Oceanobacillus chironomi sp. nov., a halotolerant and facultatively alkaliphilic species isolated from a chironomid egg mass

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Chironomids (Diptera; Chironomidae) are the most abundant insects in freshwater aquatic habitats. Females of the genus *Chironomus* lay egg masses containing hundreds of eggs embedded in a gelatinous matrix. A bacterial strain, designated T3944D^T, was isolated from a chironomid egg mass sampled from a waste-stabilization pond in northern Israel and was found to be Gram-positive, motile by peritrichous flagella, endospore-forming, halotolerant and facultatively alkaliphilic. Comparative 16S rRNA gene sequence analysis showed that strain T3944D^T belonged to the genus *Oceanobacillus*, exhibiting the highest levels of similarity with the sequences of *Oceanobacillus oncorhynchi* subsp. *incaldanensis* DSM 16557^T (94.9%), *Oceanobacillus oncorhynchi* subsp. *oncorhynchi* JCM 12661^T (94.8%), *Oceanobacillus iheyensis* JCM 11309^T (94.7%) and *Oceanobacillus picturae* LMG 19416 (94.5%). Strain T3944D^T grew optimally at 1–3% NaCl, pH 8.5 and 37 °C. The major cellular fatty acids were anteiso-C_{15:0} (60.0%) and anteiso-C_{17:0} (12.9%) and the DNA G + C content was 38.1 mol%. On the basis of its phenotypic properties and phylogenetic distinctiveness, strain T3944D^T represents a novel species in the genus *Oceanobacillus*, for which the name *Oceanobacillus chironomi* sp. nov. is proposed. The type strain is T3944D^T (=LMG 23627^T = DSM 18262^T).

The genus *Oceanobacillus* was first described by Lu *et al.* (2001, 2002) and at the time of writing comprises three species: *O. iheyensis* (Lu *et al.*, 2001), whose entire genome sequence has been determined and is now available (Takami *et al.*, 2002), *O. oncorhynchi* (Yumoto *et al.*, 2005) and *O. picturae*, which was recently transferred from the genus *Virgibacillus* (Lee *et al.*, 2006). All of the species belonging to this genus are halophilic or halotolerant and alkaliphilic or alkalitolerant.

Chironomids (Diptera; Chironomidae) are the most abundant insects in freshwater aquatic habitats. Females of the genus *Chironomus* lay egg masses at the water's edge. Each egg mass contains hundreds of eggs embedded in a thick, gelatinous matrix. Chironomid egg masses harbour *Vibrio cholerae* and provide a natural reservoir for this bacterium (Broza & Halpern, 2001; Halpern *et al.*, 2003, 2004, 2006). All *Vibrio cholerae* isolates have the capacity to degrade chironomid egg masses and prevent the eggs from hatching (Halpern *et al.*, 2003, 2004, 2006). Strain T3944D^T was isolated in the course of screening of the microbial population of chironomid egg masses (Halpern *et al.*, 2007). The egg masses were sampled in mid-September 2004 from

the Tivon waste-stabilization pond near Haifa (northern Israel), using Styrofoam boards as artificial oviposition sites for the adult female chironomids. The egg masses were thoroughly washed and then cultured directly on three bacteriological media, thiosulphate-citrate-bile salts agar (selective for *Vibrio cholerae*), LB agar and MacConkey agar. Strain T3944D^T was one of the colonies that was randomly picked and streaked from the LB agar. More than 10² faecal coliforms ml⁻¹ were counted in the water samples from the Tivon waste-stabilization pond (Halpern *et al.*, 2004), but no faecal coliforms were isolated from the egg mass even though MacConkey agar (selective for *Escherichia coli*) was used. This confirmed that the egg masses had been rinsed properly and that strain T3944D^T and the other isolates had been tightly attached to the egg mass.

Diverse types of bacteria have been isolated from the egg masses and characterized (Halpern *et al.*, 2007). One of these isolates, a Gram-positive, halotolerant, facultatively alkaliphilic, spore-forming, motile bacillus, designated strain T3944D^T, is described here. Its exact taxonomic position was determined by using a polyphasic approach that included phenotypic analysis and a phylogenetic investigation based on 16S rRNA gene sequences.

For electron microscopy, bacteria from LB agar were suspended in saline. The samples were attached to a

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain T3944D^T is DQ298074.

