Exiguobacterium oxidotolerans sp. nov., a novel alkaliphile exhibiting high catalase activity

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A novel alkaliphile was isolated from a drain of a fish processing plant. The isolate grew at a pH range of 7–10. Cells were Gram-positive, facultatively aerobic, motile rods with peritrichous flagella. Colonies were orange or yellow in colour. Catalase and oxidase reactions were positive. The isolate grew in 0–12 % NaCl but not above 15 % NaCl. Its cell extract exhibited 567 times higher catalase activity than an *Escherichia coli* cell extract. The major cellular fatty acids were iso-C_{13:0}, anteiso-C_{13:0}, iso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0}, anteiso-C_{17:0} and iso-C_{17:1}. Its DNA G + C content was 46.7 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing and chemotaxonomic data indicated that strain T-2-2^T is a member of the genus *Exiguobacterium*. DNA–DNA hybridization revealed a low relatedness of the isolate to several phylogenetic neighbours (less than 25 %). On the basis of phenotypic characteristics, phylogenetic data and DNA–DNA relatedness data, the isolate merits classification as a novel species, for which the name *Exiguobacterium oxidotolerans* sp. nov. is proposed. The type strain is T-2-2^T (=JCM 12280^T = NCIMB 13980^T).

There are micro-organisms living in environments of extreme temperature, pH, salinity and hydropressure (Horikoshi & Grant, 1991). These micro-organisms have apparently acquired the ability to survive under such environmental conditions through long-term evolutionary processes, and they possess specific mechanisms for survival in such extreme environments. Among such adaptational processes, not only the micro-organisms themselves might be affected by environmental conditions and induced to acquire adaptation-suitable features, but so too might production of organic molecules within the micro-organisms, such as enzymes and proteins that sustain their metabolism. Although H₂O₂ production and interaction with micro-organisms often occur in nature (Haas & Goebel, 1992), there have been only a few reports of specific micro-organisms that inhabit environments

with hyperoxidative stress caused by factors such as high H₂O₂ concentrations (Yumoto *et al.*, 1999).

Aerobic organisms normally possess specific enzymes to eliminate H₂O₂, which are produced extracellularly as well as intracellularly. Catalase is one such well-known enzyme. It has been reported that catalase plays an important role in certain micro-organisms in obtaining niches in host cells. For example, in *Vibrio fischeri*, catalase KatA is required for its colonization of the light organ of squid (Visick & Ruby, 1998). The production of catalase and H₂O₂ has been reported in several other cases of either parasitic or symbiotic relationships between micro-organisms and their host (Katsuwon & Anderson, 1992; Rocha *et al.*, 1996). If there is a micro-organism that is able to survive in an environment with high H₂O₂ concentrations, it can be expected that this micro-organism will possess an enzyme (e.g. catalase) exhibiting a high H₂O₂-degrading ability. In the present study, we isolated a micro-organism exhibiting a high tolerance to H₂O₂ and an extraordinarily high catalase activity. It was of considerable interest to identify the taxonomic position of such a new type of extremophile. Phenotypic and chemotaxonomic characteristics were examined, as well as the

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Details of the catalase activity of strain T-2-2^T and reference strains are available as supplementary material in IJSEM Online.

phylogenetic position of the isolate, and it was found that the strain should be classified as representing a novel species belonging to the genus *Exiguobacterium*.

A water sample was obtained from a drain pool of a fish processing plant in Hokkaido in which H_2O_2 is used as a bleaching agent. It was plated onto a 10 mM H_2O_2 (Wako Pure Chemical)-containing PYS-2 agar plate (pH 7.5) containing 8 g polypeptone (Nihon Pharmaceuticals), 3 g yeast extract (Kyokuto) and 5 g NaCl, and incubated at 27 °C for 1 week. From the incubated plate, eight colonies were obtained, six white-coloured colonies and two orange-coloured colonies. H_2O_2 decomposition activity of the eight colonies was measured. Among them, bacterial strain T-2-2^T exhibiting unusual H_2O_2 decomposition activity was isolated. For DNA–DNA hybridization, *Exiguobacterium antarcticum* DSM 14480^T, *Exiguobacterium undae* DSM 14481^T, *Exiguobacterium acetylicum* JCM 1968^T and *Exiguobacterium aurantiacum* NCIMB 11798^T were used. These micro-organisms were grown in PYS-2 medium at 27 °C until the early-stationary phase of growth.

For phenotypic characterization, PYS-2 medium was used as the basal medium. The culture was incubated at 27 °C for 2 weeks unless otherwise stated, and the experiment repeated three times. Morphological, physiological and biochemical tests were performed as described in Barrow & Feltham (1993). Carbohydrate metabolism was tested by the method of Hugh & Leifson (1953), and the result checked daily until 2 weeks after inoculation. Alginase activity was determined after an inoculated agar plate was overlaid with ethanol following cultivation for 10 days. For comparison of strain T-2-2^T with other *Exiguobacterium* species in acid production from sugars and enzymic activities, API 50 CH (bioMérieux) and API ZYM (bioMérieux) were used, respectively.

