Cellulophaga tyrosinoxydans sp. nov., a tyrosinase-producing bacterium isolated from seawater

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An aerobic, gliding, yellow-pigmented bacterium lacking flagella and showing strong tyrosinase activity, designated strain EM41^T, was isolated from seawater on the eastern coast of Jeju Island in Korea. Growth was observed at 15–35 °C (optimum, 25–30 °C) and at pH 6.5–9.0 (optimum, pH 7.0–8.5). Cells were Gram-negative, negative for flexirubin pigments and catalase- and oxidase-positive. The G + C content of the genomic DNA was 33.5 mol% and the major respiratory quinone was menaquinone-6 (MK-6). Comparative 16S rRNA gene sequence analysis showed that strain EM41^T formed a distinct phyletic line within the genus *Cellulophaga* with a 100 % bootstrap value and was most closely related to *Cellulophaga pacifica* KMM 3664^T (97.0 % sequence similarity). The level of DNA–DNA relatedness between strain EM41^T and *C. pacifica* KMM 3664^T was about 17.8 %. On the basis of phenotypic and genotypic data, strain EM41^T is considered to represent a novel species of the genus *Cellulophaga*, for which the name *Cellulophaga tyrosinoxydans* sp. nov. is proposed. The type strain is EM41^T (=KCTC 22297^T=DSM 21164^T).

The genus *Cellulophaga*, belonging to the family *Flavobacteriaceae* within the phylum *Bacteroidetes*, was first described by Johansen *et al.* (1999). At the time of writing, the genus comprises five recognized species, namely *Cellulophaga baltica*, *Cellulophaga fucicola*, *Cellulophaga lytica* (Johansen *et al.*, 1999), *Cellulophaga algicola* (Bowman, 2000) and *Cellulophaga pacifica* (Nedashkovskaya *et al.*, 2004), all isolated from marine habitats. In the present study, the taxonomic position of a novel bacterium with strong tyrosinase activity was investigated based on a polyphasic analysis. This bacterium, designated strain EM41^T, is shown to represent a novel species of the genus *Cellulophaga*.

Strain EM41^T was isolated from a seawater sample that was collected from the eastern coast of Jeju Island in Korea. Isolation was by means of the serial dilution plating method and incubation on marine agar 2216 (MA; Difco) at 25 °C for 5 days. Subcultivation was routinely performed on MA at 30 °C for 3 days under aerobic conditions.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EM41^T is EU443205.

A transmission electron micrograph of cells of strain EM41^T is available with the online version of this paper.

Sequencing of the 16S rRNA gene of strain EM41^T was carried out as described by Lane (1991). The resulting 16S rRNA gene sequence (1479 nt) was compared with available 16S rRNA gene sequences from GenBank by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation, and was aligned with sequences of closely related bacteria by using the CLUSTAL W software program (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using three different algorithms, neighbour-joining, maximum-likelihood and maximum-parsimony, available within the PHYLIP software, version 3.6 (Felsenstein, 2002). Resulting tree topologies were evaluated by bootstrap analysis based on 1000 resamplings. Sequence similarity values between the novel strain and related bacteria were computed by using the Nucleotide Similarity Search program (<http://www.ebi.ac.uk/fasta33/nucleotide.html>; Pearson & Lipman, 1988). DNA–DNA hybridization was performed to evaluate the genomic DNA relatedness between strain EM41^T and *C. pacifica* KMM 3664^T by using the fluorometric microplate method (Ezaki *et al.*, 1989). Fluorometric data recorded after 30 min reaction of the substrates were used for calculation of DNA–DNA hybridization values. The signals produced by

self-hybridization were inferred as 100 % and percentages of DNA–DNA relatedness were calculated from the mean values of five replications.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain EM41^T formed a distinct phylogenetic line within the genus *Cellulophaga* with a 100 % bootstrap value (Fig. 1). The overall topology of the neighbour-joining tree was supported by the trees built according to the maximum-likelihood and maximum-parsimony algorithms. Comparative 16S rRNA gene sequence analysis showed that strain EM41^T was related most closely to *C. pacifica* KMM 3664^T, *C. baltica* NN015840^T, *C. algicola* IC166^T, *C. fucicola* NN015860^T and *C. lytica* ATCC 23178^T with similarities of 97.0, 96.7, 94.7, 94.0 and 92.4 %, respectively. The level of DNA–DNA relatedness obtained from hybridization experiments between strain EM41^T and *C. pacifica* KMM 3664^T was 17.8 %, which is clearly below the 70 % threshold generally accepted for species delin-

eation (Rosselló-Mora & Amann, 2001; Wayne *et al.*, 1987).

