Thalassobacillus hwangdonensis sp. nov., isolated from a tidal flat sediment

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A Gram-staining-positive, endospore-forming, motile, rod-shaped bacterium, strain AD-1^T, was isolated from a tidal flat sediment of the coast of Hwangdo on the Yellow Sea, Korea. Strain AD-1^T grew optimally at pH 7.0–7.5 and 40 °C and in the presence of 5–10 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain AD-1^T was most closely related to *Thalassobacillus devorans* G-19.1^T (98.0 % sequence similarity) and *Thalassobacillus cyri* HS286^T (97.8 %). The cell-wall peptidoglycan was based on meso-diaminopimelic acid and MK-7 was the predominant menaquinone. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and two unidentified lipids. The major fatty acids (>10 % of total fatty acids) were iso-C_{15:0}, iso-C_{17:0} and anteiso-C_{15:0}. The DNA G + C content of strain AD-1^T was 45.2 mol%. It appears reasonable to classify strain AD-1^T as a member of the genus *Thalassobacillus*. There were differences in fatty acid profiles and phenotypic and genetic characteristics between strain AD-1^T and the type strains of the two *Thalassobacillus* species. On the basis of the data presented, strain AD-1^T represents a novel species within the genus *Thalassobacillus*, for which the name *Thalassobacillus hwangdonensis* sp. nov. is proposed. The type strain is AD-1^T (=KCTC 13254^T =CCUG 56607^T).

The genus *Thalassobacillus* was first described by García *et al.* (2005) with the description of a single species, *Thalassobacillus devorans*. Subsequently, a second species of the genus has been described, *Thalassobacillus cyri* (Sanchez-Porro *et al.*, 2009). Members of the genus *Thalassobacillus* have been isolated from saline soil and hypersaline lake water (García *et al.*, 2005; Sanchez-Porro *et al.*, 2009). *T. devorans* was isolated from a phenol enrichment of saline soil samples and was found to degrade phenol (García *et al.*, 2005). The genus *Thalassobacillus* is characterized chemotaxonomically by having cell-wall peptidoglycan based on meso-diaminopimelic acid, MK-7 as the predominant menaquinone, anteiso-C_{15:0} as the predominant fatty acid and a DNA G + C content of 42.4–43.0 mol% (García *et al.*, 2005; Sanchez-Porro *et al.*, 2009). Many novel bacterial strains have been isolated recently from the coast of Hwangdo (Hwang island) on the Yellow Sea, Korea (Jung *et al.*, 2010). One of these isolates, AD-1^T, which was phylogenetically most closely related to the genus *Thalassobacillus*, is described in this study.

Tidal flat sediments were collected from the coast of Hwangdo and used as the source for the isolation of bacterial strains. The sediment samples were suspended in a 0.85 % NaCl solution. The suspensions were serially diluted, spread on modified S-G medium (1⁻¹ distilled

water: 100 g NaCl, 20 g MgSO₄·7H₂O, 2 g KCl, 3 g trisodium citrate, 10 g yeast extract, 7.5 g Casamino acid, 15 g agar; pH 7.2; Sehgal & Gibbons, 1960) and incubated at 25 °C. The type strains of the two *Thalassobacillus* species were used as reference strains for the analyses of fatty acids, polar lipids and phenotypic properties and the determination of DNA–DNA relatedness. *T. devorans* DSM 16966^T was purchased from the DSMZ and *T. cyri* HS286^T was a kind gift from Professor Antonio Ventosa (University of Seville, Spain). Strain AD-1^T was routinely cultivated at 40 °C on MSG agar (modified S-G medium containing 75 g NaCl content l⁻¹) for the determination of morphological, physiological and biochemical characteristics. The two reference strains were routinely cultivated at 37 °C on MSG agar to determine phenotypic characteristics and to obtain cell mass for polar lipid analysis and DNA–DNA hybridization experiments.

Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (Philips CM-20). Flagellation was determined with transmission electron microscopy using cells from exponentially growing cultures that were negatively stained with 1 % (w/v) phosphotungstic acid and air-dried. The Gram reaction was performed by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Endospores were investigated by using Schaeffer–Fulton (Murray *et al.*, 1994) and malachite green (Smibert & Krieg, 1994) staining methods. Growth under anaerobic conditions was determined by

