Oceanobacillus oncorhynchi sp. nov., a halotolerant obligate alkaliphile isolated from the skin of a rainbow trout (*Oncorhynchus mykiss*), and emended description of the genus *Oceanobacillus*

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A halotolerant, obligately alkaliphilic bacterium, R-2^T, was isolated from the skin of a rainbow trout (*Oncorhynchus mykiss*), a freshwater fish. The strain is Gram-positive, ferments several carbohydrates, is rod-shaped and motile by peritrichous flagella and produces ellipsoidal spores. The isolate grows at pH 9–10 but not at pH 7–8. This micro-organism grows in 0–22% (w/v) NaCl at pH 10. Its major cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and anteiso-C_{17:0}, the major isoprenoid quinone is MK-7 and the DNA G + C content is 38.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicates that strain R-2^T is a member of the genus *Oceanobacillus*. DNA–DNA hybridization reveals low relatedness between the isolate and *Oceanobacillus iheyensis* (21.0%). On the basis of phenotypic characteristics, phylogenetic data and DNA–DNA relatedness data, the isolate should be designated as a novel species, for which the name *Oceanobacillus oncorhynchi* sp. nov. is proposed. The type strain is R-2^T (=JCM 12661^T = NCIMB 14022^T).

Alkaliphilic micro-organisms are very important in basic research as well as in industrial applications. These organisms have been studied for the industrial application of their enzymes, to understand the physiology of alkali adaptation and to elucidate their taxonomy and ecology (Horikoshi, 1991; Duckworth *et al.*, 1996; Takami *et al.*, 1997; Krulwich & Guffanti, 1989; Krulwich *et al.*, 2001; Yumoto, 2002, 2003; Thongaram *et al.*, 2003). Alkaliphiles are widely distributed in soils and related environments, in marine settings and in freshwater. Many of the alkaliphiles encountered belong to the genus *Bacillus*, which clusters into several phylogenetic groups. Recently, several *Bacillus* species have been reclassified into several novel genera, or novel genera of strains related to the genus *Bacillus* have been discovered, such as *Gracilbacillus* (Wainø *et al.*, 1999), *Halobacillus* (Spring *et al.*, 1996), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Salibacillus* (Wainø *et al.*, 1999), *Geobacillus* (Nazina *et al.*, 2001), *Anoxybacillus* (Pikuta *et al.*, 2000) and

Oceanobacillus (Lu *et al.*, 2001). Of these, the genera *Anoxybacillus* and *Oceanobacillus* contain alkaliphilic strains. As reports on alkaliphiles from freshwater and related environments are scant (Horikoshi, 1991; Nielsen *et al.*, 1995), we initiated a study to investigate such micro-organisms. A halotolerant obligate alkaliphile not belonging to the genus *Bacillus* was isolated from a freshwater fish. Phenotypic and chemotaxonomic characterization, phylogenetic analysis based on the 16S rRNA gene sequence and DNA–DNA hybridization with close neighbours showed that the isolate merited classification as a novel *Oceanobacillus* species.

The sample fish used, a rainbow trout (approx. 40 cm long), was obtained from a freshwater fish pond in Sapporo (Hokkaido, Japan). A viscous liquid (approx. 1 ml) obtained from the fish skin was inoculated into 250 ml PYA broth (pH 10) in a 500 ml flask. The medium comprised the following: 8 g peptone (Kyokuto), 3 g yeast extract (Merck), 1 g K₂HPO₄, 3.5 mg EDTA, 3 mg ZnSO₄·7H₂O, 10 mg FeSO₄·7H₂O, 2 mg MnSO₄·5H₂O, 1 mg CuSO₄·5H₂O, 2 mg Co(NO₃)₂·6H₂O and 1 mg H₃BO₃ in 1 l NaHCO₃/Na₂CO₃ buffer (100 mM in

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deionized water; pH 10) and was incubated with reciprocal shaking (140 r.p.m.) at 27 °C for 30 h. A loopful of culture broth was spread on a PYA agar plate. The isolate obtained from the plate was reisolated five times and maintained on a PYA agar slant. Cells for chemotaxonomic analysis were harvested in the late exponential phase of growth during cultivation with reciprocal shaking (140 r.p.m.) at 27 °C. In addition to the isolate, *Oceanobacillus iheyensis* JCM 11309^T, *Virgibacillus pantothenicus* IAM 11061^T and *Virgibacillus picturae* DSM 14867^T were used as reference strains for DNA–DNA hybridization. These micro-organisms were cultivated using PYA broth containing 100 mM NaHCO₃/Na₂CO₃ buffer (pH 10) and the same broth medium containing 100 mM NaH₂PO₄/Na₂HPO₄ (pH 7).