Growth was tested at 30 °C on MA, R2A agar (Difco), laboratory-prepared Luria–Bertani (LB) agar, trypticase soy agar (TSA; Difco) and nutrient agar (NA; Difco). Physiological characteristics of strain EM41^T were examined by growing the isolate on MA at different temperatures (5–50 °C at 5 °C intervals) and in marine broth adjusted to different pH values (5.0–10.0 at 0.5 pH unit intervals) as described by Gomori (1955). Requirement for and tolerance to NaCl were determined with 0–10 % (w/v) NaCl (increments of 1 %) in nutrient broth (NB) supplemented with modified artificial seawater (per litre: 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂). Gram staining was performed by using a bioMérieux Gram stain kit according to the manufacturer's instructions. Cell morphology and flagellar and gliding motility were studied by using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described by Bernardet *et al.* (2002) and Jeon *et al.* (2004). Antibiotic susceptibility tests were performed in duplicate as described by Reva *et al.* (1995) by using filter-paper discs (diameter 8 mm; Whatman) containing the following antibiotics: ampicillin (10 µg), polymyxin B (100 U), streptomycin (50 µg), penicillin G (10 U), chloramphenicol (100 µg), gentamicin (30 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg), carbenicillin (100 µg) and novobiocin (50 µg). Oxidase activity was tested based on oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated based on the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. The production of flexirubin-type pigments was investigated by using the KOH test following the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Hydrolysis of casein, Tweens 20 and 80, aesculin, urea, tyrosine, carrageenan, starch and carboxymethylcellulose was investigated on MA after 7 days incubation at 30 °C according to the methods of Lányi (1987) and Smibert & Krieg (1994). The ability of strain EM41^T to oxidize tyrosine was evaluated based on the production of black diffusible pigments on MA containing 1 % (w/v) tyrosine. The test for nitrate reduction was performed according to the method of Lányi (1987) and acid production from carbohydrates was tested as described by Leifson (1963). Additional enzymic activities and biochemical features were determined by using API ZYM and API 20NE kits at 30 °C as recommended by the manufacturer (bioMérieux). Anaerobic growth was assessed on MA under anaerobic conditions (with 4–10 % CO₂) by using the GasPak Plus system (BBL) at 30 °C for 20 days.

Cells of strain EM41^T were Gram-negative straight rods of 0.4–0.6 µm wide and 1.3–2.2 µm long (see Supplementary Fig. S1 in IJSEM Online). Strain EM41^T was able to grow in NB supplemented with modified artificial seawater containing 1–7 % (w/v) NaCl; optimal growth occurred in the

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain EM41^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates; only values >50 % are shown. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Bergeyella zoohelcum* ATCC 43767^T was used as an outgroup. Bar, 0.1 changes per nucleotide position.

presence of 2–4 % NaCl. The phenotypic characteristics of strain EM41^T are detailed in Table 1 and in the species description below. Some of these characteristics are in accordance with those of recognized members of the genus *Cellulophaga*, whereas others, such as hydrolysis of agar, can be used to differentiate strain EM41^T from closely related species of the genus *Cellulophaga* (Table 1).

Whole-cell fatty acids of strain EM41^T were analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.) on cells grown on MA at 30 °C for 3 days. Analysis of isoprenoid quinones was carried out by using the methods described by Komagata & Suzuki (1987). The DNA G+C content was determined by using an HPLC system fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka &

Komagata (1984). The major respiratory lipoquinone was menaquinone-6 (MK-6). The major cellular fatty acids of strain EM41^T were iso-C_{15:0} (17.0 % of the total), C_{15:0} (15.2 %), summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 11.2 %), iso-C_{15:1} G (9.5 %), iso-C_{16:0} 3-OH (9.0 %), iso-C_{17:0} 3-OH (8.9 %), C_{15:0} 3-OH (5.3 %), C_{15:1}ω6c (5.0 %), C_{16:0} 3-OH (2.2 %), C_{10:0} (2.2 %), iso-C_{16:0} (1.8 %), iso-C_{17:1}ω9c (1.6 %), iso-C_{16:1} H (1.6 %), C_{15:0} 3-OH (1.5 %), C_{17:1}ω6c (1.2 %), C_{16:0} (1.1 %) and iso-C_{14:0} (1.0 %). Trace amounts (<1 %) of the following fatty acids were also found: anteiso-C_{15:0}, unknown ECL 16.582, C_{18:1}ω5c, C_{18:0}, iso-C_{14:0} 3-OH, C_{16:1}ω5c, C_{17:0} 3-OH and 10 methyl C_{18:0}. The G+C content of the genomic DNA of strain EM41^T was 33.5 mol%. The fatty acid composition, major lipoquinone and DNA G+C content of strain EM41^T were in accordance with those of members of the genus *Cellulophaga* (Johansen *et al.*, 1999; Bowman, 2000; Nedashkovskaya *et al.*, 2004).

The data obtained from the present polyphasic study suggest that strain EM41^T represents a novel species of the genus *Cellulophaga*, for which the name *Cellulophaga tyrosinoxydans* sp. nov. is proposed.