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incubation in a Forma anaerobic chamber on MSG agar and on MSG agar supplemented with 0.1 % (w/v) potassium nitrate, both of which had been prepared under a nitrogen atmosphere. Growth at 4, 10, 15, 20, 25, 28, 30, 35, 37 and 40–50 °C (at intervals of 1 °C) was determined on MSG agar. Growth with 0–25 % (w/v) NaCl (in increments of 1.0 %) was investigated by using liquid media prepared according to the formula of MSG agar except that NaCl and agar were excluded. Growth in the absence of NaCl was also investigated using trypticase soy broth prepared according to the formula of the Difco medium except that 0.45 % (w/v) MgCl₂ · 6H₂O was included and NaCl was excluded. The pH range for growth was determined in MSG broth adjusted to pH 4.5–9.5 (at intervals of 0.5 pH units) by using sodium acetate/acetic acid and Na₂CO₃ buffers. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Nitrate reduction, hydrolysis of aesculin, gelatin, urea and Tweens 20, 40, 60 and 80, indole production and methyl red and Voges–Proskauer reactions were tested as described by Lányi (1987) using artificial seawater (Bruns *et al.*, 2001) for media preparation. Hydrolysis of casein, hypoxanthine, starch and xanthine was tested on MSG agar with the substrate concentrates described by Cowan & Steel (1965). DNase activity was examined by using DNase test agar with methyl green (Difco) and supplementation with 10 % (w/v) NaCl. Degradation of phenol was tested as described by García *et al.* (2005); *T. devorans* DSM 16966^T was used as a positive control. H₂S production was tested as described by Bruns *et al.* (2001). Utilization of various substrates for growth was tested as described by Choi *et al.* (2006) with the modification that Noble agar (Difco) was used instead of Bacto agar. Carbon sources were sterilized by filtration and added to a final concentration of 0.2 % (w/v). Acid production from carbohydrates was investigated according to the method of Leifson (1963). Enzyme activities were determined by using the API ZYM and API 20E systems (bioMérieux). Susceptibility to antibiotics was investigated on MSG agar by using antibiotic discs with the following amounts (µg per disc unless otherwise indicated): ampicillin (10), carbenicillin (100), cephalothin (30), chloramphenicol (100), gentamicin (30), kanamycin (30), lincomycin (15), neomycin (30), novobiocin (5), oleandomycin (15), penicillin G (20 U), polymyxin B (100 U), streptomycin (50) and tetracycline (30).

To collect cell biomass for DNA extraction and analysis of the cell-wall peptidoglycan, isoprenoid quinones and polar lipids, strain AD-1^T was cultivated in MSG broth at 40 °C for 3 days. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination by RNA. The 16S rRNA gene was amplified by PCR using universal primers 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGAAAGGAGGTGATCCAGCC-3', as described previously (Yoon *et al.*, 1998). Sequencing of the amplification products and phylogenetic analysis were performed as described by Yoon *et al.* (2003). The isomer type of the diamino acid in

the cell-wall peptidoglycan was analysed using TLC according to the method described by Komagata & Suzuki (1987). Isoprenoid quinones were analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column, as described by Komagata & Suzuki (1987). For cellular fatty acid analysis, strain AD-1^T and the two reference strains were cultivated under two different culture conditions: on MSG agar at 37 °C for 3 days and on solid SW-7.5 medium (Ventosa *et al.*, 1982) at 37 °C for 5 days. Fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values.

The morphological, cultural, physiological and biochemical characteristics of strain AD-1^T are given in the species description and in Table 1. The almost-complete 16S rRNA gene sequence of strain AD-1^T determined in this study comprised 1522 nucleotides, representing approximately 96 % of the 16S rRNA gene sequence of *Escherichia coli*. In the phylogenetic tree based on the neighbour-joining algorithm (Fig. 1), strain AD-1^T joined the cluster comprising *T. devorans* G-19.1^T and *T. cyri* HS286^T. The cluster was supported by a bootstrap resampling value of 99.6 % and was also found in the trees constructed using the maximum-likelihood and maximum-parsimony algorithms. Strain AD-1^T exhibited 16S rRNA gene sequence similarities of 98.0 and 97.8 % with *T. devorans* G-19.1^T and *T. cyri* HS286^T, respectively, and less than 96.6 % with the other sequences included in the phylogenetic analysis.