For the phenotypic characterization, PYA medium was used as the basal medium. All of the experiments were performed under aerobic conditions (except the tests on carbohydrate metabolism under anaerobic conditions). The culture was incubated at 27 °C for 2 weeks and the experiment was performed three times. Acid production from carbohydrates was determined by the method of Hugh & Leifson (1953) using thymol blue (0.008 %, w/v) instead of bromothymol blue at pH 10. Growth experiments at pH 7–10 were performed using PYA broth containing 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7–8) or 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–10). Other physiological and biochemical characteristics were examined according to the methods of Yumoto *et al.* (1998) and as described by Barrow & Feltham (1993). For observation of negatively stained cells by transmission electron microscopy (H-800 apparatus; Hitachi), cells were grown on a PYA agar slant. The transmission electron microscopy preparations and observations were performed as described previously (Yumoto *et al.*, 2001). The morphological, physiological and biochemical characteristics of the isolate are given in the species description. The isolate was revealed to be Gram-positive and to produce ellipsoidal spores positioned subterminally within a swollen sporangium. Electron microscopy showed that the cells were peritrichously flagellated rods (0.4–0.6 × 1.1–1.4 µm).

Analyses of whole-cell fatty acids and isoprenoid quinones were performed as described previously (Yumoto *et al.*, 2001). GLC (GC-353 apparatus; GL Sciences) analysis of the fatty acids of strain R-2^T revealed that the measurable components were iso-C_{14:0} (1.1 %), iso-C_{15:0} (22.7 %), anteiso-C_{15:0} (49.3 %), iso-C_{16:0} (3.2 %), C_{16:0} (2.1 %), iso-C_{17:0} (4.3 %) and anteiso-C_{17:0} (18.0 %). The major isoprenoid quinone was MK-7.

Bacterial DNA was prepared according to the method of Marmur (1961). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984): the value for strain R-2^T was 38.5 mol%, which is higher than that of *O. iheyensis* JCM 11309^T (35.8 mol%) (Lu *et al.*, 2001).

The 16S rRNA gene sequence of strain R-2^T was analysed to determine its phylogenetic position. The 16S rRNA gene was

amplified using the PCR method, with primers 9F (5'-GAGTTTGATCCTGGCTCAG) and 1541R (5'-AAGG-AGGTGATCCAGCC). The PCR product (approximately 1.5 kb) was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100) with BigDye Termination RR mix version 3.1 (Applied Biosystems) according to the manufacturer's instructions. Primers 9F, 339F, 785F, 1224F and 802R were used in the gene sequencing reaction. Multiple alignments of the sequence 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). Sequence similarity was calculated using the GENETYX computer program (Software Development). The sequence of 1451 bases of the 16S rRNA gene of strain R-2^T was compared with those of previously reported strains. A phylogenetic tree constructed using these data (Fig. 1) showed that strain R-2^T formed a monophyletic group with *O. iheyensis*. Strain R-2^T showed the highest similarity with *O. iheyensis* JCM 11309^T (96.7 %). The similarities between strain R-2^T and other phylogenetic neighbours were as follows: 94.3 % (*Virgibacillus halodenitrificans* ATCC 49067^T), 93.8 % (*V. pantothenicus* IAM 11061^T), 93.3 % (*Bacillus lentus* NCIMB 8773^T) and 93.3 % (*Bacillus niacini* NBRC 15566^T). Other taxa exhibited even lower similarities to strain R-2^T.

The level of DNA–DNA relatedness was determined fluorometrically by using the method of Ezaki *et al.* (1989) with photobiotin-labelled DNA probes, prepared using Photoprobe biotin (SP1000; Vector Laboratories) and black microplates (F16 Black Maxisorp; Nage Nunc International). The results of sequence similarity and phylogenetic analyses based on 16S rRNA gene sequences

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences of *O. oncorhynchi* R-2^T and other related organisms using the neighbour-joining method. Bootstrap values from 1000 replications are shown at branching points. Bar, 0.1 K_{nuc} .

show that strain R-2^T is closely related to *O. iheyensis* JCM 11309^T. Therefore, DNA–DNA hybridization between strain R-2^T and *O. iheyensis* JCM 11309^T and the phylogenetic neighbours *V. pantothenicus* IAM 11061^T and *V. picturae* DSM 14867^T was performed. The DNA–DNA hybridization data indicated that the isolate is distinct from *O. iheyensis* JCM 11309^T (21.0 % relatedness), *V. pantothenicus* IAM 11061^T (12.1 % relatedness) and *V. picturae* DSM 14867^T (5.4 % relatedness).

GLC analysis of the fatty acids of *O. iheyensis* JCM 11309^T revealed that the measurable components were iso-C_{14:0} (13.0 %), iso-C_{15:0} (34.3 %), anteiso-C_{15:0} (38.7 %), iso-C_{16:0} (7.8 %), C_{16:0} (1.1 %), iso-C_{17:0} (1.1 %) and anteiso-C_{17:0} (4.1 %). Obvious differences in iso-C_{14:0} and anteiso-C_{17:0} content between strain R-2^T and *O. iheyensis* JCM 11309^T were observed. Strain R-2^T can be also differentiated from *O. iheyensis* JCM 11309^T on the basis of several phenotypic and chemotaxonomic characteristics (Table 1).

