Corynebacterium humireducens sp. nov., an alkaliphilic, humic acid-reducing bacterium isolated from a microbial fuel cell

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A novel halotolerant, alkaliphilic, humic acid-reducing bacterium, designated MFC-5T, was isolated from a microbial fuel cell that was fed continuously with artificial wastewater (pH 10.0). Cells were Gram-positive-staining, facultatively anaerobic, non-fermentative, non-motile rods and had a G+C content of 59.0 mol%. Microbial growth was observed with <13 % (w/v) NaCl (optimum 10 %), at pH 7.0–11.0 (optimum pH 9.0) and at 25–45 °C (optimum 37 °C). Strain MFC-5T was active in the anaerobic reduction of a humic acid analogue, anthraquinone-2,6-disulphonate, with lactate, formate, acetate, ethanol or sucrose as the electron donor. The major cellular fatty acids were C18 : 1ω9c (42.68 %), C16 : 0 (33.69 %), C18 : 0 (7.56 %), C17 : 1ω6c (5.14 %) and C17 : 0 (3.39 %). Phylogenetic analysis demonstrated that strain MFC-5T displayed >3 % 16S rRNA gene sequence divergence from its closest relatives. Based on phenotypic, genetic and phylogenetic analysis, a novel species, Corynebacterium humireducens sp. nov., is proposed. The type strain is MFC-5T (=NBRC 106098T =CGMCC 2452T =DSM 45392T).

Abbreviations: AH2DS, reduced anthraquinone-2,6-disulphonate; AQDS, anthraquinone-2,6-disulphonate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MFC-5T is G0421281.

Three supplementary figures are available with the online version of this paper.

Microbe-mediated humic substance reduction has been widely recognized, and quinone moieties have been shown to be the redox-active compounds in humic compounds (Lovley et al., 1996; Scott et al., 1998; Straub et al., 2005). Humic substance reduction may play a significant role in the anaerobic biotransformation of both organic and inorganic compounds. A wide variety of organic compounds, including priority pollutants, can be mineralized by micro-organisms under anaerobic conditions with humic substances serving as the terminal electron acceptor (Bradley et al., 1998; Cervantes et al., 2000, 2001; Finneran & Lovley, 2001). In addition, humic substances, once reduced, can shuttle electrons to insoluble electron acceptors (e.g. ferric oxides) and several distinct contaminants (e.g. azo dyes, chlorinated organic contaminants) by acting as a redox mediator (Bond & Lovley, 2002; Kappler et al., 2004; Hong et al., 2007; Wang et al., 2009). Wang et al. (2009) found that the reduction rate of 2,4-dichlorophenoxyacetic acid (2,4-D) was increased significantly by the addition of quinone, which was reduced microbially to the corresponding hydroquinone, which could then reduce 2,4-D chemically.

A large number of humic acid-reducing micro-organisms have been isolated from a broad range of environments (Field & Cervantes, 2005) and the humic acid reductions mediated by these micro-organisms are commonly studied at circumneutral pH. To the best of our knowledge, no studies have reported humic acid reduction under alkaline conditions or by an alkaliphilic humic acid-reducing bacterium. In this study, we successfully isolated a new alkaliphilic bacterium, strain MFC-5T, from an alkaline microbial fuel cell and identified its ability to reduce humic acids (quinone) under alkaline conditions.

Strain MFC-5T was isolated from the anode of a wastewater-fed microbial fuel cell in our laboratory that was continuously operated at pH 10.0. A piece of anode (0.5 cm3) was transferred to a 25.2 ml sterile bottle containing 20 ml sterilized mineral salts medium [MSM, supplemented with (per litre deionized water) 0.6 g NaH2PO4, 0.25 g NH4Cl, 0.1 g KCl, 0.2 g yeast extract, 10.0 ml vitamin stock solution and 10.0 ml mineral stock solution (Zachara et al., 1998); pH 10.0] supplemented with 0.5 mM anthraquinone-2,6-disulphonate (AQDS; electron acceptor), 5 mM sodium lactate (electron donor) and...
10 mM carbonate buffer. The bottle was purged with O₂-free N₂/CO₂ (80:20, v/v) for 15 min and sealed with a butyl-rubber stopper and an aluminium cap. After 10 days of incubation, the colour of the contents of the bottle changed to red and the enriched population was serially diluted and plated onto MSM agar (pH 10.0) supplemented with (1⁻¹) 18 g agar, 1 mmol AQDS and 5 mmol sodium lactate. The plates were incubated in an anaerobic chamber (ShelLab; Sheldon Manufacturing) with N₂/CO₂ (80:20, v/v). Strain MFC-5T formed red colonies owing to its ability to reduce AQDS.

