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Brevibacterium celere sp. nov., isolated from degraded thallus of a brown alga

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Two whitish yellow, Gram-positive, non-motile, aerobic bacteria were isolated from enrichment culture during degradation of the thallus of the brown alga *Fucus evanescens*. The bacteria studied were chemo-organotrophic, mesophilic and grew well on nutrient media containing up to 15 % (w/v) NaCl. The DNA G+C content was 61 mol%. The two isolates exhibited a conspecific DNA–DNA relatedness value of 98 %, indicating that they belong to the same species. A comparative analysis of 16S rRNA gene sequences revealed that strain KMM 3637^T formed a distinct phyletic lineage in the genus *Brevibacterium* (family *Brevibacteriaceae*, class *Actinobacteria*) and showed the highest sequence similarity (about 97 %) to *Brevibacterium casei*. DNA–DNA hybridization experiments demonstrated 45 % binding with the DNA of *B. casei* DSM 20657^T. Physiological and chemotaxonomic characteristics (*meso*-diaminopimelic acid in the peptidoglycan, major cellular fatty acids 15 : 0ai and 17 : 0ai) of the bacteria studied were consistent with the genomic and phylogenetic data. On the basis of the results of this study, a novel species, *Brevibacterium celere* sp. nov., is proposed. The type strain is KMM 3637^T (=DSM 15453^T=ATCC BAA-809^T).

The genus *Brevibacterium* was proposed by Breed (1953) for some Gram-positive, non-spore-forming, non-branching rods formerly classified as members of the genus '*Bacterium*'. A number of species with diverse morphological, physiological and biochemical properties were subsequently included in the genus (Breed, 1957). The description of the genus was later emended and restricted only to the species that correspond to the type species *Brevibacterium linens* in terms of morphological and chemotaxonomic characteristics (Collins *et al.*, 1980). Along with *B. linens*, the following *Brevibacterium* (*sensu stricto*) species are currently recognized within the genus: *Brevibacterium casei*, *Brevibacterium epidermidis* (Collins *et al.*, 1983), *Brevibacterium iodinum* (Collins *et al.*, 1980), *Brevibacterium mcbrellneri* (McBride *et al.*, 1993), *Brevibacterium otitidis* (Pascual *et al.*, 1996), *Brevibacterium avium* (Pascual & Collins, 1999), *Brevibacterium paucivorans* (Wauters *et al.*, 2001) and *Brevibacterium luteolum* (Wauters *et al.*, 2003; Euzéby & Tindall, 2004). Brevibacteria are isolated from dairy milk products, encountered in humans as commensals or opportunistic pathogens, are found to be residents of poultry and also occur in marine and terrestrial environments, as reported by Collins (1992) and Jones & Keddie (1986), and are available from public databases.

Here we describe two strains, KMM 3637^T and KMM 6008, isolated from degraded thallus of the brown alga *Fucus evanescens*. Algae were collected (by scuba-diving) in midsummer (July 1999) at Kraternaya Bay, Kuril Islands, in the Pacific Ocean, during the 23rd scientific expedition of the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Brevibacterium celere* KMM 3637^T is AY228463.

A consensus tree including '*B. sanguinis*' and the fatty acid compositions of *B. celere* sp. nov. strains are available as supplementary material in IJSEM Online.

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R/V *Akademician Oparin*. The set-up of the enrichment experiments and bacterial isolation were as described elsewhere (Ivanova *et al.*, 2002), with the modification that a protein inhibitor of endo-(1,3)-β-D-glucanases (Yermakova *et al.*, 2002) was added to the enrichment culture (E. P. Ivanova, unpublished). Cultures were maintained on marine agar (Difco) plates and medium B [0·2 % (w/v) Bacto peptone (Difco); 0·2 % (w/v) casein hydrolysate (Merck); 0·2 % (w/v) Bacto yeast extract (Difco); 0·1 % (w/v) glucose; 0·02 % (w/v) KH_2PO_4; 0·005 % (w/v) $MgSO_4.7H_2O$; 1·5 % (w/v) Bacto agar (Difco); 50 % natural sea water; 50 % distilled water at pH 7·5–7·8] and in marine broth supplemented with 30 % (v/v) glycerol at −80 °C. All isolates were streaked on agar plates from broth cultures every 6 months to ensure purity and viability.

