Psychrobacter piscatorii sp. nov., a psychrotolerant bacterium exhibiting high catalase activity isolated from an oxidative environment

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A Gram-negative, non-motile, psychrotolerant bacterium exhibiting high catalase activity, designated strain T-3-2^T, was isolated from a drain of a fish-processing plant. Its catalase activity was 12 000 U (mg protein)$^{-1}$, much higher than the activity of the other *Psychrobacter* strains tested. The strain grew at 0–30 °C and in the presence of 0–12 % NaCl. The predominant isoprenoid quinone was ubiquinone-8 (Q-8), and $C_{16:1}\omega9c$ and $C_{18:1}\omega9c$ were the predominant cellular fatty acids. The DNA G+C content of strain T-3-2^T was 43.9 mol%. 16S rRNA gene sequence phylogeny suggested that strain T-3-2^T is a member of the genus *Psychrobacter*, with the closest relatives being the type strains of *Psychrobacter nivimaris* (99.2 % similarity), *P. aquimaris* (98.7 %) and *P. proteolyticus* (98.5 %). DNA–DNA hybridization showed less than 65 % relatedness with these strains. A phylogenetic tree based on *gyrB* gene sequences was more reliable, with higher bootstrap values than the 16S rRNA gene sequence-based tree. The result also differentiated the isolate from previously reported *Psychrobacter* species. Owing to the significant differences in phenotypic and chemotaxonomic characteristics and the phylogenetic and DNA–DNA relatedness data, the isolate merits classification within a novel species, for which the name *Psychrobacter piscatorii* sp. nov. is proposed. The type strain is T-3-2^T (=JCM 15603^T =NCIMB 14510^T).

Micro-organisms living under extreme conditions such as high and low temperature and pH, high salinity and high hydrothermal pressure are called extremophiles and have acquired the ability to adapt to an extreme environment (Gerday & Glansdorff, 2007). Although there are several examples of interactions between oxidative stress produced by host micro-organisms and parasitic or symbiotic micro-organisms (Katsuwon & Anderson, 1992; Rocha *et al.*, 1996; Visick & Ruby, 1998; Jamet *et al.*, 2003; Merle *et al.*, 2007), there have been only a few reports of specific micro-organisms that inhabit environments with hyperoxidative stress caused by factors such as high H_2O_2 concentrations.

We previously isolated *Exiguobacterium oxidotolerans* T-2-2^T, exhibiting very strong H_2O_2 tolerance, from the upstream part of a H_2O_2-containing wastewater in a fish egg-processing plant in Hokkaido, Japan, in which H_2O_2 is used as a bleaching agent (Yumoto *et al.*, 2004). During the search for H_2O_2-tolerant micro-organisms and micro-organisms possessing high catalase activity, we have found that the wastewater environment in this fish egg-processing plant is appropriate for the isolation of such micro-organisms. This micro-organism also exhibits high catalase activity. As a consequence, both the micro-organisms and their enzymes might be induced to adapt to the environment.

~~During screening for micro-organisms with high catalase activity from the H_2O_2-containing wastewater, strain T-3-2^T was isolated.~~ It is of considerable interest to identify the taxonomic position of such a unique extremophile. The isolate was examined on the basis of its phenotypic and chemotaxonomic characteristics; phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA hybridization showed that the isolate can be identified as belonging to a novel species of the genus *Psychrobacter*.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *gyrB* sequences of strain T-3-2^T are AB453700 and AB490498.