The source of the first isolate belonging to the genus *Oceanobacillus* was deep-sea sediment (Lu *et al.*, 2001). Although strain R-2^T grows better with NaCl and tolerates very high NaCl concentrations, it can grow well in culture broth without NaCl. This means that the presence of NaCl in the medium is not essential for growth of strain R-2^T. In summary, we have isolated a novel *Oceanobacillus* species from a fish living in fresh water. On the basis of the characteristics of the first isolate identified as belonging to the genus *Oceanobacillus*, *O. iheyensis* JCM 11309^T, this genus has been considered to contain only obligately aerobic, facultative alkaliphiles. However, a facultatively anaerobic and obligately alkaliphilic strain was isolated in the present study, which means that the genus *Oceanobacillus* contains species exhibiting a variety of phenotypic characteristics.

On the basis of the above results, the isolate was designated

as a novel species, for which the name *Oceanobacillus oncorhynchi* sp. nov. is proposed.

Emended description of genus *Oceanobacillus* Lu *et al.* 2002

Oceanobacillus (O.ce.a.no.ba.cil'lus. L. n. *oceanus* the ocean; L. dim. n. *bacillus* a small rod; N.L. masc. n. *Oceanobacillus* the ocean bacillus/rod).

Gram-positive, spore-forming rods, motile by means of peritrichous flagella. Ellipsoidal spores are subterminal or terminal within swollen sporangia. Colonies are circular and white. Obligately aerobic or facultatively anaerobic, obligately or facultatively alkaliphilic and grows at 0–22 % (w/v) NaCl. Catalase and oxidase reactions are positive. Growth occurs at temperatures of 15–42 °C. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}. The major isoprenoid quinone is MK-7. The DNA G + C content is 35.8–38.5 mol%. The type species is *Oceanobacillus iheyensis*.

Description of *Oceanobacillus oncorhynchi* sp. nov.

Oceanobacillus oncorhynchi (on.co.rhyn'chi. N.L. gen. n. *oncorhynchi* of *Oncorhynchus*, named after the rainbow trout, *Oncorhynchus mykiss*, from which the type strain was isolated).

Cells are Gram-positive, peritrichously flagellated straight rods (0.4–0.6 × 1.1–1.4 µm) and produce ellipsoidal spores subterminally positioned within swollen sporangia. Colonies are circular and white. Ferments several carbohydrates and is obligately alkaliphilic. Grows at pH 9–10 but not at pH 7–8. Catalase and oxidase reactions are positive. Growth occurs at 15–40 °C, with the optimum at 30–36 °C. Negative for indole production, ONPG hydrolysis and deamination of phenylalanine. Growth occurs at 0–22 %

Table 1. Differentiating characteristics of *O. oncorhynchi* and *O. iheyensis*

Data for *O. iheyensis* are from Lu *et al.* (2001).

Characteristic	<i>O. oncorhynchi</i> R-2 ^T	<i>O. iheyensis</i> JCM 11309 ^T
Cell size (µm)	0.4–0.6 × 1.1–1.4	0.6–0.8 × 2.5–3.5
Growth pH range	9.0–10.0	6.5–10.0
Anaerobic growth	+	–
Hydrolysis of:		
Gelatin	–	+
Casein	–	+
DNA	–	+
Tween 60	–	+
Reduction of NO ₃ [–] to NO ₂ [–]	+	–
Major cellular fatty acids	iso-C _{15:0} , anteiso-C _{15:0} , anteiso-C _{17:0}	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{14:0}
DNA G + C content (mol%)	38.5	35.8

(w/v) NaCl, with the optimum at 7% (w/v). Nitrate is reduced to nitrite. Acid is produced from D-glucose, D-fructose, maltose, D-mannose, melibiose, sucrose, raffinose, D-galactose and trehalose. No acid is produced from D-arabinose, D-xylose, myo-inositol, sorbitol or lactose. Hydrolyses Tween 40. Negative for hydrolysis of casein, gelatin, starch, DNA, lipid (tributyrin) and Tweens 20, 60 and 80. The fatty acid components are iso-C_{14:0} (1.1%), iso-C_{15:0} (22.7%), anteiso-C_{15:0} (49.3%), iso-C_{16:0} (3.2%), C_{16:0} (2.1%), iso-C_{17:0} (4.3%) and anteiso-C_{17:0} (18.0%). The major isoprenoid quinone is MK-7. The DNA G+C content of the type strain is 38.5 mol%.

The type strain, strain R-2^T (=JCM 12661^T=NCIMB 14022^T), was isolated from the skin of a rainbow trout (*Oncorhynchus mykiss*) living in freshwater.

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