Fig. 1. Electron micrograph of a negatively stained cell of strain T3944D^T, showing the peritrichous flagellation of the rod. Bar, 200 nm.

carbon-coated grid, stained with 2 % uranyl acetate and then photographed under a JEM-1200EX electron microscope (JEOL). Electron microscopy showed that the cells were peritrichously flagellated rods ($0.8\text{--}1.0 \times 1.3\text{--}3.0\text{ }\mu\text{m}$) (Fig. 1).

The 16S rRNA gene was analysed to determine the phylogenetic position of strain T3944D^T. Universal bacterial primers 8f and 1512r, based on *E. coli* positions, were used to amplify internal fragments of the 16S rRNA gene, according to Felske *et al.* (1997). The amplified PCR product (approx. 1.5 kb) was purified with a Wizard PCR product-purification kit (Promega). Purified PCR products were sequenced directly by the dideoxynucleotide chain-termination method, using a DNA sequencer (ABI PRIZM 3100) with

BigDye terminator reagents (Applied Biosystems), according to the instructions of the manufacturer (at Technion Medical School, Haifa, Israel). Sequencing was performed using primers 8f (5'-CACGGATCCAGACTTTGATYMTGGCTCAG-3'), 519f (5'-CAGCMGCCGCGGTAATWC-3'), 534r (5'-ATTACCGCGGCTGCTGG-3') and 968f (5'-AACGCGAAGAACCTTAC-3'). This resulted in data relating to approximately 1471 bp. To identify the closest relatives, the newly determined sequence was compared with those available in GenBank (<http://www.ebi.ac.uk>) by using the WU BLAST program (Washington University Basic Local Alignment Search Tool, version 2.0). Sequence alignment was performed using the CLUSTAL W program, and the phylogenetic tree was generated using the neighbour-joining method in the MEGA3 software (Kumar *et al.*, 2004). Bootstrap values (from 1000 replicates) greater than 50 % are shown at the branch points in the tree presented in Fig. 2.

The 1471-base sequence of the 16S rRNA gene of strain T3944D^T was compared with the sequences of previously reported strains. Strain T3944D^T showed the highest levels of similarity with *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T (94.9 %), *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T (94.8 %), *O. iheyensis* JCM 11309^T (94.7 %) and *O. picturae* LMG 19416 (94.5 %). The similarities between strain T3944D^T and other phylogenetic neighbours within the genera *Virgibacillus* and *Bacillus* were less than 94.5 % (Fig. 2).

For the phenotypic characterization, LB agar was used as the basal medium, except for the determination of the pH range, for which peptone-yeast extract medium supplemented with 1 % NaCl was used (according to Lu *et al.*, 2001). Salt tolerance was determined at 37 °C on LB agar containing varying concentrations of NaCl. Growth at various temperatures (4, 6, 10, 11, 12, 15, 25, 30, 32, 35, 37, 39, 40, 44, 46, 47, 48 and 50 °C) was measured on LB agar (pH 8.5) supplemented with 1 % NaCl. Growth under anaerobic conditions was determined after incubation of the novel strain, in an anaerobic chamber, on LB agar

Fig. 2. Neighbour-joining tree (obtained with MEGA3, based on an alignment from CLUSTAL W), based on 16S rRNA gene sequence data, showing the phylogenetic position of strain T3944D^T with respect to species of the genus *Oceanobacillus* and other related taxa. Percentage bootstrap values are indicated. Bar, 0.005 accumulated changes per nucleotide.

containing 1 % NaCl (pH 8.5) and on LB agar supplemented with nitrate. Strain T3944D^T showed optimal growth at 1–3 % NaCl, pH 8.5 and 37 °C (Table 1).

Biochemical tests were performed by using the API 20E system (micromethod tests for the identification of Gram-negative rods; bioMérieux). Carbon assimilation was analysed using Biolog GP microwell plates according to the manufacturer's instructions (release 3.50, version DE; Biolog). The plates were incubated for 48 h at 37 °C. Wells that changed to purple were scored as being positive for metabolic activity. The sensitivity of strain T3944D^T to antibiotics was tested by using LB agar and Sensi-Discs (BBL) after incubation for 48 h.