H_2O_2-resistant isolates were selected by ~~plating 0.2 ml wastewater sample onto a 10 mM H_2O_2-supplemented PYS-2 agar plate (pH 7.5)~~ containing 8 g polypeptone (Nihon Pharmaceutical), 3 g yeast extract (Kyokuto) and 5 g NaCl, followed by incubation at 27 °C for 1 week. From the incubated plate, one colony was picked and transferred to a PYS-2 agar plate. Single isolated colonies were serially transferred five times on PYS-2 agar. *Psychrobacter nivimaris* DSM 16093^T, *P. proteolyticus* DSM 13887^T and *P. aquimaris* DSM 16329^T were used as reference strains for DNA–DNA hybridization. The micro-organisms were cultivated using 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 and the experiment was performed three times. Morphological, physiological and biochemical tests were performed as described by Barrow & Feltham (1993). Carbohydrate metabolism was tested by the method of Hugh & Leifson (1953), and the result was checked daily until 2 weeks after inoculation. Alginase activity was determined after an inoculated agar plate was overlaid with ethanol after 10 days of cultivation.

For the comparative study of catalase activity, activity was estimated as described previously (Yumoto *et al.*, 2004). The catalase activities of strain T-3-2^T, *P. nivimaris* DSM 16093^T, *P. proteolyticus* DSM 13887^T and *P. aquimaris* DSM 16329^T were respectively 12 000, 15, 29 and 1800 U (mg protein)$^{-1}$.

To observe negatively stained cells by transmission electron microscopy, strain HT-3^T was grown on a PYS-2 agar slant. The procedures for preparation and observation by transmission electron microscopy were described previously (Yumoto *et al.*, 2001). The morphological, physiological and biochemical characteristics of the isolate are given in the species description. The isolate was a Gram-negative and non-motile coccobacillus (0.9–1.1 × 1.3–1.6 μm). The genus *Psychrobacter* is described as comprising psychrophilic to psychrotolerant, halotolerant, aerobic, non-motile, Gram-negative coccobacilli. The characteristics of the isolate were similar to those of the genus *Psychrobacter*.

Whole-cell fatty acids and isoprenoid quinones were analysed as described previously (Yumoto *et al.*, 2001). The fatty acids of strain HT-3^T comprised $C_{10:0}$ (1.7 %), $C_{12:0}$ (2.6 %), $C_{16:0}$ (2.2 %), $C_{16:1}\omega7c$ (1.2 %), $C_{16:1}\omega9c$ (20.4 %), $C_{17:0}$ (1.0 %), $C_{17:1}\omega8c$ (5.9 %), $C_{18:0}$ (8.9 %), $C_{18:1}\omega9c$ (53.7 %) and $C_{12:0}$ 3-OH (2.8 %). The major isoprenoid quinone was Q-8.

Bacterial DNA was prepared according to the method of Marmur (1961). The DNA base composition was determined by the method of Tamaoka & Komagata (1984). The DNA G+C content of strain HT-3^T was 43.9 mol%, which is similar to those of the type strains of *P. nivimaris* (42 mol%), *P. proteolyticus* (43.6 mol%) and *P. aquimaris* (43.2 mol%).

The 16S rRNA gene was amplified by PCR using primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′). The PCR product was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100; Applied Biosystems). A 16S rRNA gene sequence of 1510 bp was obtained from strain T-3-2^T and analysed. Multiple alignments of the sequences were performed and the nucleotide-substitution rate (K_{nuc} value) was calculated. The 16S rRNA gene sequence similarity of T-3-2^T with previously reported strains was determined, and a

