Thalassobacillus devorans gen. nov., sp. nov., a moderately halophilic, phenol-degrading, Gram-positive bacterium

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A novel moderately halophilic bacterium, strain G-19.1^T, has been isolated from a phenol enrichment of samples collected in hypersaline habitats of southern Spain. This enrichment culture was a part of a screening programme to isolate halophilic bacteria able to degrade various aromatic compounds. Strain G-19.1^T has been characterized as a potential phenol-degrader over a wide range of saline conditions. Strain G-19.1^T was found to be an aerobic, Gram-positive, endospore-forming, non-pigmented, moderately halophilic rod that grew optimally in media containing 7.5–10% NaCl at pH 7.0. The DNA G + C content was 42.4 mol%. Phylogenetic analysis based on comparison of 16S rRNA gene sequences indicated that the closest relatives were *Halobacillus* species (96.2–97.0%), although this novel isolate constitutes a separate line of descent within the radiation of Gram-positive rods. The cell-wall peptidoglycan contained meso-diaminopimelic acid, indicating that this strain does not share the main characteristic that differentiates members of the genus *Halobacillus* (which contain Orn–D–Asp) from other related genera. The predominant cellular fatty acids were anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{15:0}. On the basis of phenotypic, genotypic and phylogenetic analyses, this isolate should be classified in a novel genus and species, for which the name *Thalassobacillus devorans* gen. nov., sp. nov. is proposed. The type strain is strain G-19.1^T (=DSM 16966^T=CECT 7046^T=CCM 7282^T).

Aromatic hydrocarbons from both natural and anthropogenic sources are abundant organic compounds in saline habitats. Because of environmental problems, various approaches designed to eliminate or reduce the presence of aromatic hydrocarbons have been pursued using bioremediation processes. However, the presence of salt constitutes a problem in bioremediation programmes in these contaminated habitats, constituting a limiting factor in such processes (Oren *et al.*, 1992). In this respect, the study of halophilic communities able to degrade aromatic compounds over a wide range of salinities is of great importance and will help in the characterization and identification of novel efficient pollutant-degrading micro-organisms.

Halophilic communities in saline and hypersaline

environments are composed of species that fall mainly into two physiological groups, the extremely halophilic and the moderately halophilic bacteria and archaea (Ventosa *et al.*, 1998), representing a promising ecosystem for biodegradation purposes. In recent years, efforts have been made to isolate halophilic bacteria able to degrade aromatic compounds (Margesin & Schinner, 2001; Mellado & Ventosa, 2003). However, little progress has been made in identifying the strains isolated. Of the moderately halophilic bacteria, a few members of the genera *Halomonas*, *Marinobacter* and *Arhodomonas* have been described as species able to degrade organic compounds (Adkins *et al.*, 1993; Muñoz *et al.*, 2001; Alva & Peyton, 2003; Nicholson & Fathepure, 2004; García *et al.*, 2004; Huu *et al.*, 1999; Hedlund *et al.*, 2001). Less well known is the capacity of Gram-positive halophilic bacteria to degrade aromatic compounds, although other Gram-positive, non-halophilic genera are associated with degradation of these compounds, including both high-G + C Gram-positive bacteria, such as species of the genera *Rhodococcus* (Rast *et al.*, 1980; Grund *et al.*, 1992; Briglia *et al.*, 1996; Eulberg *et al.*, 1997; Yoon *et al.*, 2000a, b), *Arthrobacter* (Eck & Belter, 1993; Westerberg *et al.*, 2000) and *Microbacterium* (Arrault *et al.*, 2002; Gauthier *et al.*, 2003), and low-G + C Gram-positive

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Abbreviation: *m*-Dpm, *m*-diaminopimelic acid.

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Maximum-likelihood and maximum-parsimony trees are available as supplementary figures in IJSEM Online.

bacteria, such as species of the genus *Bacillus* (Zhuang *et al.*, 2002; Yumoto *et al.*, 2003).