For the comparative study of catalase activity, *Micrococcus luteus* JCM 1464^T, *Exiguobacterium aurantiacum* NCIMB 11798^T, *Exiguobacterium acetylicum* JCM 1968^T, *Aeromonas hydrophila* JCM 1027^T, *Alcaligenes faecalis* (laboratory strain), *Pseudomonas fluorescens* JCM 5963^T, *Staphylococcus aureus* IAM 12544^T, *Escherichia coli* IAM 1264 and *Vibrio parahaemolyticus* JCM 2147 were used. Cells for catalase activity determination were incubated in a 500 ml flask containing 100 ml PYSG medium (pH 7.5) containing PYS-2 plus 0.5 % sodium glutamate, which was set on a rotary shaker (140 r.p.m.) and maintained at 27 °C until growth reached the late-exponential to early-stationary phase. A cell extract was obtained as follows. Cells were harvested by centrifugation and disrupted with a French pressure cell (SLM-AMINCO) at 20 000 p.s.i. (138 MPa). After removing unbroken cells by centrifugation (10 000 g for 15 min), the resulting fluid was used as the cell extract. Catalase activity was assayed at pH 7 using 30 mM H_2O_2 measured spectrophotometrically as described previously (Yumoto *et al.*, 1999). The amount of enzyme activity that decomposed 1 μmol H_2O_2 min⁻¹ was defined as 1 U of

activity. Enzyme activity was estimated five times for each sample, using at least three independent samples.

For electron microscopic observation, cells were cultivated on PYS-2 agar at 27 °C for 1 day. A small drop of the suspension was placed on a carbon-coated copper grid, negatively stained with 1 % phosphotungstic acid and observed using transmission electron microscopy (H-800, Hitachi) (Yumoto *et al.*, 2002).

Analyses of whole-cell fatty acids and isoprenoid quinones were performed as described previously (Yumoto *et al.*, 2001, 2002). Analysis of cell wall amino acids was performed using the methods of Schleifer & Kandler (1972). Polar lipids were analysed by the methods of Minnikin *et al.* (1979) and Collins & Jones (1980).

Bacterial DNA was prepared according to the method of Marmur (1961). DNA base composition was determined by the method of Tamaoka & Komagata (1984). DNA–DNA relatedness was determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and black microplates.

The 16S rRNA gene sequence corresponding to positions 27–1519 in the 16S rRNA gene sequence of *Escherichia coli* (Brosius *et al.*, 1978) was amplified by PCR. The PCR product (about 1.5 kb) was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (PRISM 377; Applied Biosystems). Closest relatives to the sequence were retrieved from GenBank. Multiple alignments of the sequences were performed and the nucleotide substitution rate (K_{nuc} value) was calculated. A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987) using the CLUSTAL W program (Thompson *et al.*, 1994). Similarity values for sequences were calculated using the GENETYX computer program (Software Development).

Morphological, physiological and biochemical characteristics of the isolate are given in the species description. Phenotypic characterization of the isolate showed that it formed an orange or yellow colony, with Gram-positive, non-spore-forming rods (0.6–0.7 × 0.6–1.8 μm) with peritrichous flagella (Fig. 1). It exhibited positive catalase and oxidase reactions and produced acid from several carbohydrates under aerobic conditions. It grew at 4–40 °C (optimum growth temperature was 34 °C), pH 7–10 and 0–12 % NaCl.

GLC analysis of strain T-2-2^T revealed that the fatty acids comprised iso-C_{12:0} (1.4 %), iso-C_{13:0} (8.5 %), anteiso-C_{13:0} (9.0 %), iso-C_{14:0} (2.7 %), iso-C_{15:0} (20.7 %), anteiso-C_{15:0} (4.2 %), iso-C_{16:0} (7.1 %), iso-C_{16:1} (2.4 %), C_{16:0} (2.9 %), C_{16:1} (3.0 %), iso-C_{17:0} (23.3 %), anteiso-C_{17:0} (6.1 %), iso-C_{17:1} (6.2 %), C_{17:1} (1.2 %) and C_{18:1} (1.6 %); the major isoprenoid quinone was menaquinone-7. DNA G + C content was 46.7 mol%. Cell wall amino acid was lysine as the diagnostic amino acid (Lys–Gly type). Polar lipids consisted of phosphatidylglycerol,

Fig. 1. Transmission electron micrograph of *Exiguobacterium oxidotolerans* T-2-2^T incubated on PYS-2 agar for 24 h at 27 °C. Negatively stained cells of T-2-2^T showing peritrichous flagellation. Bar, 1 µm.

diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. These chemotaxonomic characteristics indicated that the isolate belonged to the genus *Exiguobacterium*.

