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Thalassobacillus pellis sp. nov., a moderately halophilic, Gram-positive bacterium isolated from salted hides

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A Gram-positive, moderately halophilic and endospore-forming bacterium, designated strain 18OM^T, was isolated from salted animal hides. The cells were rods and produced ellipsoidal endospores at a terminal position. Strain 18OM^T was motile, strictly aerobic and grew at 0.5–25 % (w/v) NaCl [optimal growth at 10 % (w/v) NaCl], at between pH 5.0 and 9.0 (optimal growth at pH 7.5) and at temperatures between 15 and 45 °C (optimal growth at 37 °C). Phylogenetic analysis based on 16S rRNA gene sequence comparisons showed that strain 18OM^T was closely related to species of the genus *Thalassobacillus* within the phylum *Firmicutes*. The closest phylogenetic similarity was with *Thalassobacillus devorans* G-19.1^T (98.4 %), *Thalassobacillus cyri* HS286^T (97.9 %) and *Thalassobacillus hwangdonensis* AD-1^T (97.4 %). The major cellular fatty acids were anteiso-$C_{15:0}$ (57.9 %), anteiso-$C_{17:0}$ (14.0 %), iso-$C_{15:0}$ (10.8 %) and iso-$C_{16:0}$ (8.1 %). The respiratory isoprenoid quinones were MK-7 (98.5 %) and MK-6 (1.5 %). The DNA G+C content was 42.9 mol%. These features confirmed the placement of strain 18OM^T within the genus *Thalassobacillus*. The DNA–DNA hybridization values between strain 18OM^T and *T. devorans* G-19.1^T, *T. cyri* HS286^T and *T. hwangdonensis* AD-1^T were 49 %, 9 % and 15 %, respectively, showing unequivocally that strain 18OM^T constituted a novel genospecies. On the basis of phylogenetic analysis and phenotypic, genotypic and chemotaxonomic characteristics, strain 18OM^T is considered to represent a novel species of the genus *Thalassobacillus*, for which the name *Thalassobacillus pellis* sp. nov. is proposed. The type strain is 18OM^T (=CECT 7566^T=DSM 22784^T=JCM 16412^T).

The genus *Thalassobacillus* was proposed by García *et al.* (2005) for Gram-positive, endospore-forming motile rods that were moderately halophilic and unable to grow in media without NaCl. The cell-wall peptidoglycan of members of the genus contains *meso*-diaminopimelic acid as the diagnostic diamino acid. The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{15:0}$. The predominant quinone is MK-7. This genus was described on the basis of a single species, *Thalassobacillus devorans* (García *et al.*, 2005), isolated from a saline soil in southern Spain. Recently, two other species have been described within this genus: *Thalassobacillus cyri* (Sanchez-Porro *et al.*, 2009a), isolated from a hypersaline lake in Iran, and *Thalassobacillus hwangdonensis* (Lee *et al.*, 2010), isolated from a tidal flat sediment of the Yellow Sea, Korea. During the course of studies focused on the determination of the biodiversity of bacteria from salted animal hides, we isolated a novel endospore-forming, moderately halophilic, Gram-positive bacterium, strain 18OM^T. The aim of the present study was to determine the taxonomic position of strain 18OM^T by using a polyphasic approach. Our results show that this isolate represents a novel species of the genus *Thalassobacillus*.

Strain 18OM^T was isolated on R2A medium (Difco) supplemented with a mixture of 15 % salts (Ventosa *et al.*, 1982) after incubation at 37 °C for 7 days. For routine growth, the strain was cultivated in SW10 medium with 10 % (w/v) total salts [8.1 % NaCl, 0.7 % $MgCl_2$, 0.96 % $MgSO_4 . 7H_2O$, 0.036 % $CaCl_2$, 0.2 % KCl, 0.006 % $NaHCO_3$, 0.0026 % NaBr, 0.5 % yeast extract (Difco)] (Ventosa *et al.*, 1982). The pH was adjusted to 7.2 with 1 M

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 18OM^T is FN690978.

Supplementary figures are available with the online version of this paper.

