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Corynebacterium marinum sp. nov. isolated from coastal sediment in Qingdao, China

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Running title: *Corynebacterium marinum* sp. nov.

The GenBank accession number for the 16S rRNA gene sequence of *Corynebacterium marinum* D7015^T is DQ219354. A representative phase-contrast micrograph of strain D7015^T and a table showing the cellular fatty acid profiles of strain D7015^T and related species are available as supplementary material in IJSEM Online.

A taxonomic study was performed on strain D7015^T, which was isolated from coastal sediment close to a coal-fired power station in Qingdao, China. Strain D7015^T comprised Gram-positive, non-motile diphtheroid rods, which grew in the presence of 0-8% (w/v) NaCl and at 4-37°C, with optimum growth at 1% (w/v) NaCl and 30-32°C. The G+C content was 65.0 mol%**.** The major fatty acids were $C_{18:1}\omega9c$ (56.18%), $C_{16:0}$ (38.02%), $C_{16:1}\omega7c$ (4.45%), $C_{18:0}$ (1.0%) and $C_{14:0}$ (0.35%). On the basis of the morphological, physiological and phylogenetic characteristics, strain D7015^T was classified in the genus *Corynebacterium*. It exhibited a 16S rRNA gene sequence similarity of 95.9% and a DNA:DNA relatedness value of 20.4% with *Corynebacterium halotolerans* DSM 44683^T. Strain D7015^T was sufficiently different from hitherto described *Corynebacterium* species to be considered as a novel species. The name *Corynebacterium marinum* sp. nov. is proposed, with strain D7015^T (=CGMCC 1.6998^T =NRRL B-24779^T) as the type strain.

The genus *Corynebacterium* was proposed by Lehmann & Neumann (1896) and represents a large group of Gram-positive, asporogenous, rod-shaped bacteria with high DNA G+C content. Corynebacteria have been isolated from a wide range of environments, namely dairy products, soil, sewage, sediments, plant materials and aquatic sources, but the majority of novel species described in recent years have originated from human or animal clinical samples (e.g. Renaud *et al.*, 2007; Yassin & Siering, 2008; Yassin, 2009; Funke *et al.*, 2009). However, some corynebacterial species have been isolated from the marine environment, and they may occur as part of the indigenous flora of marine animals. For example, *Corynebacterium phocae* and *Corynebacterium caspium* were isolated from seals (Pascual *et al.*, 1998; Collins *et al.*, 2004), *Corynebacterium spheniscorum* and *Corynebacterium sphenisci* were recovered from wild penguins (Goyache *et al.*, 2003a, 2003b), and *Corynebacterium maris* Coryn-1T was found in the mucus of the coral, *Fungia granulose* (Ben-Dov *et al.*, 2009). Here, we report the taxonomic characteristics of a novel *Corynebacterium* species that originated from coastal sediment close to a coal-fired power station in Qingdao, China.

Strain D7015^T was isolated from coastal sediment in 2000 (Du *et al*., 2002). The isolate was grown aerobically at 28°C on marine 2216E agar (MA; Difco) for 48 h. Cultures were maintained on MA slants at room temperature, and stock cultures were kept in tryptone soya broth (Oxoid) supplemented with 1% (w/v) NaCl (= TNB) and 20% (v/v) glycerol at -70°C. Identification of strain D7015^T was performed as described by Jordan *et al*. (2007). For phenotypic tests, the strain was grown on MA for 48 h at 28°C, and cells were resuspended in saline for use as an inoculum. Tolerance of 1, 3, 5, 7 , 8 and 10% (w/v) NaCl was assessed on appropriately modified tryptone soya agar (TSA; Oxoid). Growth in the absence of NaCl was assessed on plate count agar (PCA; Oxoid). Inoculated plates were incubated at 28°C for up to 5 days. The effects of different temperatures on growth were assessed on TSA plates supplemented with 1.0% (w/v) NaCl (TNA) with incubation at 4, 10, 15, 28, 30, 32, 37, 42 and 45°C. The reduction of nitrate was assessed in nitrate broth, prepared according to the method of Cowan & Steel (1974), and incubated at room temperature for 10 days. Oxidase and catalase activities were determined by using standard methods. The culture was characterized biochemically using the API Coryne, API 50CH and API ZYM systems according to the manufacturer's instructions (bioMérieux). Measuring turbidity was used to evaluate the growth of strain D7015^T in the API 50CH and the API 20NE systems. The API 50 CH strips were read after 7 days incubation at 28°C.