For a long time, the group formed by the moderately halophilic, Gram-positive, endospore-forming bacteria was very limited and most of these micro-organisms were assigned to the genus *Bacillus* (Slepecky & Hemphill, 1991). However, in recent years a number of novel genera and species have been described, such as the genus *Halobacillus*, which accommodates six species. This genus includes Gram-positive, spore-forming, moderately halophilic, motile bacteria possessing peptidoglycan of the Orn–D–Asp type. The type species of the genus is *Halobacillus halophilus*, previously described as *Sporosarcina halophila* (Claus *et al.*, 1983; Ventosa *et al.*, 1983); the other *Halobacillus* species are *Halobacillus trueperi*, *H. litoralis* (Spring *et al.*, 1996), *H. karajensis* (Amoozegar *et al.*, 2003), *H. salinus* (Yoon *et al.*, 2003) and *H. locisalis* (Yoon *et al.*, 2004). In addition to species belonging to the genus *Halobacillus*, other spore-forming and halophilic species are found within the genera *Virgibacillus* (Heyndrickx *et al.*, 1999; Heyrman *et al.*, 2003), *Gracilibacillus* (Wainø *et al.*, 1999), *Filobacillus* (Schlesner *et al.*, 2001), *Jeotgalibacillus* (Yoon *et al.*, 2001), *Lentibacillus* (Yoon *et al.*, 2002; Namwong *et al.*, 2005; Jeon *et al.*, 2005), *Pontibacillus* (Lim *et al.*, 2005a, b) and *Tenuibacillus* (Ren & Zhou, 2005).

Recently, we characterized an active and acclimatized bacterial population able to degrade aromatic compounds in saline habitats in southern Spain. In this study, we isolated several strains that represented the dominant, culturable, moderately halophilic bacteria by using an enrichment culture method previously used for phenol and *p*-coumaric acid (García *et al.*, 2004). For the enrichment strategy, cultures of sterile modified mineral medium M63 [KOH, 0.075 M; KH₂PO₄, 0.1 M; (NH₄)₂SO₄, 0.015 M; 1% (v/v) MgSO₄/FeSO₄ solution (MgSO₄, 1.6 mM; FeSO₄, 39 µM)] (Cohen & Rickenberg, 1956) supplemented with 10% NaCl and 0.005% yeast extract were used. Phenol was added to this medium at a concentration of 0.05% (w/v). Enrichments were incubated at 37 °C in a shaker incubator at 200 r.p.m. After 4 days incubation, 1 ml enrichment was transferred into fresh medium and the medium was reincubated. Bacterial growth was monitored by observing increasing turbidity of the medium. After three successive transfers, the enriched cultures were plated on a solid version (18 g agar l⁻¹) of modified M63 medium and incubated at 37 °C. A negative control containing no substrate was also included. Each individual colony was again checked for its ability to grow on phenol, after inoculation in the liquid selective medium described previously.

The enrichment procedure selected a limited number of fast-growing, phenol-degrading bacteria, which were mainly Gram-negative. However, a Gram-positive, rod-shaped strain, G-19.1^T, showing a high level of degradative activity was isolated. The aim of the present study is to unravel the taxonomic and phylogenetic status of G-19.1^T, using a

combination of phenotypic and phylogenetic analyses. To our knowledge, this is the first Gram-positive, moderately halophilic, phenol-degrading bacterium to be subjected to phylogenetic analysis.

The isolate was routinely grown on a complex saline medium (SW) with a final concentration of 10% (w/v) total salts (SW-10) supplemented with 0.5% (w/v) yeast extract (Nieto *et al.*, 1989). The strain was cultivated at 37 °C in an orbital shaker (New Brunswick Scientific) at 200 r.p.m. When necessary, solid medium was prepared by adding 20 g Bacto agar l⁻¹ (Difco).

Strain G-19.1^T was examined for a range of phenotypic properties using standard procedures (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; García *et al.*, 1987). Cells of strain G-19.1^T were found to be Gram-positive rods that were oxidase-negative, catalase-positive and strictly aerobic. On SW-10 medium incubated at 37 °C for 48 h, the strain produced cream-coloured, circular, convex and uniformly round colonies with a diameter of 1–2 mm. Ellipsoidal endospores were observed in a central position. No pigment was produced in the medium. Most species of the genus *Halobacillus* are characterized by their orange-pigmented colonies (Spring *et al.*, 1996; Yoon *et al.*, 2003, 2004). In contrast to species of *Halobacillus*, strain G-19.1^T does not produce orange colonies, is negative for oxidase and is able to reduce nitrate to nitrite. Several other features that distinguish strain G-19.1^T from its close phylogenetic relatives are shown in Table 1.