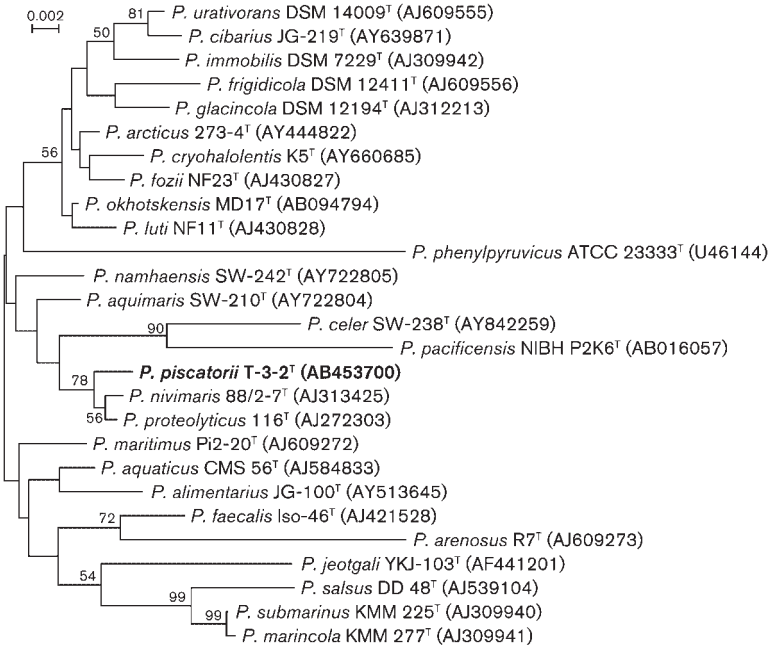

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences of *Psychrobacter piscatorii* sp. nov. T-3-2^T and related members of the genus *Psychrobacter*. Bootstrap values (expressed as percentages of 1000 replicates) ⩾50 % are shown at branch points. Bar, 0.002 expected base substitutions per nucleotide position.

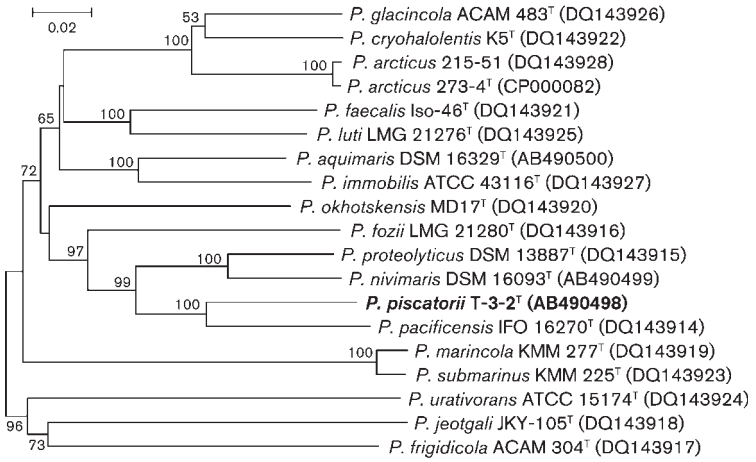

Fig. 2. Neighbour-joining phylogenetic tree derived from *gyrB* gene sequences of *P. piscatorii* sp. nov. T-3-2^T and related members of the genus *Psychrobacter*. Bootstrap values (expressed as percentages of 1000 replicates) >50 % are shown at branch points. Bar, 0.02 expected base substitutions per nucleotide position.

phylogenetic tree of the strain together with its closely related neighbours was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987) using the program CLUSTAL W (Thompson *et al.*, 1994) in MEGA4 (Tamura *et al.*, 2007) (Fig. 1). The similarity between sequences was calculated using the GENETYX computer program (Software Development). The strain showed the highest similarity to *P. nivimaris* DSM 16093^T (99.2 %) among strains of reported species. It also showed high sequence similarity to its phylogenetic neighbours *P. proteolyticus* DSM 13887^T (98.5 %) and *P. aquimaris* DSM 16329^T (98.7 %). The *gyrB* gene was amplified and sequenced as described by Bakermans *et al.* (2006). Sequences were determined and trees constructed as described above (Fig. 2). The result showed a resolution greater than that based on 16S rRNA gene sequence analyses. The strain showed the highest similarity to *Psychrobacter pacificensis* IFO 16270^T (89.8 %) among strains of reported species. It also showed high sequence similarity to *P. nivimaris* DSM 16093^T (86.9 %) and *P. proteolyticus* DSM 13887^T (86.6 %).

The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and black microplates. The hybridization temperature was 38.5 °C. According to the sequence similarities and phylogenetic analysis based on the 16S rRNA gene sequence, strain HT-3^T is most closely related to *P. nivimaris* DSM 16093^T among strains assigned to named species. DNA–DNA hybridizations were performed between strain HT-3^T and *P. nivimaris* DSM 16093^T, *P. proteolyticus* DSM 13887^T and *P. aquimaris* DSM 16329^T; these experiments indicated that the isolate is distinct from *P. nivimaris* DSM 16093^T (64 % relatedness), *P. proteolyticus* DSM 13887^T (36 %) and *P. aquimaris* DSM 16329^T (31 %).