The almost-complete 16S rRNA gene sequence (1444 nt) of strain D7015^T was obtained using the universal primers 27f and 1492r (MWG Biotech; Lane, 1991). The 16S rRNA gene sequence of strain D7015^T was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm. A phylogenetic dendrogram of strain D7015^T and some closely related members of the genus *Corynebacterium* based on 16S rRNA gene homology was constructed using the neighbor-joining method of the MEGA software version 4.1 (Tamura *et al*., 2007). The

resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates.

Cellular fatty acids were determined on a 3 day old culture grown on marine 2216E agar plates after incubation at 28°C. The fatty acids were extracted, methylated and analyzed using the standard MIDI (Microbial Identification) system (Sasser, 1990). The G+C content of the DNA was determined directly by high pressure liquid chromatography according to a method described previously (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989). DNA:DNA hybridization between strain D7015^T and *Corynebacterium halotolerans* DSM 44683^T was carried out by applying the optical renaturation method (De Ley *et al.*, 1970; Huss *et al.*, 1983; Jahnke, 1992) under optimal hybridization conditions.

Microscopic examination of strain D7015^T suggested that it belonged to the genus *Corynebacterium*, as cells stained Gram-positive and produced short, diphtheroid rods with some of the cells arranged in a V formation due to their snapping division (Collins & Cummins, 1986). A representative phase-contrast micrograph of strain D7015^T is available as supplementary material in IJSEM Online. Strain D7015^T was non-motile and non-endospore-forming. The isolate was catalase-positive and oxidase-negative. The following results of carbon source assimilation were positive in API 50CH: aesculin ferric citrate, salicin, D-maltose and glycogen. Strain D7015^T displayed a numerical profile of 3200127 with the commercial API Coryne system, which corresponded to a “doubtful” identification as *Corynebacterium glucuronolyticum* (with a confidence level of 99.2%). However, further biochemical analyses, using API ZYM and additional phenotypic tests revealed that strain D7015^T could be distinguished from C. *glucuronolyticum* on the basis of its ability to produce α-chymotrypsin and its inability to produce esterase lipase (C4). Strain D7015^T produced acid from glucose, maltose, sucrose and glycogen, but not from ribose, xylose, mannitol and lactose. Strain D7015^T was also different from *C.*

spheniscorum notably in the inability of the latter to reduce nitrate, or produce β-glucuronidase, α-chymotrypsin or naphthol-AS-BI-phosphohydrolase. The complete morphological and biochemical characteristics for strain D7015^T are given in the species description.

Phylogenetic analyses performed with nearly complete sequences of members of closely related species (Fig. 1) showed that no sequence available in the GenBank database exhibited more than 96% similarity. The sequence similarity observed between the new isolate and its closest relative, *C. halotolerans*, was 95.9%, which is a lower value than the borderline used for defining bacterial species (i.e. 97%) as proposed by Stackebrandt & Goebel (1994). *C. halotolerans* was isolated from a saline soil sample in China (Chen *et al*., 2004). For strain D7015^T, growth was not observed at 10% (w/v) NaCl. However, optimum growth of *C. halotolerans* was in 10% (w/v) KCl, NaCl or $MgCl_2 \cdot 6H_2O$. These two strains were also different biochemically, with strain D7015^T able to hydrolyse starch and Tween 20-80, but unable to produce esterase lipase (C4). The DNA:DNA relatedness value of 20.4 ± 0.1% (experiment repeated twice) was significantly lower than 70%, which is considered to be the threshold value for the delineation of genomic species (Wayne *et al*., 1987). Clearly, strain D7015^T differed biochemically from all of its closest relatives, both in its ability to hydrolyse casein and in its inability to produce esterase lipase C4. The phenotypic features that differentiate strain D7015^T from its closest phylogenetic relatives are provided in Table 1.