To identify morphological features, strain MFC-5T was cultivated aerobically on LB agar overnight at 30 °C. Cell morphology and dimensions were determined by scanning electron microscopy (ESEM-30; Philips). Cell motility was tested by the hanging-drop method. Physiological characteristics were determined by standard methods (Buchanan & Gibbons, 1974). Salt tolerance was determined in MSM supplemented with 0.5 mM AQDS and 5 mM sodium lactate and on LB agar supplemented with 0–15 % (w/v) NaCl. The isolate was characterized biochemically using the API Coryne and API ZYM systems (bioMérieux), according to the manufacturer’s instructions. Cellular fatty acids were extracted from dried cells and methylated and analysed as described by Kämpfer & Kroppenstedt (1996). Extraction of genomic DNA was performed according to Chun & Goodfellow (1995). The G+C content of the DNA was determined by HPLC as described by Mesbah et al. (1989).

The 16S rRNA gene of the isolate was amplified by PCR using conserved primers that bind close to the 3' and 5' ends of the gene, as described by Finneran et al. (2003). The purified PCR product was sequenced using an automated sequencer (ABI 3730; Applied Biosystems). The sequence was aligned with related 16S rRNA gene sequences from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch) using CLUSTAL X (Thompson et al., 1997) and gaps at the 5' and 3' ends were removed. Phylogenetic trees were constructed with MEGA version 4.0 using the maximum-parsimony and neighbour-joining methods (Kumar et al., 2004) according to Kimura’s two-parameter model and bootstrap analyses were based on 1000 resamplings (Kimura, 1980; Saitou & Nei, 1987). 16S rRNA gene sequence similarities between strain MFC-5T and its closest known relatives were calculated using the EzTaxon server.

To investigate AQDS reduction by strain MFC-5T, batch experiments were conducted in a 25.2 ml serum bottle using cells grown aerobically in LB medium to late-exponential phase, harvested by centrifugation (8000 g at 4 °C for 10 min), washed twice and resuspended in sterilized MSM. To determine the pH range for AQDS reduction, 20 ml MSM (containing 1 × 10⁷ cells ml⁻¹,

### Table 1. Characteristics that can be used to differentiate strain MFC-5T from alkalophilic and halotolerant corynebacteria

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Strains: 1, Corynebacterium humireducens sp. nov. MFC-5T; 2, C. halotolerans YIM 70093T (data from Chen et al., 2004); 3, C. marinum D7015T (Du et al., 2010); 4, C. maris Coryn-1T (Ben-Dov et al., 2009); 5, C. matruchotii (n=6; Barrett et al., 2001); 6, C. testudinoris M935/96/4T (Collins et al., 2001); 7, C. urealyticum (n=6; Pitcher et al., 1992). All strains were negative for aesculin hydrolysis, β-galactosidase and N-acetyl-β-glucosaminidase (no data available for C. urealyticum) and acid production from mannitol, xylose and lactose. ND, No data available.
MFC-5T produced acid from glucose and ribose, but not and methyl red-negative. With the API systems, strain 13 % NaCl. The isolate was catalase-positive and oxidase-
(optimum pH 9.0) and with 0–12 % NaCl, but not with halotolerant species of the genus Fig. 1(a) shows the reduction of AQDS by strain MFC-5T genus suggested that the isolate represented a novel species of the

Vegetative cells of strain MFC-5T were straight to curved rods, 1.0–2.0 μm long and 0.5–0.7 μm wide (Supplementary Fig. S1, available in IJSEM Online). Cells occurred singly or in pairs, were non-motile and stained Gram-positive during exponential and stationary growth. After 24 h on LB agar, colonies were uniformly round, 0.5–1.0 mm in diameter, yellowish, dry and rough with a distinct wrinkled morphology.

