Euzebyella marina sp. nov., isolated from seawater

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Abstract
A Gram-stain-negative, aerobic, yellow-pigmented, non-flagellated, non-gliding, oxidase- and catalase-positive bacterium, designated CY01†, was isolated from seawater of the Yellow Sea. CY01† grew at 15–37 °C (optimum, 30 °C), pH 5–8 (optimum, 6.5–7.5) and with 0.5–12 % (w/v) NaCl (optimum, 0.5–3.5 %). It could not produce flexirubin-type pigment or reduce nitrate to nitrite. CY01† showed the highest 16S rRNA gene sequence similarity to the type strain of Euzebyella saccharophila (97.0 %) and clustered tightly with the species of the genus Euzebyella in the phylogenetic trees based on the 16S rRNA gene sequences. The major cellular fatty acids of CY01† were iso-C15:0, iso-C15:0 3-OH and iso-C17:0 3-OH and the major respiratory quinone was menaquinone MK-6. Polar lipids included phosphatidylethanolamine (PE), four unidentified lipids and one unidentified aminolipid. The genomic DNA G+C content was 38.2 mol%. Based on the results of the polyphasic characterization of CY01†, it represents a novel species of the genus Euzebyella, for which the name Euzebyella marina sp. nov. is proposed. The type strain is CY01† (=CCTCC AB 2014348=CCTC 42440†).

The genus Euzebyella, belonging to the family Flavobacteriaceae in the phylum Bacteroidetes, was originally proposed by Lucena et al. [1] and currently contains only one species: Euzebyella saccharophila, the type strain of which was isolated from seawater from the western Mediterranean Sea. In the present study, we describe a novel bacterial strain, designated CY01†, which was isolated from seawater from the Yellow Sea.

The seawater sample was collected at the site H01 (36.0° N, 121.1° E) at a water depth of 3.9 m in the Yellow Sea using a conductivity–temperature–depth (CTD) system (Seabird 911 Plus) during a cruise in the Bohai Sea and Yellow Sea of R. V. Dongfang Hong 2 organized by the National Natural Science Foundation of China (NSFC) in April, 2014. Strain CY01† was isolated from a seawater sample using the dilution plating technique on a solid medium [containing 0.2 % yeast extract, 0.3 % casein, 0.5 % gelatin, 1.5 % agar and artificial sea water (designated ASW1), containing 2.8 % NaCl, 0.5 % MgCl2, 0.2 % MgSO4, 0.1 % CaCl2, 0.1 % KCl, 0.001 % FeSO4 and distilled water; [2]), pH=7.5] at 15 °C. CY01† was routinely cultivated in TYS broth [containing 0.5 % tryptone, 0.1 % yeast extract and artificial sea water prepared according to another formula (designated ASW2: containing 0.0002 % NH4NO3, 0.0027 % H3BO3, 0.1 % CaCl2, 0.0001 % FePO4, 0.5 % MgCl2, 0.01 % KBr, 0.1 % KCl, 0.02 % NaHCO3, 2.4 % NaCl, 0.0003 % NaF, 0.0002 % Na2SiO3, 0.4 % Na2SO4, 0.0026 % SrCl and distilled water; [2]), pH=7.5] or on TYS agar (1.5 % agar) at 30 °C and preserved in TYS broth supplemented with 20 % (v/v) glycerol at −80 °C. The reference strain E. saccharophila KCTC 22655T (obtained from the Korean Collection for Type Cultures) was also routinely cultivated on TYS agar or in TYS broth at 30 °C.

Genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA gene were performed as described previously [3]. The obtained 16S rRNA gene sequence of CY01† was compared with those of species with validly published names through the EzTaxon server (old.ezbiocloud.net/ [4]) using the BLASTN program [5]. Phylogenetic trees were generated with MEGA version 6 [6] using neighbour-joining [7], maximum-parsimony [8] and maximum-likelihood [9] methods. Evolutionary distance was computed with the Jukes–Cantor model [10] for the neighbour-joining tree and the maximum-likelihood tree. The default parameter options were used for maximum-parsimony tree construction. The topologies of the

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CY01† is KU174197.