Strain AD-1^T contained *meso*-diaminopimelic acid as the diamino acid in the cell-wall peptidoglycan. The predominant menaquinone detected in strain AD-1^T was MK-7 (peak area ratio of approximately 95 %). The cell-wall peptidoglycan and predominant menaquinone type of strain AD-1^T were the same as those of the two reference strains (García *et al.*, 2005; Sanchez-Porro *et al.*, 2009). Strain AD-1^T contained large amounts of branched fatty acids: the major fatty acid was iso-C_{15:0} (48.9 %), and significant amounts (>10 % of total fatty acids) of iso-C_{17:0} and anteiso-C_{15:0} were detected (Table 2). The cellular fatty acid profiles determined in this study for *T. devorans* DSM 16966^T and *T. cyri* HS286^T were essentially the same as those determined previously, although there

Table 1. Differential characteristics of strain AD-1^T and the type strains of *Thalassobacillus* species

Strains: 1, *Thalassobacillus hwangdonensis* sp. nov. AD-1^T; 2, *T. devorans* DSM 16966^T (unless indicated, data from García *et al.*, 2005); 3, *T. cyri* HS286^T (Sanchez-Porro *et al.*, 2009). All strains were positive for nitrate reduction, utilization of L-arabinose*, D-fructose, D-glucose†, maltose, D-mannose, trehalose, L-malate* and pyruvate, acid production from D-fructose, D-glucose, D-mannose, maltose and trehalose and activity of alkaline phosphatase, esterase (C4) and α -glucosidase. All strains were negative for the Voges–Proskauer test, phenylalanine deaminase*, production of indole and H₂S‡, hydrolysis of urea, utilization of D-xylose, formate and L-glutamate, acid production from L-arabinose, myo-inositol, lactose, raffinose, L-rhamnose, D-ribose, D-sorbitol and D-xylose, and activity of lipase (C14), valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, α - and β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase.

Characteristic	1	2	3
Anaerobic growth	+	–	–
Optimum growth temperature (°C)	40	37	40
Growth with NaCl (%)			
Range	0–20	0.5–20	1–15
Optimum	5–10	7.5–10	8
Oxidase	+	–	+
Methyl red test	+	–	+
Hydrolysis of:			
Aesculin	–	–	+
Casein	+	–	+
Gelatin	–	+	–
Starch	–	–	+
Utilization of:§			
D-Galactose	–	+†	+
Sucrose	–	+	+
Citrate	–	+	+
Benzoate	–	+	+
Acid produced from:§			
D-Galactose	–	–	+
D-Mannitol	–	+	–
Melezitose	–	+	–
Melibiose	–	–	+
Sucrose	–	+	–
Enzyme activity (API ZYM)§			
Esterase lipase (C8)	+	–	+
Leucine arylamidase	+	–	–
Naphthol-AS-BI-phosphohydrolase	–	+	–
DNA G + C content (mol%)	45.2	42.4	43.0

*Data from this study; opposite result was obtained for *T. devorans* DSM 16966^T by García *et al.* (2005).

†Data from this study; opposite result was obtained for *T. cyri* HS286^T by Sanchez-Porro *et al.* (2009).

‡Data for *T. devorans* DSM 16966^T from this study.

§Data for reference strains from this study.

were differences in the contents of some fatty acids (García *et al.*, 2005; Sanchez-Porro *et al.*, 2009). The fatty acid profiles of the three strains were similar although there

were differences in the proportions of the major fatty acids. Intragenetic differences in fatty acid profiles have been reported for members of some genera (Yoon *et al.*, 2001; Hanada *et al.*, 2002; Hua *et al.*, 2007; Nam *et al.*, 2008). The major polar lipids detected in strain AD-1^T were diphosphatidylglycerol, phosphatidylglycerol and two unidentified lipids. The major polar lipids detected in *T. devorans* DSM 16966^T in this study were diphosphatidylglycerol, phosphatidylglycerol, an unidentified lipid and an unidentified phospholipid. The major polar lipids detected in *T. cyri* HS286^T by Sanchez-Porro *et al.* (2009) were diphosphatidylglycerol, phosphatidylglycerol, two unidentified phospholipids and an unidentified glycolipid. The DNA G + C content of strain AD-1^T was 45.2 mol%, which was a little higher than those reported for *T. devorans* DSM 16966^T and *T. cyri* HS286^T (42.4 and 43.0 mol%, respectively; Table 1). Taking these data into account, it appears appropriate to classify strain AD-1^T as a member of the genus *Thalassobacillus*.

Strain AD-1^T exhibited mean DNA–DNA relatedness of 5 and 9% to *T. devorans* DSM 16966^T and *T. cyri* HS286^T, respectively. Strain AD-1^T was distinguishable from *T. devorans* DSM 16966^T and *T. cyri* HS286^T by differences in several phenotypic properties, including absence of endospores, anaerobic growth, oxidase activity and hydrolysis, utilization and acid production from carbohydrates and enzyme activities (Table 1). Therefore, the genetic distinctiveness and differential phenotypic properties are sufficient to categorize strain AD-1^T in a novel species within the genus *Thalassobacillus* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), for which the name *Thalassobacillus hwangdonensis* sp. nov. is proposed.

Description of *Thalassobacillus hwangdonensis* sp. nov.