KOH. When necessary, solid media were prepared by adding 2.0 % (w/v) Bacto-agar (Difco). For comparative purposes, the following type strains were used in this study: *T. devorans* G-19.1^T, *T. cyri* HS286^T and *T. hwangdonensis* AD-1^T. They were cultivated under the same conditions as strain 18OM^T.

The phenotypic characterization of strain 18OM^T was carried out following the recommendations of the proposed Minimal Standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009). For the determination of cellular morphology and motility, a culture from liquid 10 % Halophiles Moderate (HM) medium was examined by light microscopy under a phase-contrast microscope. The composition of the 10 % HM medium was (w/v): 8.1 % NaCl, 0.7 % $MgCl_2$, 0.96 % $MgSO_4$, 0.036 % $CaCl_2$, 0.2 % KCl, 0.006 % $NaHCO_3$, 0.0026 % NaBr, 0.5 % proteose peptone (Difco), 1.0 % yeast extract (Difco), 0.1 % glucose and 1.5 % agar (Ventosa *et al.*, 1982). The morphology of colonies, their size and pigmentation were observed on 10 % HM solid medium after 48 h incubation at 37 °C. Optimal conditions for growth were determined by growing the strain in SW medium at 0, 0.5, 3, 5, 7.5, 10, 12.5, 15, 20, 25 and 30 % (w/v) NaCl and at temperatures of 4, 15, 20, 28, 30, 37, 40 and 45 °C, respectively. The pH range for the isolate was tested in SW10 medium adjusted to the following pH values: 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 10.0 with the addition of the appropriate buffering capacity to each medium (Sánchez-Porro *et al.*, 2009b). All biochemical tests were carried out with 10 % NaCl and at 37 °C, unless stated otherwise. Growth under anaerobic conditions was determined by incubating strain 18OM^T in an anaerobic chamber in SW10 medium. Catalase activity was determined by adding a 1 % (w/v) H_2O_2 solution to colonies on SW10 agar medium. The oxidase test was performed using the Dry Slide Assay (Difco). Hydrolysis of aesculin, casein, DNA, gelatin, starch, Tween 80, pullulan and xylan, Voges–Proskauer, the methyl red test, production of indole, arginine, lysine and ornithine decarboxylase, phenylalanine deaminase, phosphatase, urease and nitrate reduction were determined as described by Cowan & Steel (1965) with the addition of 10 % total salts to the medium (Ventosa *et al.*, 1982; Quesada *et al.*, 1984). Citrate utilization was determined on Simmons' citrate medium supplemented with SW10. Acid production from carbohydrates was determined using phenol red base supplemented with 1 % of the carbohydrate and SW10 medium (Ventosa *et al.*, 1982). For determining the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, the classical medium of Koser (1923) as modified by Ventosa *et al.* (1982) was used: 75 g NaCl l^{-1}, 2 g KCl l^{-1}, 0.2 g $MgSO_4 . 7H_2O$ l^{-1}, 1 g KNO_3 l^{-1}, 1 g $(NH_4)_2HPO_4$ l^{-1}, 0.5 g KH_2PO_4 l^{-1} and 0.05 g yeast extract (Difco) l^{-1}. Substrates were added as filter-sterilized solutions to give a final concentration of 1 g l^{-1}, except for carbohydrates, which were used at 2 g l^{-1}. When the substrate was an amino acid, it was tested as carbon, nitrogen and energy source and the basal medium was therefore prepared without KNO_3 and $(NH_4)_2HPO_4$. The susceptibility of the strain to antimicrobial compounds was determined according to the conventional Kirby-Bauer method (Bauer *et al.*, 1966).

Strain 18OM^T was Gram-positive, motile and strictly aerobic. It was able to grow in media containing 0.5–25 % (w/v) NaCl and grew optimally in medium containing 10 % (w/v) NaCl. No growth was observed in the absence of NaCl. Thus this bacterium can be considered as a moderately halophilic micro-organism (Ventosa *et al.*, 1998). Optimal temperature and pH were 37 °C and pH 7.5. Other phenotypic features of strain 18OM^T are detailed in the species description and Table 1.