The salt requirements of strain G-19.1^T were determined in the complex medium SW with different salt concentrations: optimal growth occurred in media containing 7.5–10% (w/v) salt, although growth occurs in a wide range of salinities (from 0.5 to 20%, w/v, salt). No growth was observed in the absence of NaCl.

The nutritional features of G-19.1^T were determined by using GP MicroPlates (Biolog). Strains were grown on isolate medium (Biolog) at 37 °C for 24 h. Suspensions of the strains were made using sterile saline medium (3% NaCl). Immediately after the cells had been suspended in the saline solution, Biolog GP MicroPlates were inoculated and incubated at 37 °C for 24 h. The results were read with a MicroPlate Reader using MICROLOG 3.59 computer software to perform automated reading. The results of the nutritional tests are given in the species description.

Genomic DNA was prepared using the method described by Marmur (1961). A PCR was carried out for the amplification of the almost-complete 16S rRNA gene (1506 bp) by using primers 16F27 and 16R1488 (Mellado *et al.*, 1995). Sequencing was performed using an automated DNA sequencer (model 3100; Applied Biosystems). The DNA sequences were analysed using the ARB software package (Ludwig & Strunk, 1996). The 16S rRNA gene sequences were aligned and the alignment confirmed and checked against both primary and secondary structure of

Table 1. Characteristics useful for distinguishing *Thalassobacillus devorans* gen. nov., sp. nov. from related taxa

Taxa: 1, *Thalassobacillus devorans*; 2, *Halobacillus*; 3, *Virgibacillus*; 4, *Gracilibacillus*; 5, *Filobacillus*; 6, *B. haloalkaliphilus* DSM 5271^T; 7, *Lentibacillus*; 8, *Pontibacillus*; 9, *Tenuibacillus*. Data are from Spring *et al.* (1996), Amoozegar *et al.* (2003), Yoon *et al.* (2002, 2003, 2004), Heyndrickx *et al.* (1999), Heyrman *et al.* (2003), Wainø *et al.* (1999), Schlesner *et al.* (2001), Fritze (1996), Namwong *et al.* (2005), Jeon *et al.* (2005), Lim *et al.* (2005a, b), Ren & Zhou (2005) and this study. Symbols: +, positive; –, negative; v, variable; w, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9
Spore shape*	E	E/S	E/S	E/S	S	S	S/O	S	S
Optimal growth temperature (°C)	37	30–38	25–37	45–47	33–38	ND	30–37	30–35	36–41
Optimal NaCl concentration (%)	7.5–10	2–10	4–10	0–15	8–14	ND	4–14	2–5	5–8
NaCl growth range (%)	0.5–20	0.5–30	0–25	0–20	2–23	0–25	1–30	1–15	1–20
Growth in medium without NaCl	–	–	v	+	–/w	–/w	–	–	–
Oxidase	–	+	v	+	–	+	v	v	+
Nitrate reduced to nitrite	+	–	v	+	–	–	+	v	–
Acid produced from glucose	+	v	v	+	–	–	v	v	–
Hydrolysis of:									
Aesculin	–	v	+	+	–	ND	v	v	+
Starch	–	v	v	+	–	w	–	v	–
Casein	–	v	+	–	–	–/w	v	v	+
Diamino acid in murein	<i>m</i> -Dpm	Orn–D-Asp	<i>m</i> -Dpm	<i>m</i> -Dpm	L-Orn	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm
DNA G + C content (mol%)	42.4	40–45	36–43	38–39	35	37–38	43–44	41–42	36.5–37
Major fatty acid(s)	anteiso-C _{15:0} , iso-C _{16:0} , iso-C _{15:0}	anteiso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0}	anteiso-C _{15:0} , anteiso-C _{17:0}	anteiso-C _{15:0} , iso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0}	anteiso-C _{15:0} , iso-C _{16:0}	iso-C _{15:0} , anteiso-C _{15:0}	iso-C _{15:0} , anteiso-C _{15:0}

*E, Ellipsoidal; S, spherical; O, oval.

the 16S rRNA molecule by using the alignment tool of the ARB software package. The aligned sequences were subjected to different phylogenetic methods integrated into the ARB software for phylogenetic inference. These methods included maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) procedures. Base-frequency filters were applied in the sequence comparison analysis and the effects on the results evaluated.