Unless otherwise indicated, the phenotypic properties used for characterization of the two strains were tested by following established procedures (Smibert & Krieg, 1994) and as described elsewhere (Ivanova *et al.*, 1996). The cell morphology was determined on cultures grown for 12, 24, 36 and 72 h on trypticase soy agar (Oxoid) and marine agar at 28 °C. The following physiological and biochemical properties were examined: oxidation/fermentation of glucose (Hugh & Leifson, 1953), denitrification (Azegami *et al.*, 1987), catalase activity, gelatin liquefaction, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activity, indole and H_2S production and the ability to hydrolyse starch, Tween 80 and casein. Alginate (sodium salt) (0·1 %, w/v) hydrolysis was determined by assessing the development of clear zones around the colonies. The haemolytic activity of the strains studied was detected on blood agar comprising 40 g trypticase soy agar l^{-1}, 50 ml sheep blood and 950 ml water. Haemolytic activity on mouse erythrocytes and cytotoxicity on Ehrlich cells were tested on butanol extracts of the strains, as described earlier (Ivanova *et al.*, 2001). Pyrazinamidase and acid production from 2,3-butylene glycol were detected as described by Wauters *et al.* (2001). Oxidative utilization of 95 carbon sources was tested by using Biolog GN Microplates (Rüger & Krambeck, 1994). Growth at different temperatures, NaCl concentrations or pH values was measured using optical density at 660 nm after 24 h incubation in medium B. The incubation temperatures employed ranged from 5 to 45 °C. The NaCl concentrations used were in the range 0–15 % (w/v). The pH was adjusted within the range 4·5–12·0 by using HCl and NaOH. Susceptibility to antibiotics was tested by using the routine diffusion plate method, employing medium B agar and discs impregnated with the antibiotics listed in the species description. The results of examination of the morphological and physiological properties are given in Table 1 and in the species description.

Table 1. Differential phenotypic characteristics of *B. celere* sp. nov. and other species of the genus

Taxa: 1, *B. celere* sp. nov.; 2, *B. avium*; 3, *B. mcbrellneri*; 4, *B. casei*; 5, *B. epidermidis*; 6, *B. otitidis*; 7, *B. iodinum*; 8, *B. linens*; 9, *B. paucivorans*; 10, *B. luteolum*. Symbols: +, positive; −, negative, V(+), variable, mostly positive; W, weak reaction; ND, no data available. Data are from this study, Jones & Keddie (1986), Pascual & Collins (1999) and Wauters *et al.* (2001, 2003).

Characteristic	1	2	3	4	5	6	7	8	9	10
Colonies	Whitish yellow, smooth	Whitish-grey, smooth	Greyish, dry	Whitish-grey, smooth	Whitish-yellow, smooth	Yellowish, smooth	Whitish-grey, smooth	Yellow-orange smooth	Greyish, smooth or sticky	Yellowish, smooth
Growth at:										
12 °C	+	−	−	−	−	−	−	−	−	−
20 °C	+	ND	−	+	+	−	+	+	−	+
37 °C	+	+	+	+	+	+	+	W	+	+
Oxidase	+	+	ND	−	−	ND	+	W	ND	ND
NaCl tolerance (%, w/v)	15	ND	ND	15	15	ND	ND	15	ND	10
Hydrolysis of:										
Casein	−	+	ND	+	+	+	W	+	−	+
Gelatin	+	+	ND	+	+	+	+	+	W	+
Nitrate reduction	−	+	−	V(+)	V(+)	−	+	+	−	−
Acid production from 2,3-butylene glycol	−	ND	+	−	−	−	−	−	−	−
Utilization of:										
D-Arabinose	−	−	−	+	−	−	−	−	−	−
Mannitol	−	+	−	−	+	−	−	−	−	−
Gluconate	−	−	−	+	+	−	−	−	−	−
Pyrazinaminidase	−	ND	−	+	+	+	+	+	−	+
α-Glucosidase	+	−	−	V(+)	−	−	−	−	−	−