Four supplementary figures and one supplementary table are available with the online Supplementary Material.
phylogenetic trees were evaluated by bootstrap analyses (1000 replications).

Cellular fatty acids analysis for CY01\textsuperscript{T} and \textit{E. saccharophila} KCTC 22655\textsuperscript{T} was performed using 6890N gas chromatograph (Agilent) and the acids were identified using the Sherlock Microbial Identification System (MIDI) (version 4.5 and the TSBA40 database) at Shanghai Public Health Clinical Center (Shanghai, PR China). Cell biomasses of the two strains for the analysis were both harvested from cultures incubated in TYS broth at 30 °C for 24 h.

Polar lipids and respiratory quinones analyses were carried out as described previously [11]. Genomic DNA G+C content was determined using a HPLC method [12] by the Institute of Microbiolog, Chinese Academy of Sciences (Beijing, China).

Cellular morphology and the presence of flagella were observed using a transmission electron microscope (HT7700; Hitachi) and scanning electron microscope (7500F; JEOL). Colony morphology was observed after incubation on TYS agar at 30 °C for 4 days. Gram reaction was performed using the standard Gram procedure [13] and the non-staining method [14]. The presence of flexirubin-type pigment was examined using 20 % KOH (w/v) as described by Bernardet \textit{et al.} [15]. The gliding motility was examined using the hanging drop technique [15]. The temperature range and optimal temperature for growth were measured in TYS broth at 5–40 °C (5, 10, 15, 20, 25, 30, 35, 37 and 40 °C). Growth with 0–5 % NaCl (at intervals of 0.5 %, w/v) and 6–16 % (at intervals of 2 %, w/v) was performed in NP broth (containing 0.5 % tryptone, 0.1 % yeast extract, 0.5 % MgCl\textsubscript{2}, 0.2 % MgSO\textsubscript{4}, 0.1 % CaCl\textsubscript{2}, 0.1 % KCl and distilled water, pH=7.5). The pH range [5–10.0, at intervals of 0.5 pH units, buffered with MES (pH 5.0–6.0, 50 mM), MOPS (pH 6.5–7.5, 50 mM), Tris (pH 8.0–8.5, 50 mM) and CHES (pH 9.0–10.0, 50 mM)] were determined in NP broth with added 2.8 % NaCl. Capnophilic growth was examined on TYS agar under both aerobic and microaerobic conditions (5 % O\textsubscript{2}, 10 % CO\textsubscript{2} and 85 % N\textsubscript{2}). Anaerobic growth was examined for 10 days on TYS agar supplemented with electron acceptors/donors (potassium nitrate, sodium nitrite, ferric citrate, sodium thiosulfate, fumaric acid, sodium sulfate, glycine, dimethyl sulfoxide and ferric phosphate, each 0.1 %, w/v) in an anaerobic jar [containing Anaerocult A (Merck) to produce anaerobic conditions].