Genomic DNA from strain 18OM^T was prepared using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer 16F27 and the reverse primer 16R1488 (Márquez *et al.*, 2008). Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3130XL; Applied Biosystems). The 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig *et al.*, 2004). The 16S rRNA gene sequence was aligned with the published sequences of closely related bacteria and the alignment was confirmed and checked against both the primary and secondary structures of the 16S rRNA molecule using the alignment tool in the ARB software package. Phylogenetic trees were constructed using three different methods: maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987). These algorithms are integrated into the ARB software for phylogenetic inference. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1.

The almost-complete 16S rRNA gene sequence (1470 bp) of strain 18OM^T was obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the ARB software package (Ludwig *et al.*, 2004) and the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). The phylogenetic analysis, based on the neighbour-joining algorithm, revealed that strain 18OM^T formed a phyletic group with species of the genus *Thalassobacillus* (Fig. 1). Maximum-parsimony and maximum-likelihood methods resulted in highly similar tree topologies (see Supplementary Figs S1 and S2 in IJSEM Online). The closest phylogenetic similarity (obtained with the ARB software package) was with *T. devorans* G-19.1^T (98.4 % 16S rRNA sequence similarity), *T. cyri* HS286^T (97.9 %) and *T. hwangdonensis* AD-1^T (97.4 %). Neighbour-joining and maximum-likelihood methods resulted in highly similar tree topologies (Supplementary Figs S1 and S2). In order to determine whether the new isolate constituted a novel species, we performed DNA–DNA hybridization studies

Table 1. Characteristics that distinguish strain 18OM^T from other species of the genus *Thalassobacillus*

Taxa: 1, 18OM^T (data from this study); 2, *T. devorans* G-19.1^T (García *et al.*, 2005); 3, *T. cyri* HS286^T (Sanchez-Porro *et al.*, 2009a); 4, *T. hwangdonensis* AD-1^T (Lee *et al.*, 2010 and this study). +, Positive; −, negative.

Characteristic	1	2	3	4
Cell size (μm)	1.0 × 1.5–6.0	1.0–1.2 × 2.0–4.0	0.6–0.8 × 1.3–8.5	0.3–0.7 × 1.2–3.5
Oxidase	+	−	+	+
NaCl range for growth (%, w/v)	0.5–25	0.5–20	1–15	0–20
Optimum NaCl (%, w/v)	10	7.5–10	8	5–10
Temperature range (°C)	15–45	15–45	5–45	15–47
Optimum temperature (°C)	37	37	40	40
pH range	5.0–9.0	6.0–10.0	7.0–9.5	6.0–9.0
Optimum pH	7.5	7.0	7.5	7.0–7.5
Nitrate reduction	−	+	+	+
Methyl red test	−	−	+	−*
Hydrolysis of:				
Casein	+	−	+	+
Gelatin	+	+	−	+*
Aesculin	+	−	+	−
Starch	−	−	+	−
Tween 80	−	+	+	+
Acid production from:				
D-Galactose	−	−	+	−
D-Mannitol	−	+	−	−
Sucrose	+	+	−	−
DNA G+C content (mol%)	42.9	42.4	43.0	45.2

*Different result was obtained in this study to that reported by Lee *et al.* (2010).

between strain 18OM^T and the type strains of the other species of the genus *Thalassobacillus.*

DNA–DNA hybridization studies were conducted according to the competition procedure of the membrane filter method (Johnson, 1994). The hybridization temperature was 47.3 °C, which was within the limit of validity for the filter method (De Ley & Tijtgat, 1970) and the percentage hybridization was calculated according to Johnson (1994). The experiments were performed in triplicate. The DNA–DNA hybridization values between strain 18OM^T and *T. devorans* G-19.1^T, *T. cyri* HS286^T and *T. hwangdonensis* AD-1^T were 49 %, 9 % and 15 %, respectively. These levels of DNA–DNA hybridization were sufficiently low to classify strain 18OM^T as a genotypically distinct species within the genus *Thalassobacillus* (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

The G+C content of the genomic DNA was determined from the midpoint value of the thermal denaturation profile