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The 16S rRNA gene was amplified and sequenced by MIDI Labs. Briefly, the primers used for the amplification corresponded to *Escherichia coli* positions 5 and 1540. Amplification products were purified using Microcon 100 (Millipore) molecular mass cut-off membranes and were checked for quality and quantity on an agarose gel. Cycle sequencing of the 16S rRNA gene amplification products was carried out using AmpliTaq ES DNA polymerase (PE Biosystems) and rhodamine dye terminators (PE Biosystems). The samples were electrophoresed on an ABI Prism 377 DNA sequencer (PE Biosystems).

Phylogenetic analyses were done as reported previously (Ivanova *et al.*, 2004) and are explained in detail at http://bioinfo.unice.fr (Publications section, document: Phylogeny_How). The domains used to construct the final phylogenetic trees were positions 75–996 and 1013–1413 of the strain KMM 3637^T sequence, excluding domains for which alignments were insecure. Phylogenetic trees were constructed according to three methods, BIONJ (Gascuel, 1997), maximum likelihood and maximum parsimony, as described by Ivanova *et al.* (2004). Fig. 1 shows a consensus tree for neighbour-joining (bootstrap analysis, 1000 replications), maximum-likelihood and maximum-parsimony analyses. The 16S rRNA gene sequence analyses revealed that strain KMM 3637^T is a member of the genus *Brevibacterium*, *B. casei* being the closest phylogenetic neighbour (with a similarity value of 97 %). However, because eight positions of 16S RNA gene sequence of KMM 3637^T were not identified, this percentage may be greater (up to 98 %). According to published data, bacteria that differ by more than 2·5 % at the 16S rRNA gene sequence level are unlikely to exhibit more than 60–70 % similarity at the genomic DNA level (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001): we were therefore at the threshold level for the definition of a novel species. Additional DNA–DNA hybridization experiments were performed to resolve this issue (see below). It should also be stated that, during the revision of the manuscript, a new sequence, which was the closest to that of strain KMM 3637^T, appeared in the public database ('*Brevibacterium sanguinis*', accession no. AJ564859; see the Supplementary Figure in IJSEM Online). However, we did not take it into account since the species does not yet have a validly published name.

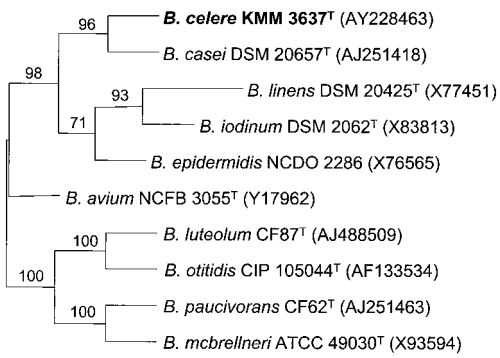

Fig. 1. Phylogenetic position of *B. celere* sp. nov. KMM 3637^T according to 16S rRNA gene sequence analysis. The topology shown was obtained using the BIONJ algorithm (Gascuel, 1997) and 1000 bootstrap replications with Kimura's two-parameter correction (Kimura, 1980) for the distances. Bootstrap percentages are indicated only for branches that were also retrieved by maximum parsimony and maximum likelihood ($P<0·01$); these branches should be considered as the only robust clusters identified by this analysis.

The DNA was isolated according to the method of Marmur (1961). The G+C content of the DNA (61–62 mol%) was determined by using the thermal denaturation method of Marmur & Doty (1962). DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970). The level of DNA relatedness between the two strains from the alga was 98 %. DNA hybridization analysis also indicated that there was 45 % hybridization between strain KMM 3637^T and *B. casei* DSM 20657^T. These data clearly indicate that the novel strains belong to a separate genomic species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

The amino acid composition of the peptidoglycan was determined using TLC on cellulose (modified method of Hasegawa *et al.*, 1983; Rhuland *et al.*, 1955). The peptidoglycan contained *meso*-diaminopimelic acid, which is characteristic of the genus *Brevibacterium* [peptidoglycan type A1γ (*m*-Dpm direct, type A31); DSMZ, 2001].