Ionic requirements were examined in TYS broth (containing 0.5 % tryptone, 0.1 % yeast extract and distilled water, pH 7.5) supplemented with the following salt combinations (w/v): (i) 0 % NaCl, (ii) 2.8 % NaCl, (iii) 1 % MgCl\textsubscript{2}·6H\textsubscript{2}O, (iv) 0.1 % CaCl\textsubscript{2}, (v) 0.1 % CaCl\textsubscript{2} and 1 % MgCl\textsubscript{2}·6H\textsubscript{2}O, (vi) 2.8 % NaCl and 0.1 % CaCl\textsubscript{2}, (vii) 2.8 % NaCl and 1 % MgCl\textsubscript{2}·6H\textsubscript{2}O, (viii) 2.8 % NaCl, 1 % MgCl\textsubscript{2}·6H\textsubscript{2}O and 0.1 % CaCl\textsubscript{2}. Carbon utilization was determined using Biolog GEN III according to the manufacturer’s instructions except that inoculation fluid A for the cell suspension was supplemented with 2.8 % NaCl, 1 % MgCl\textsubscript{2}·6H\textsubscript{2}O and 0.1 % CaCl\textsubscript{2}. Acid production from carbohydrates were detected using API 50CH strips (bioM\textregistered{}rieux) according to the manufacturer’s instructions except that API 50 CHB/E medium for the cell suspension was supplemented with 2.8 % NaCl, 1 % MgCl\textsubscript{2}·6H\textsubscript{2}O and 0.1 % CaCl\textsubscript{2}. Oxidase activity was determined by using commercial oxidase test strips (Tianhe Microorganism Reagent). Catalase activity was detected by bubble production in a 3 % (v/v) hydrogen peroxide solution. Hydrolysis of starch and Tween 40, 60 and 80 were tested on TYS agar supplemented with 0.2 % (w/v) soluble starch or 1 % (v/v) Tween 40, 60 or 80. Hydrolysis of casein was tested on a medium containing 0.2 % yeast extract, 1.5 % agar and ASW2 with 0.5 % (w/v) casein. Antibiotic susceptibility testing was performed using the disc-diffusion method on TYS agar and the growth inhibition zones were observed after 3 days incubation at 30 °C. Other enzymatic activities and other physiological and biochemical characteristics were detected using API ZYM and API 20NE strips (bioM\textregistered{}rieux) according to the manufacturer’s instructions with ASW1 as the cell suspension solution.

The almost complete 16S rRNA gene sequence (1489 nucleotides) of CY01\textsuperscript{T} was obtained. Comparative analysis of the sequence showed that CY01\textsuperscript{T} had the highest 16S rRNA gene sequence similarity to the type strain of \textit{E. saccharophila} KCTC 22655\textsuperscript{T} (97.0 %), followed by \textit{Pseudobellia thermophila} KMM 3531\textsuperscript{T} (95.0 %) and less than 94.0 % sequence similarity to type strains of other known species of the family \textit{Flavobacteriaceae}. In the neighbour-joining tree (Fig. 1), the maximum-parsimony tree (see Fig. S1, available in the online Supplementary Material) and maximum-likelihood tree (see Fig. S2), CY01\textsuperscript{T} and \textit{E. saccharophila} KCTC 22655\textsuperscript{T} formed a coherent branch supported by high bootstrap values (96–100 %), indicating that strain CY01\textsuperscript{T} represents a member of the genus \textit{Euzebyella}.

The DNA G+C content of CY01\textsuperscript{T} was 38.2 mol% (by HPLC), close to the value reported for type strain of \textit{E. saccharophila} (40 mol%, [11]). The major cellular fatty acids (>5 %) of CY01\textsuperscript{T} were iso-C\textsubscript{15:0} (48.1 %), iso-C\textsubscript{15:1}G (14.6 %), iso-C\textsubscript{17:0} 3-OH (12.5 %) and C\textsubscript{15:0} (5.2 %), which were similar to those of \textit{E. saccharophila} KCTC 22655\textsuperscript{T} (see Table S1). Polar lipids of CY01\textsuperscript{T} included phosphatidylethanolamine (PE), four unidentified lipids and one unidentified aminolipid (see Fig. S3). The major respiratory quinone of CY01\textsuperscript{T} was menaquinone MK-6, typical in members of the family \textit{Flavobacteriaceae} [16, 17].

Cells of CY01\textsuperscript{T} were Gram-stain-negative, aerobic, non-flagellated rods (see Fig. S4). Resistant to bacitracin (10 U), kanamycin (30 µg), neomycin (30 µg), nystatin (100 µg), polymyxin B (300 U), streptomycin (10 µg) and tobramycin (10 µg). Susceptible to amoxycillin (10 µg), carbenicillin (100 µg), cefotaxime (30 µg), cefoxitin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), novobiocin (30 µg) and rifampicin (5 µg). CY01\textsuperscript{T} did not grow without NaCl or in TYS broth supplemented with only NaCl or MgCl\textsubscript{2} or CaCl\textsubscript{2}, could grow slightly when both CaCl\textsubscript{2} and MgCl\textsubscript{2} or NaCl and CaCl\textsubscript{2} or NaCl and MgCl\textsubscript{2} were added to the
medium and showed good growth when NaCl, MgCl₂ and CaCl₂ were added to TY broth. Other morphological, physiological and biochemical data for CY01ᵀ are given in the species description. Characteristics that differentiate CY01ᵀ from E. saccharophila KCTC 22655ᵀ are listed in Table 1. Based on the results of the phenotypic and phylogenetic analysis, CY01ᵀ represents a novel species within the genus Euzebyella, for which the name Euzebyella marina sp. nov. is proposed.