A comparison using 16S rRNA gene sequences available in the databases revealed that the 16S rRNA gene sequence of strain G-19.1^T displays a high level of similarity to those from *Halobacillus* species with validly published names (96.2–97.0 % sequence similarity). Other spore-forming and halophilic or halotolerant taxa (*Virgibacillus*, *Gracilibacillus*, *Lentibacillus* and *Pontibacillus* species, *Filobacillus milensis*, *Bacillus haloalkaliphilus* and *Tenuibacillus multivorans*) were more distantly related to strain G-19.1^T, displaying 16S rRNA gene sequence similarity below 95 %.

In the tree based on the neighbour-joining algorithm (Saitou & Nei, 1987), strain G-19.1^T falls within the radiation of the cluster comprising members of the genus *Halobacillus*, but emerges as a separate entity. These results are congruent with those obtained using the maximum-likelihood and maximum-parsimony algorithms. The neighbour-joining tree in Fig. 1 shows the relationship between strain G-19.1^T and the other species of the genus *Halobacillus* and other related bacteria. Maximum-likelihood and maximum-parsimony trees are available as supplementary figures in IJSEM Online.

The genotypic relatedness between isolate G-19.1^T and phylogenetically closely related species was determined by DNA–DNA hybridization. These studies were carried out by following the competition procedure of Johnson (1994), described in detail in Mormile *et al.* (1999). The levels of DNA–DNA hybridization between strain G-19.1^T and the phylogenetically related *Halobacillus* species were low and, in any case, below 70 % (*H. trueperi* DSM 10404^T, 32 %;

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence comparison, showing the phylogenetic position of *Thalassobacillus devorans* G-19.1^T among representatives of some other related Gram-positive bacteria. The accession numbers of the sequences used in this study are shown in parentheses. Bar, 1% sequence divergence.

H. litoralis DSM 10405^T, 13%; *H. salinus* KCCM 41590^T, 31%; *H. karajensis* DSM 14948^T, 25%), providing decisive evidence that strain G-19.1^T is not genotypically related to the *Halobacillus* species tested.

The G+C content of the genomic DNA, determined by using the method of Marmur & Doty (1962) with the equation of Owen & Hill (1979), was 42.4 mol%.

The cell-wall peptidoglycan was analysed according to Schleifer & Kandler (1972) by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The analysis showed that strain G-19.1^T possessed peptidoglycan type A1 γ with *m*-diaminopimelic acid (*m*-Dpm) as the diagnostic diamino acid. The major isoprenoid quinone was MK-7. The cellular fatty acids of strain G-19.1^T were analysed with the MIDI system (Microbial ID). Cells were cultured in SW-7.5 medium (Ventosa *et al.*, 1982) at 37 °C for 24 h. The predominant fatty acids of strain G-19.1^T were anteiso-C_{15:0} (52.3%), iso-C_{16:0} (10.5%), iso-C_{15:0} (10.2%), anteiso-C_{17:0} (8.3%), anteiso-C_{17:1} (5.1%) and iso-C_{14:0} (4.8%). Strain G-19.1^T and the phylogenetically related genera contained similar fatty acid profiles, having anteiso-C_{15:0} as a major fatty acid (Table 1).

Overall, our results showed that strain G-19.1^T exhibited the closest phylogenetic affiliation to *Halobacillus* species; however, comparative 16S rRNA gene sequence analysis showed that this strain represents a novel branch within the category of Gram-positive spore-forming rods. A cell-wall

type based on Orn-D-Asp constitutes a key characteristic that differentiates members of the genus *Halobacillus* from other phylogenetically related genera of endospore-forming rods (Arahal & Ventosa, 2000). The results obtained in our chemotaxonomic analysis, revealing a cell-wall type based on *m*-Dpm, support the conclusion that strain G-19.1^T cannot be assigned to the genus *Halobacillus*. In addition, some phenotypic features differentiate isolate G-19.1^T from species of *Halobacillus* (Table 1), a genus that was defined as being oxidase-positive and unable to reduce nitrate to nitrite (Spring *et al.*, 1996), two features that differ from those of strain G-19.1^T.