The cellular fatty acids in strain D7015^T were $C_{18:1}\omega9c$ (56.18%), $C_{16:0}$ (38.02%), $C_{16:1}\omega7c$ (4.45%), $C_{18:0}$ (1.0%) and $C_{14:0}$ (0.35%). The genomic DNA G+C content was 65.0 mol%.

Based on these molecular, chemotaxonomic and phenotypic results, it is proposed that strain D7015^T should be classified as a new species of the genus *Corynebacterium*, for which the name

Corynebacterium marinum sp. nov. is proposed.

Description of *Corynebacterium marinum* sp. nov.

Corynebacterium marinum (ma.ri′num. L. neut. adj. *marinum* of the sea, marine)

Cells are short Gram-positive, non-motile, diphtheroid rods; some of the cells are arranged in a V formation. Colonies on marine 2216E agar medium are circular, erose, convex, yellow and of a creamy consistency and are 0.5-1.5 mm in diameter after 48 h at 28°C. Facultatively anaerobic, catalase-positive and oxidase-negative. Bacteria are methyl-red negative, Voges-Proskauer positive, reduce nitrate, and lyse horse blood cells. Aesculin and urea are not hydrolysed, but casein is digested and starch and pullulan are hydrolysed. Gelatin is not liquefied. Tween 20-80 are hydrolysed. Cells grow in the presence of 0-8% (w/v) NaCl and at 4-37°C. Prolific growth occurs at 30-32°C in media that contain 1% (w/v) NaCl. Using the API 50CH system, aesculin ferric citrate, salicin, D-maltose, and glycogen are utilized. Acid is produced from glucose, maltose, sucrose and glycogen, but not from ribose, xylose, mannitol or lactose. Activities for esterase lipase (C8), leucine arylamidase, α-chymotrypsin, naphthol-as-bi-phosphohydrolase, pyrazinamidase and β-glucuronidase are positive. Esterase lipase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, alkaline phosphatase, pyrrolidonyl arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. The type strain is resistant to nalidixic acid (30 μg), nitrofurantoin (50 μg), sulphamethizole (200 μg), tetracycline (100 μg) and cotrimoxazole (25 μg), but sensitive to ampicillin (25 μg), chloramphenicol (50 μg), gentamycin (10 μg), kanamycin (30 μg), carbenicillin (100 μg) and streptomycin (25 μg) as determined by antibiotic discs. Major fatty acids produced are $C_{18:1}\omega9c$ (56.18%), $C_{16:0}$ (38.02%), $C_{16:1}\omega7c$ (4.45%), $C_{18:0}$ (1.0%) and $C_{14:0}$ (0.35%). The percentage whole-cell fatty acid compositions of strain D7015^T and related species is

146 available as supplementary material in IJSEM Online. The genomic DNA G+C content is 65.0
147 mol% for the type strain.
148
149 The type strain is D7015^{T} (=CGMCC 1.6998^{T} = NRRL B-24779^{T}), which was isolated from coastal
150 sediment close to a coal-fired power station in Qingdao, China.
151
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Table 1. Comparison of strain D7015^T with the phylogenetically related species of the genus *Corynebacterium*

Species: 1, Strain D7015^T, 2, *Corynebacterium efficiens* DSM 44549^T (Fudou *et al.*, 2002); 3, *Corynebacterium halotolerans* DSM 44683^T (Chen *et al*., 2004). +, positive; -, negative; ND, no data available.

Characteristic	**1**	**2**	**3**
Starch hydrolysis	+	ND	-
Esterase lipase (C4)	-	ND	+
Growth at 45 °C	-	+	ND
Growth in 10% (w/v) NaCl	-	+	+
Acid produced from:			
ribose	-	+	-
maltose	+	+	-
glycogen	+	-	-
DNA G+C content (mol%)	65.0	59.0-60.2	63.0

Figure legend:

Fig. 1. Neighbor-joining phylogeny of *Corynebacterium marinum* and closely related species. Numbers along branches represent bootstrap values; only values greater than 50% are shown. GenBank accession numbers for the 16S rRNA sequences used in the tree reconstruction are given in the parentheses next to each taxon. Bar, 0.01 substitutions per nucleotide.