**DESCRIPTION OF EUZEBYELLA MARINA SP. NOV.**

Euzebyella marina (ma.ri’na. L. fem. adj. marina of the sea, marine).

Cells are Gram-stain-negative, non-flagellate, non-gliding, non-capnophilic, oxidase- and catalase-positive rods (0.3–0.6×0.8–4.3 µm). Colonies are yellow, circular (1.2–2 mm in diameter), slightly convex and smooth with entire edges after 4 days of incubation on TYS agar at 30 °C. Flexirubin-type pigment is not produced. Growth occurs at 15–37 °C (optimum, 30 °C), at pH 5–8 (optimum, pH 6.5–7.5) and in the presence of 0.5–12% (w/v) NaCl (optimum, 0.5–3.5%). Anaerobic growth is not observed. Hydrolyses aesculin (API 20NE) but not gelatin (API 20NE), casein, Tween 40, Tween 60, Tween 80 and starch. Using API ZYM strips, the following 16 enzymic activities are detected: alkaline and acid phosphatase, esterase (C4), weak, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase (weak), trypsin, naphthol-AS-BI-phosphohydrolase (weak), α-galactosidase (weak), β-galactosidase (weak), α-glucosidase (weak), β-glucosidase (weak), α-mannosidase (weak), N-acetyl-β-glucosaminidase and α-fucosidase, but the enzymic activities of lipase (C14), α-chymotrypsin and β-glucuronidase are not detected. In API 20NE tests, positive for β-galactosidase but negative for the following reactions or enzymes: nitrate reduction to nitrites or nitrogen, indole production, acid production from glucose, arginine dihydrolase, urease, assimilattion of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, glucanate, caprate, adipate, malate, citrate and phenylacetate. In API 50 CH tests (48 h), the following carbohydrates are acidified: glycerol, L-arabinose, D-xyllose, D-glucose, D-fructose, D-mannose, L-ribose, L-rhamnose, D-mannitol, aesculin, cellobiose, maltose, melibiose, sucrose, trehalose, starch, glycogen, turanose and D-fucose. The following carbohydrates are slightly acidified: D-arabinose, D-ribose, D-galactose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, lactose, melezitose, raffinose, gentiobiose, D-lyxose, D-tagatose, L-fucose, D-arabitol and L-arabitol. Negative responses are obtained with the following carbohydrates: erythritol, L-xyllose, D-adonitol, methyl β-D-xylpyranoside, L-sorbos, dulcitol, inositol, D-sorbitol, N-acetylglucosamine, inulin, xylitol, glucanate, 2-ketogluconate and 5-ketogluconate. On Biolog GEN III microplates (48 h), the following carbohydrates are oxidized: dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, α-D-lactose, melibiose, methyl β-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, L-rhamnose, glycyrl-L-proline, L-arginine, L-aspartic acid, L-glutamic acid, pectin, D-galacturonic acid, D-
MK-6 and polar lipids include phosphatidylethanolamine (PE), four unidentified lipids and one unidentified aminolipid. The dominant cellular fatty acids (>10%) are iso-C₁₅:₀, iso-C₁₆:₁, G and iso-C₁₇:₀ 3-OH.

The type strain is CY01ᵀ (=CCTCC AB 2014348ᵀ=KCTC 42440ᵀ), isolated from seawater from the Yellow Sea. The DNA G+C content of the type strain is 38.2 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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