For analysis of the cellular fatty acids, cells were cultured on tryptic soy agar at 28 °C before extraction of the fatty acids. The microbial fatty acid profile was analysed using the MIDI/Hewlett Packard microbial identification system (Analytical Services), which uses GC profiles of fatty acid methyl esters. The measurable fatty acid components of strain T3944D^T were as follows: iso-C_{14:0} (5.03 %), C_{14:0} (2.39 %), iso-C_{15:0} (6.08 %), anteiso-C_{15:0} (59.97 %), iso-C_{16:0} (5.92 %), C_{16:0} (7.72 %) and anteiso-C_{17:0} (12.89 %).

For determination of the DNA G+C content, genomic DNA of strain T3944D^T was prepared according to a modified version of the procedure of Wilson (1987). The G+C content of the DNA sample was determined in three independent analyses using the HPLC technique (Mesbah *et al.*, 1989) and was performed by the BCCM/LMG Bacteria Collection Identification Service (Laboratory of Microbiology, Ghent University, Ghent, Belgium). The DNA G+C content of strain T3944D^T was found to be 38.1 mol%.

Strain T3944D^T was isolated from chironomid egg masses, along with *Vibrio cholerae* and other culturable bacterial populations that inhabit this environment (Halpern *et al.*, 2007). The eggs are embedded in a gelatinous matrix and, although strain T3944D^T is able to degrade gelatin (Table 1), unlike all *Vibrio cholerae* isolates from the same niche (Halpern *et al.*, 2003, 2006), it does not degrade the egg mass.

The genus *Oceanobacillus* was created by Lu *et al.* (2001, 2002) and at the time of writing contains three species, which were isolated from quite different environments: (i) *O. iheyensis*, which is of marine origin; (ii) *O. oncorhynchi* subsp. *oncorhynchi*, *O. oncorhynchi* subsp. *incaldanensis* and

Table 1. Differential characteristics of strain T3944D^T and *Oceanobacillus* species

Strains: 1, *O. chironomi* sp. nov. strain T3944D^T; 2, *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T; 3, *O. oncorhynchi* subsp. *oncorhynchi* JCM 12661^T; 4, *O. iheyensis* JCM 11309^T; 5, *O. picturae* LMG 19492^T. Data were obtained from Lu *et al.* (2001), Heyrman *et al.* (2003), Yumoto *et al.* (2005), Lee *et al.* (2006), Romano *et al.* (2006) and this study. ND, Not determined; W, weakly positive reaction.

Characteristic	1	2	3	4	5
Cell size (µm)	0.8–1.0 × 1.3–3.0	0.5–0.8 × 1.2–2.0	0.4–0.6 × 1.1–1.4	0.6–0.8 × 2.5–3.5	0.5–0.7 × 2.0–6.0
Colony colour	Cream–beige	Cream–beige	White	Cream–white	Cream
Spore formation	+	–	+	+	+
Anaerobic growth	–	–	+	–	–
Hydrolysis of:					
Gelatin	+	–	–	+	–
Tween 60	–	ND	–	+	ND
Casein	–	–	–	+	W
Reduction of nitrate to nitrite	+	+	+	–	+
Reduction of nitrite to N ₂	+	+	–	–	–
NaCl range for growth (%)	0–11	5–20	0–22	0–21	0–10
NaCl optimum (%)	1–3	10	3	3	7
Growth temperature range (°C)	12–46	10–40	15–40	15–42	5–40
Optimum growth temperature (°C)	37	37	30–36	30	25–35
pH range for growth	6.5–10.0	6.5–9.5	9.0–10.0	6.5–10.0	7.0–10.0
pH optimum	8.5	9.0	9.0–10.0	7.0–9.5	ND
DNA G+C content (mol%)	38.1	40.1	38.5	35.8	39.5
Major cellular fatty acids (> 10 % of total)*	anteiso-C15:0, anteiso-C17:0 ^a	iso-C14:0, iso-C15:0, anteiso-C15:0 ^b	iso-C15:0, anteiso-C15:0, anteiso-C17:0 ^b	iso-C14:0, iso-C15:0, anteiso-C15:0 ^c	iso-C14:0, anteiso-C15:0, anteiso-C17:0 ^d

*Growth conditions for fatty acids analysis: *a*, tryptic soy agar, 28 °C; *b*, no data available; *c*, marine broth and 10 % moderate halophile medium, 37 °C (Lu *et al.*, 2001) and tryptic soy agar, 28 °C (this study); *d*, tryptic soy agar, 30 °C.