Analysis of fatty acid methyl ethers was performed by GLC as described by Svetashev *et al.* (1995). The most relevant cellular fatty acids were saturated, anteiso- and iso-methyl-branched acids, namely 12-methyltetradecanoic (15 : 0ai) and 14-methylhexadecanoic (17 : 0ai) fatty acids, which comprised up to 80 % of the total, while 15 : 0i and 16 : 0i were present as minor components (Supplementary Table in IJSEM Online).

Phenotypically, the two strains differed from other species of the genus *Brevibacterium* by the characteristics presented in Table 1. The strains are delineated from the species known to be tolerant of up to 15 % (w/v) NaCl (*B. linens*, *B. casei* and *B. epidermidis*) by colony colour, the presence of oxidase activity, the lack of nitrate reduction, the inability to hydrolyse casein and the utilization of different carbon sources. The novel organisms exhibited the ability to hydrolyse some algal polysaccharides (laminaran, alginate) and did not exhibit haemolytic, cytotoxic or antibacterial activities. The growth temperature range, the salt tolerance and the inability to reduce nitrate or hydrolyse casein differentiate the novel organisms from other *Brevibacterium* species. Consequently, we propose that the two strains isolated from brown algae be classified as a novel species, for which the name *Brevibacterium celere* sp. nov. is proposed.

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Description of *Brevibacterium celere* sp. nov.

Brevibacterium celere (ce′le.re. L. neut. adj. *celere* rapid, indicating the rapid growth on nutrient media).

Cells are Gram-positive, non-motile, non-acid-fast, non-spore-forming rods with coryneform morphology. Colonies are circular, convex, with entire margins, whitish yellow in colour and of a smooth and butyrous consistency. Salt-tolerant. Growth occurs at 0–15 % (w/v) NaCl. Growth occurs at 12–42 °C. No growth is detected at 4 °C or at 45 °C. Alkali-tolerant. Growth occurs at pH 5–10, with optimum growth at pH 8·5–9·0. The organism is catalase- and oxidase-positive and exhibits aerobic metabolism. Gelatin, laminaran and alginate are hydrolysed, but casein and starch are not. Nitrate is not reduced to nitrite. Tests for urease and pyrazinamidase are negative. No acid is formed from glucose, maltose, lactose, sucrose, galactose, xylose or sorbose. According to the Biolog results, the following substrates are utilized: dextrin, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-fucose, α-D-glucose, maltose, sucrose, D-trehalose, turanose, methylpyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-gluconic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketobutyric acid, DL-lactic acid, propionic acid, quinic acid, sebacic acid, succinic acid, bromosuccinic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, L-histidine, hydroxyproline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, phenylethylamine, putrescine and glycerol. In addition, strain KMM 3581 utilizes D-arabitol and i-erythritol. Susceptible to carbenicillin (10 μg) and oleandomycin (30 μg). Not susceptible to ampicillin (10 μg), lincomycin (15 μg), kanamycin (30 μg), benzylpenicillin (10 μg), neomycin (30 μg), streptomycin (30 μg), gentamicin (10 μg) or polymyxin B (25 μg). The predominant cellular fatty acids are odd-numbered, i.e. 15 : 0ai and 17 : 0ai. The peptidoglycan contains *meso*-diaminopimelic acid (peptidoglycan type A1γ). The G+C content of the DNA is 61·4 mol%.

The type strain is KMM 3637^T (=DSM 15453^T=ATCC BAA-809^T), isolated from the brown alga *Fucus evanescens*.

Note added in proof

After this paper had been accepted for publication, four more novel species of *Brevibacterium* were reported: *Brevibacterium picturae* (Heyrman *et al.*, 2004), '*Brevibacterium antiquum*', '*Brevibacterium aurantiacum*' and '*Brevibacterium permense*' (Gavrish *et al.*, 2004).

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