The temperature for growth of strain MFC-5T was 25–40 °C (optimum 37 °C); no growth was observed at 45 or below 10 °C. Growth was observed at pH 7.0–11.0 (optimum pH 9.0) and with 0–12 % NaCl, but not with 13 % NaCl. The isolate was catalase-positive and oxidase- and methyl red-negative. With the API systems, strain MFC-5T produced acid from glucose and ribose, but not from maltose, sucrose, mannose, xylose or lactose, and was positive for alkaline phosphatase, esterase, esterase lipase, lipase, pyrazinamidase and pyrrolidonyl arylamidase, but not for leucine arylamidase, N-acetyl-β-glucosaminidase, β-glucuronidase or β-galactosidase. In terms of overall phenotypic characteristics, the isolate resembled corynebacteria. Comparing the biochemical characteristics of strain MFC-5T with representatives of alkaliophilic and halotolerant species of the genus Corynebacterium (Table 1) suggested that the isolate represented a novel species of the genus Corynebacterium.

Fig. 1(a) shows the reduction of AQDS by strain MFC-5T using lactate as the electron donor at different pH. After 10 days, the AH2DS concentration at pH 7, 8, 9, 10 and 11 was 0.23, 0.14, 0.48, 0.31 and 0.16 mM, respectively. Reduction of AQDS at pH 6.0 was negligible. In the abiotic control, <0.07 mM AH2DS was formed (data not shown). Thus, the pH range for AQDS reduction by strain MFC-5T was pH 7.0–11.0 and the optimum pH was pH 9.0. To the best of our knowledge, the reduction of humic acids and quinone by alkaliophilic bacteria has not been reported, and the identification of strain MFC-5T raises the upper pH limit for microbial quinone reduction. As shown in Fig. 1(b), AH2DS was produced with formate, acetate, lactate, ethanol and sucrose as electron donors, but not with citrate, propionate or glycerol (not shown). The electron donors could be ranked according to the final concentration of AH2DS, in descending order, as sucrose, acetate, lactate, ethanol and formate. In the abiotic control, almost no AH2DS was detected (not shown). As shown in Fig. 1(c), significant microbial growth and AQDS reduction were observed with lactate as the electron donor over 10 days. Cell density in the active test approached a maximum of 13.7×10⁷ cells ml⁻¹, which was approximately 14 times greater than the initial density. Data showed that AQDS reduction and the increase in cell numbers occurred at the same time throughout the

![Fig. 1. Characteristics of AQDS reduction by strain MFC-5T over 10 days. (a) Production of AH2DS with lactate as electron donor at pH 6.0–11.0. (b) Production of AH2DS with various electron donors (5 mM l⁻¹) at pH 9.0. (c) Cell growth with AQDS and lactate as electron donor at pH 9.0. Experiments were performed in MSM medium under anaerobic conditions at 30 °C with 1×10⁷ cells ml⁻¹ initial cell density. Error bars represent standard deviations (n=3).](https://www.microbiologyresearch.org)
incubation. Cell growth was negligible in controls without AQDS or lactate, which indicated that strain MFC-5T could not ferment lactate and only grew in the presence of both AQDS and lactate. As far as is known, this is the first report of a member of the genus *Corynebacterium* that can reduce extracellular quinones and harness energy for microbial growth by quinone reduction.