Details of the catalase activity of strain T-2-2^T and reference strains are available as supplementary material in IJSEM Online. The catalase activity of T-2-2^T exhibited approximately 25 000 U (mg protein)⁻¹, two to three orders of magnitude higher than those of the other reference strains. Catalase activity of T-2-2^T is 567-fold higher than that of *Escherichia coli* [44·1 U (mg protein)⁻¹].

The almost-complete 16S rRNA gene sequence of T-2-2^T, comprising 1515 nt, was compared with all other known 16S rRNA gene sequences, and a phylogenetic tree was constructed using related taxa. The phylogenetic tree indicated that T-2-2^T falls within the genus *Exiguobacterium* (Fig. 2). 16S rRNA gene sequence similarities of T-2-2^T to *Exiguobacterium antarcticum*, *Exiguobacterium undae*, *Exiguobacterium acetylicum* and *Exiguobacterium aurantiacum* were 98·6, 98·4, 98·1 and 93·6 %, respectively. On the basis of 16S rRNA gene sequence analysis, T-2-2^T is a member of the genus *Exiguobacterium*.

Levels of DNA–DNA relatedness were estimated by using all four recognized species of *Exiguobacterium*. *Exiguobacterium antarcticum* DSM 14480^T, *Exiguobacterium undae*

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequence data of *Exiguobacterium oxidotolerans* T-2-2^T, other *Exiguobacterium* species and some other related organisms using the neighbour-joining method. Numbers indicate bootstrap values greater than 500. Bar, 0.01 *K_{nuc}* units.

DSM 14481^T, *Exiguobacterium acetylicum* JCM 1968^T and *Exiguobacterium aurantiacum* NCIMB 11798^T revealed a DNA–DNA relatedness range of 20–44 %. On the basis of these results, T-2-2^T was considered to represent a novel species.

Strain T-2-2^T also differed from the other relatively closely related *Exiguobacterium* species in terms of the following physiological and biochemical characteristics: growth temperature range, acid production from several substrates and enzymic activities (Table 1).

On the basis of the above results, the isolate is considered to represent a novel species, for which the name *Exiguobacterium oxidotolerans* sp. nov. is proposed.

Description of *Exiguobacterium oxidotolerans* sp. nov.

Exiguobacterium oxidotolerans (ox.id.o.tol.e'rans. N.L. v. *oxido* to oxidize; L. part. adj. *tolerans* tolerating; N.L. part. adj. *oxidotolerans* oxidation-tolerant).

Cells are rod-shaped (0.6–0.7 × 0.6–1.8 µm), Gram-positive and with peritrichous flagella. Colonies are circular (1–5 mm in diameter), with entire margins and are orange- or yellow-coloured. Catalase and oxidase reactions are positive. Growth occurs at pH 7–10. Grows at 12 % NaCl but not at 15 % NaCl. Grows at 4–40 °C (optimum 34 °C). The organism is positive for methyl red, ONPG test, β-galactosidase and acid phosphatase activities, but negative for Voges–Proskauer test, reduction of NO₃[−] to

Table 1. Characteristics of *Exiguobacterium oxidotolerans* sp. nov. and related species

1, *E. oxidotolerans* T-2-2^T; 2, *E. acetylicum* JCM 1968^T; 3, *E. aurantiacum* DSM 6208^T; 4, *E. undae* DSM 14481^T; 5, *E. antarcticum* DSM 14480^T. +, Positive; −, negative; V, variable; ND, no data.

Characteristic	1	2	3	4	5
Growth temperature range (°C)	4–36	7–43*	ND	20–41*	20–41*
Acid produced from:					
Galactose	−	−	−	+	+
D-Mannose	+	+	−	+	+
Melibiose	+	−	−	+	−
D-Raffinose	−	−	−	V	−
Rhamnose	+	−	−	+	−
Methyl α-D-glucoside	−	−	+	+	−
Amygdalin	+	−	+	+	+
Ribose	+	−	+	+	+
Mannitol	+	+	+	+	−
Enzymic activities					
β-Galactosidase	+	+	−	+	+
Acid phosphatase	+	−	−	+	+

*Data from Collins *et al.* (1983) and Frühling *et al.* (2002).

NO₂[−], and indole and H₂S production. It hydrolyses casein, gelatin, starch and DNA, but not chitin, alginic acid, and Tweens 20, 40, 60 and 80. It produces acid from D-fructose, D-maltose, D-mannose, melibiose, raffinose, rhamnose, amygdalin, ribose and mannitol (weak), but not from L-arabinose, sucrose, D-xylose, *myo*-inositol, sorbitol, D-galactose, L-rhamnose or trehalose. The major isoprenoid quinone is menaquinone-7. The peptidoglycan type is Lys–Gly. Polar lipids consist of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Major fatty acids are iso-C_{13:0}, anteiso-C_{13:0}, iso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0}, anteiso-C_{17:0} and iso-C_{17:1}. The DNA G + C content is 46.7 %.

The type strain is T-2-2^T (=JCM 12280^T=NCIMB 13980^T).

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