Thalassobacillus hwangdonensis (hwang.do.nen'sis. N.L. masc. adj. *hwangdonensis* pertaining to Hwangdo, the Korean island from where the type strain was isolated).

Cells are Gram-stain-positive rods, 0.3–0.7 × 1.2–3.5 µm. Motile by means of peritrichous flagella. Central or subterminal ellipsoidal endospores are observed in swollen sporangia. Colonies are circular, semi-translucent, slightly convex and moderate yellow, 1.2–2.0 mm in diameter after incubation on MSG agar for 3 days at 40 °C. Growth occurs at 15–47 °C (optimum 40 °C), at pH 6.0–9.0 (optimum pH 7.0–7.5) and with 0–20% (w/v) NaCl (optimum 5–10% NaCl). Mg²⁺ ions are required for growth. Anaerobic growth occurs on MSG agar and on MSG agar supplemented with nitrate. Oxidase-positive. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and phenylalanine deaminase activities are absent. Methyl red test is positive. Voges–Proskauer test is negative. H₂S and indole are not produced. Casein, DNA and Tweens 20, 40, 60 and 80 are hydrolysed, but aesculin, gelatin, hypoxanthine, xanthine, starch, L-tyrosine and urea are not. Phenol is not degraded. L-Arabinose, D-fructose, D-glucose, maltose,

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain AD-1^T and related taxa. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Alicyclobacillus acidocaldarius* DSM 446^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. Note that the sequence corresponding to accession number X62174 is from strain NCIMB 2269^T (Farrow *et al.*, 1992), not NCIMB 9251 as given in GenBank.

Table 2. Cellular fatty acid compositions of strain AD-1^T and the type strains of *Thalassobacillus* species

Strains: 1, *T. hwangdonensis* sp. nov. AD-1^T; 2, *T. devorans* DSM 16966^T; 3, *T. cyri* HS286^T. All data were taken from this study after cultivation on MSG agar or solid SW-7.5 medium as described in the text. Fatty acids that represented <1.0% in all strains were omitted. –, Not detected.

Fatty acid (%)	1		2		3	
	MSG	SW-7.5	MSG	SW-7.5	MSG	SW-7.5
Straight-chain						
C _{16:0}	1.0	3.6	2.0	1.4	1.4	1.8
Unsaturated						
C _{16:1} ω7c alcohol	0.4	0.3	0.4	0.2	0.8	1.4
Branched						
iso-C _{14:0}	—	0.5	1.2	3.3	2.1	2.7
iso-C _{15:0}	48.9	43.0	9.6	8.1	7.4	11.8
anteiso-C _{15:0}	11.4	18.5	53.6	59.2	51.6	49.3
iso-C _{16:0}	1.0	5.0	9.0	10.5	11.4	13.1
iso-C _{17:0}	18.0	13.6	2.6	0.9	2.3	2.6
anteiso-C _{17:0}	7.9	13.1	15.2	11.0	19.6	14.5
anteiso-C _{17:1}	—	—	2.9	2.0	1.9	1.6
iso-C _{17:1} ω10c	7.4	0.4	—	—	—	—
Summed features*						
2	—	—	0.7	0.9	0.4	1.1
3	—	0.1	1.4	1.0	0.5	—
4	3.6	0.4	—	—	—	—

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of iso-C_{16:1} and/or C_{14:0} 3-OH. Summed feature 3 consisted of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature 4 consisted of iso-C_{17:1} I and/or anteiso-C_{17:1} B.

carbon and energy sources, but cellobiose, D-galactose, sucrose, D-xylose, acetate, benzoate, citrate, formate, L-glutamate, succinate and salicin are not. Acid is produced from D-fructose, D-glucose, D-mannose, maltose, trehalose and cellobiose (weakly), but not from L-arabinose, D-galactose, myo-inositol, lactose, D-mannitol, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose or D-xylose. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and α-glucosidase activities are present and weak α-chymotrypsin activity is present, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, lincomycin, novobiocin, oleandomycin and penicillin G, but not to gentamicin, kanamycin, neomycin, polymyxin B, streptomycin or tetracycline. The cell-wall peptidoglycan contains meso-diaminopimelic acid. The predominant menaquinone is MK-7. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and two unidentified lipids. The major fatty acids (>10% of total fatty acids) are iso-C_{15:0}, iso-C_{17:0} and anteiso-C_{15:0}. The DNA G+C content of the type strain is 45.2 mol% (HPLC).

The type strain, AD-1^T (=KCTC 13254^T =CCUG 56607^T), was isolated from a tidal flat sediment of the coast of Hwangdo on the Yellow Sea, Korea.

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