Examination of the long-chain cellular fatty acids of strain MFC-5T showed that the non-hydroxylated long-chain cellular fatty acids were primarily straight-chain saturated and mono-unsaturated. The major acids were C14:0 (1.55 %), C16:0 (33.69 %), C17:0 (3.39 %), C18:0 (7.56 %), C17:1ω8c (5.14 %), C18:1ω9c (42.68 %) and C18:1ω7c (1.71 %), which were consistent with the genus *Corynebacterium* (Fernández-Garayzábal et al., 2004). The DNA G+C content of strain MFC-5T was 59.0 mol%, which fell within the range for the genus *Corynebacterium* (52.0–68.0 mol%). The presence of straight-chain saturated and mono-unsaturated fatty acids and the G+C content, along with the morphological and biochemical properties, strongly suggested that strain MFC-5T was a member of the genus *Corynebacterium*.

A nearly complete 16S rRNA gene sequence (1401 nt) was obtained for strain MFC-5T. The closest known relatives of strain MFC-5T were *Corynebacterium marinum* D7015T (96.9 % 16S rRNA gene sequence similarity, 1393 bp considered), *C. testudinoris* CCUG 41625T (95.7 %), *C. halotolerans* YIM 70093T (96.6 %, 1392 bp), *C. felinum* CCUG 39943T (96.0 %, 1383 bp), *C. singularare* CCUG 37330T (96.0 %, 1297 bp) and *C. freiburgense* 1045T (95.9 %, 1395 bp). A sequence alignment was created with sequences from the type strains of 98 species of the genus *Corynebacterium* and the results of phylogenetic analysis are shown in Supplementary Fig. S2. The neighbour-joining and maximum-parsimony trees depicting the phylogenetic relationships between strain MFC-5T and its nearest phylogenetic neighbours (95.3–96.9 % 16S rRNA gene sequence similarity) are shown in Fig. 2 and Supplementary Fig. S3, respectively. The results suggested that strain MFC-5T belongs to the genus *Corynebacterium*.

It is widely accepted that ≥3 % 16S rRNA gene sequence divergence between bacterial strains indicates that they represent different genomic species (Stackebrandt & Goebel, 1994). Therefore, on the basis of phenotypic, genotypic and phylogenetic properties, strain MFC-5T should be classified in a novel species of the genus *Corynebacterium*, for which the name *Corynebacterium humireducens* sp. nov. is proposed.

**Description of *Corynebacterium humireducens* sp. nov.**

*Corénybacterium humireducens* [hu.mi.re.du’cens. L. n. *humus* soil, used to refer to humic substances; L. part. adj. *reducens* leading back (to a reduced oxidation state); N.L. part. adj. *humireducens* converting humic substances to a reduced oxidation state].

Cells are Gram-positive-staining, non-motile, facultatively anaerobic, straight to curved rods (1.0–2.0 μm long and 0.5–0.7 μm wide). Colonies on LB agar are uniformly round, 0.5–1.0 mm in diameter, yellowish, dry and rough, with a distinct wrinkled morphology after 24 h. Optimum growth occurs at 37 °C, at pH 9.0 and with 10 % NaCl (aerobic conditions). Positive for catalase but negative for oxidase, urease, reduction of nitrate and hydrolysis of urea, gelatin and tyrosine. Produces acid from glucose and ribose, but not from arabinose, fructose, galactose, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sucrose, trehalose or xylose. Positive for alkaline phospha-
tase, esterase, esterase lipase, lipase, pyrazinamidase and pyrrolidonyl arylamidase, but not for leucine arylamidase, N-acetyl-β-glucosaminidase, β-glucuronidase or β-galactosidase. Grows with QAQS as the sole terminal electron acceptor at pH 7.0–11.0 (optimum pH 9.0) under anaerobic conditions. Available electron donors include formate, acetate, ethanol, lactate and succrose, but not propionate, glycerol or citrate. The major cellular fatty acids (>5%) are C18:1ω9c, C16:0, C18:0, C17:1ω8c and C17:0. The DNA G+C content of the type strain is 59.0 mol%.

The type strain, MFC-5T (\text{=NBRC 106098T =CGMCC 2452T =DSM 45392T}), was isolated from a microbial fuel cell fed with artificial wastewater and operated continuously at pH 10.0.

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