).

Fig. 1. Phylogenetic tree based on the 16S rDNA sequences of KMM strains and representative members of the family *Flavobacteriaceae*. Asterisks indicate branches that were also recovered using the maximum-likelihood algorithm. Numbers at nodes indicate bootstrap values (%) and the scale bar represents 0.01 substitutions per nucleotide position.

Analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification system (Microbial ID), except that marine agar 2216 was used to obtain the biomass.

Isoprenoid quinones were extracted from lyophilized cells and analysed as described by Akagawa-Matsushita *et al.* (1992). Menaquinones were detected by monitoring at 270 nm and were identified by comparison with known quinones from the reference strain *Salegentibacter salegens* DSM 5424^T.

All strains contained phosphatidylethanolamine as the main compound in their polar lipid patterns and MK-6 as the major isoprenoid quinone.

Strain KMM 3902^T is characterized by the predominance of branched-chain saturated and unsaturated fatty acids, namely i15:0 (12.5 %), a15:0 (7.2 %), i15:1 (13.4 %), 15:0 (13.4 %), 15:1ω6c (10.9 %), i15:0 3-OH (9.4 %) and i17:0 3-OH (9.1 %). It should be noted that the whole-cell fatty acid composition of strain KMM 3902^T differs significantly from that reported for *Formosa algae* strains (Ivanova *et al.*, 2004) by the presence of i15:0 3-OH and i17:0 3-OH fatty acids and the absence of 17:0 and 19:0 cyclo-fatty acids.

Phenotypic methods

Oxidative or fermentative utilization of glucose was determined using Hugh–Leifson medium modified for marine bacteria (Lemos *et al.*, 1985). Degradation of agar, starch, casein, gelatin, cellulose (filter paper and CM-cellulose), chitin, DNA, urea and alginic acids, flexirubin production, growth at different pH values, production of acid from carbohydrates and susceptibility to antibiotics were tested as described previously (Nedashkovskaya *et al.*, 2003a). Gram-staining reaction, hydrolysis of Tweens 20, 40 and 80, nitrate reduction, production of hydrogen sulphide and indole and β -galactosidase, oxidase, catalase and alkaline phosphatase activities were tested according to the methods of Gerhardt *et al.* (1994). To examine carbon source utilization, a medium that contained 0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract (Difco) and 0.4 % (w/v) carbon source in 1 l of artificial sea water was used. In order to determine the temperature range for growth, bacteria were cultivated on medium A, which consisted of (l⁻¹): 5 g Bacto-peptone (Difco), 2 g Bacto yeast extract (Difco), 1 g glucose, 0.02 g KH₂PO₄ and 0.05 g MgSO₄·7H₂O in 50 % (v/v) natural sea water and 50 % (v/v) distilled water. Bacterial growth at different concentrations of NaCl was checked on medium A that was prepared with distilled water and contained 0, 1, 2, 3, 5, 6, 8, 10 or 12 % (w/v) NaCl. Spreading growth was observed by cultivation on medium B, which contained (l⁻¹): 1 g Bacto-peptone (Difco), 1 g yeast extract (Difco), 15 g agar and half-strength natural sea water under high-moisture conditions. Gliding motility was determined as described by Bowman (2000). Cell morphology was observed by scanning electron microscopy (SEM) as described by Bruns *et al.* (2001).

Strains isolated in this study were Gram-negative, chemo-organotrophic, facultatively anaerobic and were motile by

gliding. Growth of strain KMM 3902^T occurred in media that contained 1–5 % NaCl, strains KMM 3914 and KMM 3970 grew in up to 6 % NaCl. Optimal growth was observed at 2–3 % NaCl. The maximum growth temperatures of strains KMM 3902^T, KMM 3914 and KMM 3970 were 33, 30 and 35 °C, respectively, with optimum growth occurring at 21–23 °C. The pH range for growth was 5.5–10.0, with optimum growth occurring between 7.5 and 8.3. Strains KMM 3902^T and KMM 3970 degraded Tween 20, in contrast with strain KMM 3914. Also, strains KMM 3902^T and KMM 3970 formed acid from *N*-acetylglucosamine and rhamnose, respectively. Other physiological and biochemical characteristics of the strains studied are listed in the species description and Table 1.

The differential features of the strains studied and related members of the family *Flavobacteriaceae* are represented in Table 1. Fermentative metabolism separates the strains studied from all relatives shown in Table 1. Also, strains KMM 3902^T, KMM 3914 and 3970 differ from the nearest neighbour *Formosa algae* by the requirement of Na⁺ for growth and hydrolysis of agar. Oxidase and agarase activities distinguish algal isolates from *Gelidibacter algens*. The presence of gliding motility, oxidase and agarase activities, acid formation from carbohydrates and casein hydrolysis allow the separation of KMM strains from the Antarctic bacterium *Psychroserpens burtonensis*.