Strain HT-3^T can also be differentiated from other phylogenetic neighbours including *P. nivimaris* on the basis of several phenotypic and chemotaxonomic characteristics (Table 1).

Table 1. Differential phenotypic characteristics of strain T-3-2^T and related *Psychrobacter* species

Strains: 1, *P. piscatorii* sp. nov. T-3-2^T; 2, *P. nivimaris* 88/2-7^T (unless indicated, data from Heuchert *et al.*, 2004); 3, *P. proteolyticus* 116^T (Denner *et al.*, 2001; Bozal *et al.*, 2003); 4, *P. aquimaris* SW-210^T (Yoon *et al.*, 2005); 5, *P. pacificensis* (data for six strains) (Maruyama *et al.*, 2000). Acid production from substrates and utilization of substrates were determined in this study under the same experimental conditions. +, Positive; W, weakly positive; V−, variable, type strain negative; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Anaerobic growth	–	ND	–	+	–
Urease	W	ND	+	–	+
Nitrate reduction	W	ND	–	–	–
Temperature for growth (°C)					
Maximum	30	35	<35	34	38
Optimum	24–26	10–15	19–25	25–30	25
Acid production from:					
D-Glucose	+	+	–	+	+
D-Xylose	+	–	–	+	+
L-Arabinose	+	–	–	+	+
D-Fructose	–	–	–	–	ND
L-Rhamnose	+	–	–	+	ND
D-Galactose	+	+	–	+	ND
Maltose	–	–	–	–	ND
Lactose	–	–	–	–	ND
Utilization of:					
Acetate	+	+	–	+	V–
Pyruvate	+	+	+	+	ND
Malate	+	–	–	+	+
Succinate	+	–	+	+	ND
DNA G+C content (mol%)	43.9	42	43.6	43.2	43–44

On the basis of the above results, the isolate was assigned to a novel species, for which the name *Psychrobacter piscatorii* sp. nov. is proposed.

Description of *Psychrobacter piscatorii* sp. nov.

Psychrobacter piscatorii (pis.ca.to′ri.i. L. n. *piscatorium* a fishing place; L. gen. n. *piscatorii* of a fishing place, because the type strain was isolated from a fish-processing factory).

Cells are coccobacilli (0.9–1.1 × 1.3–1.6 μm), Gram-negative and without flagella. Colonies are circular, convex and white with entire margins. Positive for catalase and oxidase. Produces acid from L-arabinose, ribose, D-xylose, D-glucose, D-mannose, D-galactose, L-rhamnose and D-fucose but not from fructose, maltose, sucrose, lactose, raffinose, *myo*-inositol, mannitol, sorbitol or glycerol under aerobic conditions. Growth occurs in medium supplemented with 0–12 % NaCl, but not at >15 % NaCl. Growth occurs at 0–30 °C. Positive for production of H_2S and Simmons' citrate, but negative in the Voges–Proskauer, methyl red and ONPG tests. Tributyrin and Tweens 20, 40, 60 and 80 are hydrolysed, but casein, gelatin, starch, DNA, alginic acid and aesculin are not. Utilizes L-arabinose, acetate, pyruvate, succinate and malate as sole carbon and energy sources for growth, but not D-fructose, glycerol, maltose or citrate. Whole-cell fatty acids consist predominantly of $C_{16:1}\omega9c$ and $C_{18:1}\omega9c$. The major isoprenoid quinone is Q-8. The DNA G+C content of the type strain is 43.9 mol%, as determined by HPLC.

The type strain, T-3-2^T (=JCM 15603^T =NCIMB 14510^T), was isolated from a drain of a fish-processing plant.