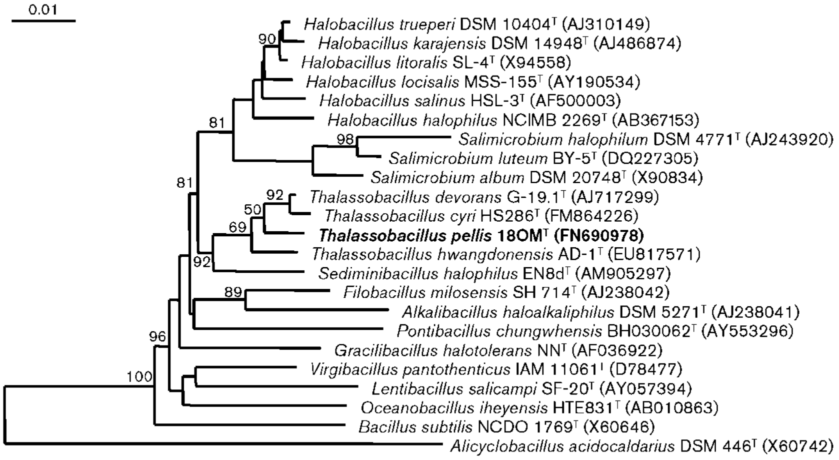

Fig. 1. Neighbour-joining phylogenetic tree, based on the 16S rRNA gene sequence comparison, showing the relationship of strain 18OM^T with related species of the genus *Thalassobacillus* and other closely related genera. The GenBank accession numbers are shown in parentheses. *Alicyclobacillus acidocaldarius* DSM 446^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

(Marmur & Doty, 1962) using the equation of Owen & Hill (1979). The G + C content of the DNA of strain 18OM^T was 42.9 mol%. This value was within the expected range for species of the genus *Thalassobacillus* (Table 1).

Fatty acid analysis was performed using the MIDI system (Microbial Identification System). Cells were cultured on SW-7.5 medium (Ventosa *et al.*, 1982) at 37 °C for 24 h. The extraction and analysis of fatty acids were performed according to the recommendations of the MIDI system. This analysis was carried out by the Identification, Characterization and Molecular Typing Service of the BCCM/LMG Bacteria Collection (Gent, Belgium). The cellular fatty acid profile of strain 18OM^T was characterized by anteiso-$C_{15:0}$ (57.9 %), anteiso-$C_{17:0}$ (14.0 %), iso-$C_{15:0}$ (10.8 %) and iso-$C_{16:0}$ (8.1 %) as the major fatty acids (Table 2). The fatty acid composition was similar to that found for the other species of the genus *Thalassobacillus*. However, when compared with the fatty acid profile of other species of the genus *Thalassobacillus*, strain 18OM^T showed a higher percentage of fatty acid $C_{16:1}\omega 17c$ than all of these species, a significantly lower percentage for iso-$C_{15:0}$ than *T. hwangdonensis* AD-1^T and a lower percentage for iso-$C_{16:0}$ than *T. cyri* HS286^T. The analysis of respiratory quinones of strain 18OM^T was carried out by the Identification Service of the DSMZ and Dr Brian Tindall, Braunschweig, Germany. Strain 18OM^T contained MK-7 (98.5 %) and MK-6 (1.5 %) as isoprenoid quinones. The major quinone reported for species of the genus *Thalassobacillus* is MK-7 (García *et al.*, 2005; Sanchez-Porro *et al.*, 2009a; Lee *et al.*, 2010).

The characteristics that differentiate strain 18OM^T from related species of the genus *Thalassobacillus* are summarized in Table 1. On the basis of the phylogenetic, genotypic, chemotaxonomic and phenotypic data, it is proposed that strain 18OM^T represents a novel species of the genus *Thalassobacillus*, *Thalassobacillus pellis* sp. nov.

Table 2. Cellular fatty acid composition (%) of strain 18OM^T and species of the genus *Thalassobacillus*

Taxa: 1, 18OM^T; 2, *T. devorans* G-19.1^T (García *et al.*, 2005, M. T. García personal communication); 3, *T. cyri* HS286^T (Sanchez-Porro *et al.*, 2009a); 4, *T. hwangdonensis* AD-1^T (Lee *et al.*, 2010). All strains were grown under the same conditions (SW-7.5 medium at 37 °C for 24 h). −, Not detected.