Phenotypic characterization of the strains studied (including their fermentative utilization of glucose, inability to grow without Na⁺ ions and presence of agar hydrolysis) in combination with phylogenetic distances and distinctiveness of cellular fatty acid composition, allow the differentiation of strains KMM 3902^T, KMM 3914 and KMM 3970 from their closest relative, *Formosa algae*. Low sequence similarities of the strains tested with other flavobacteria

Table 1. Differential characteristics of *Algibacter lectus* gen. nov., sp. nov. and close relatives of the family *Flavobacteriaceae*

Data from Bowman *et al.* (1997); Macián *et al.* (2002); Ivanova *et al.* (2004) and this study. F, Fermentative; A, aerobic; –, negative; +, positive; V, variable; ND, not determined. All taxa are negative for nitrate reduction and H₂S production.

Characteristic	<i>Algibacter lectus</i>	<i>Formosa algae</i>	<i>Gelidibacter</i>	<i>Psychroserpens burtonensis</i>
Metabolism	F	A	A	A
Gliding motility	+	+	+	–
Oxidase/catalase	+ / +	+ / +	– / +	– / +
Requirement for Na ⁺ for growth	+	–	+	+
Acid formation from carbohydrates	+	+	+	–
Growth at 37 °C	–	–	V	–
Hydrolysis of:				
Agar	+	–	–	–
Casein	–	ND	V	+
Gelatin	+	+	V	V
Starch	+	+	+	–
DNA	–	–	V	–
G + C content (mol%)	31–33	34–35	36–40	27–29

described to date (89.2–92.7%) demonstrate clearly that the bacteria isolated in this study represent a novel genus.

Thus, the polyphasic data presented in this paper support the conclusion that the bacteria studied could not be affiliated to any taxa currently included in the family *Flavobacteriaceae*. Consequently, we propose that the strains studied should be classified as *Algibacter lectus* gen. nov., sp. nov.

Description of *Algibacter* gen. nov.

Algibacter (Al.gi.bac'ter L. fem. n. *alga* seaweed; N.L. masc. n. *bacter* from Gr. n. *bacterion* rod, N.L. masc. n. *Algibacter* rod isolated from seaweed).

Rod-shaped cells, motile by gliding. Gram-negative. Do not form endospores. Facultatively anaerobic. Produce non-diffusible orange pigments. No flexirubin-type pigments are formed. Chemo-organotroph. Cytochrome oxidase-, catalase-, alkaline phosphatase- and β -galactosidase-positive. The major respiratory quinone is MK-6. The main cellular fatty acids are straight-chain unsaturated and branched-chain unsaturated fatty acids i15:0, a15:0, i15:1, 15:0, 15:1 ω 6c, i15:0 3-OH and i17:0 3-OH. Phosphatidylethanolamine is the main polar lipid compound. As determined by 16S rDNA sequence analysis, the genus *Algibacter* is a member of the family *Flavobacteriaceae*, *Cytophaga*–*Flavobacterium*–*Bacteroides* phylum. The type species is *Algibacter lectus*.

Description of *Algibacter lectus* sp. nov.

Algibacter lectus (lec'tus. L. masc. adj. *lectus* select, referring to a bacterium that forms select, beautiful colonies).

Main characteristics are as those given for the genus. In addition, cells range from 0.4 to 0.5 μ m in width and 2 to 3 μ m in length. Colonies are circular, 3–4 mm in diameter, convex, shiny, sunken into agar, bright orange in colour and translucent on solid media containing high nutrient components. Requires Na⁺ for growth. Growth occurs at 4–35 °C. Optimal temperature for growth is 21–23 °C. Growth occurs at 1–6% NaCl. Decomposes agar, gelatin, alginate, starch, Tween 20 and Tween 40. Does not hydrolyse casein, Tween 80, cellulose (CM-cellulose and filter paper), chitin or DNA. Forms acid from cellobiose, fucose, galactose, glucose, maltose, sucrose and xylose, but not from arabinose, lactose, melibiose, raffinose, glycerol, inositol or mannitol. Can oxidize rhamnose and *N*-acetylglucosamine. Utilizes lactose and mannose, but not arabinose, adonitol, dulcitol, mannitol, inositol, sorbitol, malonate or citrate. Nitrate is not reduced. Indole, H₂S and acetoin (Voges–Proskauer reaction) production are negative. Susceptible to carbenicillin, lincomycin and oleandomycin. Resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin, neomycin, polymixin B, streptomycin and tetracycline. The G+C content of the DNA is 31.0–33.0 mol%.

Type strain is KMM 3902^T (=KCTC 12103^T=DSM 15365^T). Strains have been isolated from green algae *Acrosiphonia sonderi* and *Ulva fenestrata*, Sea of Japan.

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