strain T3944D^T, which are of freshwater origin; and (iii) *O. picturae*, which was isolated from a mural painting. The phylogenetic relationships among strain T3944D^T and related taxa are shown in Fig. 2. The comparative 16S rRNA gene sequence analysis showed that the novel isolate is phylogenetically most closely related to *Oceanobacillus* species (94.5–94.9 % sequence similarity). Strain T3944D^T shared the main characteristics with *Oceanobacillus* species, for example, they were positive for catalase and oxidase, they were motile rods (having peritrichous flagella), they produced endospores and they were halotolerant (except for *O. oncorhynchi* subsp. *incaldanensis* DSM 16557^T, which is halophilic and also does not produce endospores) (Table 1). The DNA G+C content of strain T3944D^T (38.1 mol%) is within the range for *Oceanobacillus* species (35.8–40.1 mol%) and its major cellular fatty acid is anteiso-C_{15:0} (Table 1).

However, strain T3944D^T possesses a unique combination of the properties that characterize the members of the genus *Oceanobacillus*. It is halotolerant and is a facultatively alkaliphilic obligate aerobe (Table 1). It hydrolyses gelatin, is able to reduce nitrite to N₂ and has a unique fatty acid profile [including anteiso-C_{15:0} (60.0 %) and anteiso-C_{17:0} (12.9 %)] (Table 1). It grows at 46 °C, which is a higher growth temperature than that of any *Oceanobacillus* species (Table 1). In the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/blast/>), four sequences (GenBank accession numbers DQ346552, DQ346561, DQ346602 and DQ346627) of uncultured and undescribed compost bacteria showed 98.6 % 16S rRNA gene sequence similarity with respect to strain T3944D^T. These uncultured strains, which were identified during a clone library study of the bacterial diversity of compost from Korea, probably fall within the lineage of the novel species proposed here (Fig. 2).

On the basis of the phenotypic characterization and the phylogenetic analysis, strain T3944D^T should be classified as a novel species, for which the name *Oceanobacillus chironomi* sp. nov. is proposed.

Description of *Oceanobacillus chironomi* sp. nov.

Oceanobacillus chironomi [chi.ro'no.mi. N.L. gen. n. *chironomi* of *Chironomus*, named after the non-biting midge insect of the genus *Chironomus* (Chironomidae: Diptera) from which the type strain was isolated].

Cells are Gram-positive, peritrichously flagellated rods (0.8–1.0 × 1.3–3.0 µm) (Fig. 1) that sometimes form chains. Cells produce ellipsoidal spores terminally or subterminally positioned within swollen sporangia. Colonies are circular and cream–beige in colour. Colony colour darkens from the centre as the culture ages. Obligately aerobic and does not ferment carbohydrates. Facultatively alkaliphilic. Grows at pH 6.5–10, with optimal growth at pH 8.5. Halotolerant and grows with 0–11 %

NaCl, with optimal growth at 1–3 % NaCl. Catalase- and oxidase-positive. Growth occurs at 12–46 °C, with optimum growth at 37 °C. Negative for indole production, ONPG hydrolysis and deamination of phenylalanine. Cells are resistant to tetracycline but susceptible to penicillin G, ampicillin, vancomycin, streptomycin, chloramphenicol, bacitracin, novobiocin, gentamicin, neomycin and kanamycin. The following substrates are assimilated in the Biolog test: dextrin, *N*-acetyl-D-glucosamine, D-fructose, D-galactose, α-D-glucose, α-D-lactose, maltose, maltotriose, D-mannose, D-melibiose, D-ribose, α-ketoglutaric acid, α-ketovaleric acid, L-alaninamide, 2,3-butanediol, glycerol and adenosine. Cellular fatty acids are iso-C_{14:0} (5.0 %), C_{14:0} (2.4 %), iso-C_{15:0} (6.1 %), anteiso-C_{15:0} (60.0 %), iso-C_{16:0} (6.0 %), C_{16:0} (7.7 %) and anteiso-C_{17:0} (12.9 %).

The DNA G+C content of the type strain is 38.1 mol%. The type strain, T3944D^T (= LMG 23627^T = DSM 18262^T), was isolated from a chironomid egg mass sampled from a waste-stabilization pond in northern Israel.

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