Fatty acid	1	2	3	4
iso-$C_{14:0}$	2.8	4.8	9.4	0.5
Summed feature 2*	−	−	2.1	−
iso-$C_{15:0}$	10.8	10.2	7.0	43.0
anteiso-$C_{15:0}$	57.9	52.3	43.8	18.5
iso-$C_{16:0}$	8.1	10.5	21.4	5.0
$C_{16:1}\omega 17c$	2.1	0.5	1.4	0.3
$C_{16:0}$	1.9	0.6	0.4	3.6
iso-$C_{17:0}$	1.8	1.4	1.8	13.6
anteiso-$C_{17:0}$	14.0	8.3	8.7	13.1
anteiso-$C_{17:1}$	−	5.1	2.8	−

*Summed feature 2 contained iso-$C_{16:1}$/$C_{14:0}$ 3-OH.

Description of *Thalassobacillus pellis* sp. nov.

Thalassobacillus pellis (pel′lis. L. n. *pellis –is* hide, tanned hide, leather; L. gen. n. *pellis* of/from hide, tanned hide, leather, isolated from salted hides that are used in the leather industry).

Cells are Gram-positive, motile, endospore-forming rods, 1.0 × 1.5–6.0 μm in size, and occur as single cells, pairs or short chains (see Supplementary Fig. S3). Ellipsoidal endospores are produced at a terminal position in swollen sporangia. Colonies are circular, entire, smooth, convex, cream and 1–2 mm in diameter on 10 % HM agar medium after 48 h incubation at 37 °C. Strictly aerobic. Moderately halophilic, growing at 0.5–25 % (w/v) NaCl; with optimal growth at 10 % (w/v) NaCl. No growth occurs in the absence of NaCl. Grows at 15–45 °C, showing optimal growth at 37 °C, and at pH values between 5.0 and 9.0, with optimal growth at pH 7.5. Catalase- and oxidase-positive. Gelatin, casein, DNA, pullulan and aesculin are hydrolysed, but starch, Tween 80 and xylan are not hydrolysed. Nitrate is not reduced to nitrite. Acid is produced from D-glucose, D-fructose, glycerol, trehalose, sucrose and D-xylose, but not from D-arabinose, D-galactose, lactose, maltose, D-mannose or D-mannitol. Indole and H_2S are not produced. Phosphatase activity is positive. Methyl red, Voges–Proskauer, Simmons' citrate, urease, β-galactosidase arginine, lysine and ornithine decarboxylases and phenylalanine deaminase tests are negative. The following compounds are utilized as sole sources of carbon and energy: cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannose, melibiose, raffinose, ribose, salicin, sucrose, trehalose, D-xylose, dulcitol, ethanol, *myo*-inositol, D-mannitol, D-sorbitol, xylitol, acetate, benzoate, citrate, fumarate, propionate and hippurate. The following compounds are not utilized as sole sources of carbon and energy: starch, D-arabinose, fructose, D-fucose, aesculin, melezitose, butanol, glycerol, propanol, methanol, formate, succinate, valerate, malate and tartrate. The following compounds are utilized as sole sources of carbon, nitrogen and energy: L-alanine, L-arginine, phenylalanine, glutamine, L-serine, tryptophan, valine, L-lysine, isoleucine and L-threonine. The following compounds are not utilized as sole sources of carbon, nitrogen and energy: aspartate, L-cysteine, L-methionine and L-ornithine. Resistant to kanamycin (30 μg), penicillin G (10 U), nalidixic acid (30 μg) and neomycin (10 μg). Sensitive to cephalothin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), novobiocin (30 μg) and sulfamethoxazole/trimethoprim (23.75/1.25 μg). The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$. The respiratory isoprenoid quinones are MK-7 (98.5 %) and MK-6 (1.5 %).

The type strain is 18OM^T (=CECT 7566^T=DSM 22784^T=JCM 16412^T), isolated from salted animal hides.

The DNA G+C content of the type strain is 42.9 mol% (T_m).

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