Description of *Cellulophaga tyrosinoxydans* sp. nov.

Cellulophaga tyrosinoxydans (ty.ro.sin.o'xy.dans. N.L. n. *tyrosinum* tyrosine; N.L. part. adj. *oxydans* oxidizing; N.L. part. adj. *tyrosinoxydans* pertaining to the ability to oxidize tyrosine).

Cells are Gram-negative rods, 0.4–0.6 µm wide and 1.3–2.2 µm long. Cells lack flagella but gliding motility is observed. Colonies on MA are yellow, of low convexity and circular with entire margins. Grows on MA but not on R2A agar, LB agar, TSA or NA. Growth occurs at 15–35 °C (optimum, 25–30 °C), at pH 6.5–9.0 (optimum, 7.0–8.5) and in the presence of 1–7.0 % (w/v) NaCl (optimum, 2–4 %). Anaerobic growth is not observed after 20 days at 30 °C on MA. Catalase- and oxidase-positive. Nitrate is reduced to nitrite, but nitrogen gas is not produced. A very strong tyrosine-oxidizing (tyrosinase) activity is present, but tyrosine is not hydrolysed. Aesculin, Tweens 20 and 80, gelatin, starch and carboxymethylcellulose are hydrolysed, but agar, casein, urea and carrageenan are not. Flexirubin-type pigments are not produced. Acid is produced from raffinose, lactose, melibiose, D-fructose, D-galactose, D-glucose and D-mannitol, but not from *myo*-inositol, L-arabinose, D-mannose or arbutin. Positive for assimilation of D-glucose, potassium gluconate, adipic acid and malic acid, but negative for assimilation of L-arabinose, maltose, D-mannitol, *N*-acetylglucosamine, D-mannose, capric acid, trisodium citrate and phenylacetic acid (API 20NE). Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase activities are present, but α-chymotrypsin, β-glucuronidase and

Table 1. Differential phenotypic characteristics between strain EM41^T and recognized species of the genus *Cellulophaga*

Strains: 1, EM41^T; 2, *C. pacifica* KMM 3664^T (data from Nedashkovskaya *et al.*, 2004); 3, *C. baltica* NN015840^T (Johansen *et al.*, 1999); 4, *C. algicola* IC166^T (Bowman, 2000); 5, *C. fucicola* NN015860^T (Johansen *et al.*, 1999); 6, *C. lytica* ATCC 23178^T (Johansen *et al.*, 1999). All strains were positive for the following characteristics: gliding motility, oxidase and catalase production, growth in the presence of 6 % NaCl, and hydrolysis of starch and gelatin. All strains were negative for the following characteristics: flexirubin-type pigments, growth in the presence of 10 % NaCl, and susceptibility to lincomycin and oleandomycin. +, Positive; –, negative; NA, no data available.

Characteristic	1	2	3	4	5	6
Growth with/at:						
8 % NaCl	–	+	–	–	–	–
37 °C	–	–	–	–	–	+
Hydrolysis of:						
Agar	–	+	+	+	+	+
Carrageenan	–	NA	+	NA	+	+
Casein	–	–	+	–	+	–
Tyrosine	–	NA	NA	+	NA	+
Tween 20	+	+	+	–	+	+
Tween 80	+	+	+	–	+	+
CM-cellulose	+	–	+	+	+	+
Acid production from:						
Arabinose	–	+	–	–	–	–
Galactose	+	+	–	–	–	+
Mannitol	+	+	+	–	–	–
Raffinose	+	+	–	–	–	–
Susceptibility to:						
Ampicillin	+	–	–	–	–	–
Streptomycin	–	+	–	–	–	–
Tetracycline	–	–	–	+	–	–
Nitrate reduction	+	+	+	+	–	–
DNA G+C content (mol%)	33.5	31.6	34.6	33.4	34.0	33.0

β -fucosidase activities are absent; weak enzymic activities are observed for leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin and α -mannosidase (API ZYM). Resistant to polymyxin B, streptomycin, gentamicin, tetracycline, neomycin, kanamycin and carbenicillin, but sensitive to penicillin G, ampicillin, lincomycin, chloramphenicol, oleandomycin and novobiocin. The major cellular fatty acids are C_{15:0}, iso-C_{15:0}, summed feature 3 (comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH), iso-C_{15:0} G, iso-C_{16:0} 3-OH, iso-C_{17:0} 3-OH, iso-C_{15:0} 3-OH and C_{15:1} ω 6c. The major isoprenoid quinone is menaquinone-6 (MK-6).

The type strain, EM41^T (=KCTC 22297^T=DSM 21164^T), was isolated from seawater on the eastern coast of Jeju Island, Korea. The DNA G + C content of the type strain is 33.5 mol% (HPLC).

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