Strain G-19.1^T was phylogenetically related at a 16S rRNA gene sequence similarity level below 95% to other genera with a cell-wall type based on *m*-Dpm. In addition, some chemotaxonomic and phenotypic features differentiate isolate G-19.1^T from phylogenetically related spore-forming taxa (Table 1). Strain G-19.1^T is oxidase-negative, whereas other related *m*-Dpm-containing genera, such as *Gracilibacillus* and *Tenuibacillus*, are described as being oxidase-positive. The genus *Filobacillus* is defined as being oxidase-negative, like strain G-19.1^T; however, this genus does not reduce nitrate to nitrite, a characteristic that is useful for differentiating it from strain G-19.1^T. Members of other related *m*-Dpm-containing genera such as *Gracilibacillus*, *Virgibacillus* and *Tenuibacillus* hydrolyse aesculin, unlike strain G-19.1^T.

Overall, the phenotypic, genotypic and phylogenetic

analyses performed suggested that strain G-19.1^T cannot be assigned to any known taxon. We therefore propose to classify this novel isolate in a novel genus and species separate from all *m*-Dpm-containing bacilli as well as the genus *Halobacillus*, for which the name *Thalassobacillus devorans* gen. nov., sp. nov. is proposed.

Description of *Thalassobacillus* gen. nov.

Thalassobacillus (Tha.las'so.ba.cil'lus. Gr. fem. n. *thalassa* sea; L. masc. n. *bacillus* rod; N.L. masc. n. *Thalassobacillus* rod from the sea).

Gram-positive, spore-forming, rod-shaped cells. Motile. Catalase-positive, oxidase-negative and urease-negative. Nitrate is reduced to nitrite. Ellipsoidal endospores in central position. Moderately halophilic: does not grow in media without NaCl. Cell-wall peptidoglycan type A1 γ with *m*-Dpm. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{15:0}. Predominant menaquinone is MK-7. The G+C content of the DNA of the type species is 42.4 mol%. The type species is *Thalassobacillus devorans*.

Description of *Thalassobacillus devorans* sp. nov.

Thalassobacillus devorans [de.vo'rans. L. v. *devorare* to devour; L. part. adj. *devorans* devouring (organic compounds)].

Cells are 2.0–4.0 $\times$ 1.0–1.2 μ m. Motile by means of flagella. Colonies are uniformly round, circular, regular, convex and cream-coloured on SW-10 medium. Moderately halophilic, growing in a wide range (0.5–20%, w/v) of salt concentrations, with optimum growth at 7.5–10% (w/v) salts. No growth in the absence of NaCl. No other salt requirements determined. Growth occurs at 15–45 °C (optimal temperature 37 °C) and at pH 6.0–10.0 (optimal pH is 7.0). Strictly aerobic. Aesculin is not hydrolysed. Indole, methyl red and Voges–Proskauer tests are negative. Gelatin and Tween 80 are hydrolysed. Starch and casein are not hydrolysed. Acid is produced from D-glucose, D-trehalose, D-mannose and D-fructose. As determined by the Biolog GP panel, the following compounds are utilized: dextrin, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, D-fructose, α -D-glucose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, 3-methyl glucose, palatinose, D-psicose, D-sorbitol, sucrose, D-trehalose, acetic acid, β -hydroxybutyric acid, α -ketovaleric acid, pyruvic acid, thymidine and uridine. The following compounds are not utilized as sole carbon and energy sources (Biolog): α -cyclodextrin, β -cyclodextrin, glycogen, inulin, mannan, Tweens 40 and 80, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, *myo*-inositol, α -D-lactose, lactulose, D-melibiose, methyl α -D-galactoside, methyl β -D-galactoside, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, D-raffinose, L-rhamnose, D-ribose, salicin, sedoheptulosan, stachyose, D-tagatose, turanose, xylitol, D-xylose, α -hydroxybutyric acid, γ -hydroxybutyric acid,

p-hydroxyphenylacetic acid, α -ketoglutaric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, methyl pyruvate, monomethyl succinate, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglytamic acid, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2'-deoxyadenosine, inosine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate and α -DL-glycerol phosphate. Other characteristics as for the genus. The DNA G+C content of the type strain is 42.4 mol%.

The type strain, G-19.1^T (=DSM 16966^T=CECT 7046^T=CCM 7282^T), was isolated from a saline soil in southern Spain.

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