References

Bakermans, C., Ayala-del-Río, H. L., Ponder, M. A., Vishnivetskaya, T., Gilichinsky, D., Thomashow, M. F. & Tiedje, J. M. (2006). *Psychrobacter cryohalolentis* sp. nov. and *Psychrobacter arcticus* sp. nov., isolated from Siberian permafrost. *Int J Syst Evol Microbiol* **56**, 1285–1291.

Barrow, G. I. & Feltham, R. K. A. (editors) (1993). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd edn. Cambridge: Cambridge University Press.

Bozal, N., Montes, J., Tudela, E. & Guinea, J. (2003). Characterization of several *Psychrobacter* strains isolated from Antarctic environments and description of *Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov. *Int J Syst Evol Microbiol* **53**, 1093–1100.

Denner, E. B. M., Mark, B., Busse, H.-J., Turkiewicz, M. & Lubits, W. (2001). *Psychrobacter proteolyticus* sp. nov., a psychrotrophic, halotolerant bacterium isolated from the Antarctic krill *Euphausia superba* Dana, excreting a cold-adapted metalloprotease. *Syst Appl Microbiol* **24**, 44–53.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

Gerday, C. & Glansdorff, N. (editors) (2007). *Physiology and Biochemistry of Extremophiles*. Washington, DC: American Society for Microbiology.

Heuchert, A., Glöckner, F. O., Amann, R. & Fischer, U. (2004). *Psychrobacter nivimaris* sp. nov., a heterotrophic bacterium attached to organic particles isolated from the South Atlantic (Antarctica). *Syst Appl Microbiol* **27**, 399–406.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J Bacteriol* **66**, 24–26.

Jamet, A., Sigaud, S., Van de Sype, G., Puppo, A. & Hérouart, D. (2003). Expression of the bacterial catalase genes during *Sinorhizobium meliloti–Medicago sativa* symbiosis and their crucial role during the infection process. *Mol Plant Microbe Interact* **16**, 217–225.

Katsuwon, J. & Anderson, A. J. (1992). Characterization of catalase activities in a root-colonizing isolate of *Pseudomonas putida*. *Can J Microbiol* **38**, 1026–1032.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Maruyama, A., Honda, D., Yamamoto, H., Kitamura, K. & Higashihara, T. (2000). Phylogenetic analysis of psychrophilic bacteria isolated from the Japan Trench, including a description of the deep-sea species *Psychrobacter pacificensis* sp. nov. *Int J Syst Evol Microbiol* **50**, 835–846.

Merle, P.-L., Sabourault, C., Richier, S., Allemand, D. & Furla, P. (2007). Catalase characterization and implication in bleaching of a symbiotic sea anemone. *Free Radic Biol Med* **42**, 236–246.

Rocha, E. R., Selby, T., Coleman, J. P. & Smith, C. J. (1996). Oxidative stress response in an anaerobe, *Bacteroides fragilis*: a role for catalase in protection against hydrogen peroxide. *J Bacteriol* **178**, 6895–6903.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetic analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Visick, K. L. & Ruby, E. G. (1998). The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. *J Bacteriol* **180**, 2087–2092.

Yoon, J.-H., Lee, C.-H., Yeo, S.-H. & Oh, T.-K. (2005). *Psychrobacter aquimaris* sp. nov. and *Psychrobacter namhaensis* sp. nov., isolated from sea water of the South Sea in Korea. *Int J Syst Evol Microbiol* **55**, 1007–1013.

Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Suemori, A., Nakajima, K., Inoue, N. & Kawasaki, K. (2001). *Pseudomonas alcaliphila* sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. *Int J Syst Evol Microbiol* **51**, 349–355.

Yumoto, I., Hishinuma-Narisawa, M., Hirota, K., Shingyo, T., Takebe, F., Nodasaka, Y., Matsuyama, H. & Hara, I. (2004). *Exiguobacterium oxidotolerans* sp. nov., a novel alkaliphile exhibiting high catalase activity. *Int J Syst Evol Microbiol* **54**, 2013–2017.