Halobacillus alkaliphilus sp. nov., a halophilic bacterium isolated from a salt lake in Fuente de Piedra, southern Spain

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A Gram-positive, spore-forming, halophilic bacterial strain, FP5T, was isolated from a salt lake in southern Spain and subjected to a polyphasic taxonomic study. Strain FP5T was strictly aerobic. Cells were coccolidal, occurring singly or in clusters. The cell-wall peptidoglycan type of strain FP5T was A4β based on L-Orn–D-Asp. Strain FP5T was characterized chemotaxonomically by having MK-7 as the major menaquinone and anteiso-C15:0, anteiso-C17:0, iso-C16:0 and iso-C15:0 as the main fatty acids. The isolate grew optimally at 37 °C and in presence of 10 % NaCl; no growth was observed in the absence of NaCl. The DNA G+C content was 43.5 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain FP5T falls within the evolutionary radiation of species of the genus Halobacillus. Levels of 16S rRNA gene sequence similarity between strain FP5T and the type strains of nine recognized Halobacillus species were in the range 97.0–99.0 %. Levels of DNA–DNA relatedness indicated that strain FP5T represents a genomic species that is distinct from recognized Halobacillus species. Strain FP5T could be differentiated from recognized Halobacillus species based on several phenotypic characteristics. On the basis of phenotypic, phylogenetic and genomic data, strain FP5T is considered to represent a novel species of the genus Halobacillus, for which the name Halobacillus alkaliphilus sp. nov. is proposed. The type strain is FP5T (= DSM 18525T = ATCC BAA-1361T).

The genus Halobacillus was first described by Spring et al. (1996) to accommodate two novel species, Halobacillus litoralis and Halobacillus trueperi, and the transfer of Sporosarcina halophila (Claus et al., 1983) to Halobacillus as Halobacillus halophilus. At the time of writing, the genus comprises 13 species with validly published names, with the addition of Halobacillus salinus (Yoon et al., 2003), H. karajensis (Amoozegar et al., 2003), H. locisalis (Yoon et al., 2004), H. yeomjeoni (Yoon et al., 2005), H. dabanensis and H. aidingensis (Liu et al., 2005), H. profundi and H. kuroshimensis (Hua et al., 2007), H. campisalis (Yoon et al., 2007) and H. faccis (An et al., 2007).

The genus Halobacillus can be differentiated clearly from other related genera based on the cell-wall peptidoglycan type based on L-Orn–D-Asp (Spring et al., 1996; Shida et al., 1997; Yoon et al., 2001), with the exception of that for H. campisalis, which is based on meso-diaminopimelic acid (Yoon et al., 2007). The aim of the present study was to determine the exact taxonomic status of a halophilic bacterial strain, FP5T, by using a polyphasic approach, including phenotypic properties, lipid analyses, phylogenetic analysis based on 16S rRNA gene sequences and levels of genotypic relatedness.

Strain FP5T was isolated from samples collected during summer 2003 from Fuentes de Piedra saline lake, Malaga province, southern Spain (37° 6’ N 4° 44’ W). It was isolated from a saltern crystallizer pond by the dilution-plating technique. Strain FP5T represented the predominant organism in the enrichment and was the only colony-forming organism at the highest dilutions. The enrichment medium (medium A) contained the following components:

- Medium A was prepared with the following components (g l−1): NaCl, 10; MgSO4·7H2O, 0.5; CaCl2·2H2O, 0.5; KH2PO4, 0.5; K2HPO4, 1; and FeCl3·6H2O, 0.02.
- Cells were grown in a saline medium containing 10 % NaCl and 10 % MgCl2·6H2O.
- The DNA G+C content was determined using the HPLC technique.

A scanning electron micrograph of cells of strain FP5T and thin-layer chromatographs of total polar lipids of strain FP5T are available as supplementary material with the online version of this paper.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FP5T is AM295006.

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(per litre distilled water): 10.0 g yeast extract (Oxoid), 3.0 g trisodium citrate (Applichem), 2.0 g KCl (Applichem), 1.0 g MgSO4, 7H2O (Carlo Erba), 100 g NaCl (Applichem), 0.36 mg MnCl2, 4H2O (J. T. Baker), 0.05 g FeSO4, 7H2O (Carlo Erba) and 3.0 g Na2CO3 (Applichem). Solid media were prepared by the addition of 1.8% (w/v) agar. The pH of medium A was 9.0.

Growth on single carbon sources was tested on liquid media containing (per litre distilled water) 100 g NaCl (Applichem), 2.0 g KCl, 1.0 g MgSO4, 7H2O, 16.4 g MgCl2, 6H2O (Riedel-de Haeén), 0.2 g NaHCO3 (J. T. Baker), 2.29 g CaCl2, 2H2O (J. T. Baker), 152 mg NH4Cl (Applichem), 33 mg K2HPO4 (Applichem), 0.26 mg FeCl3, 4H2O (J. T. Baker) and 10.0 g of the test compound.

Reference strains H. yeomjeoni DSM 17110T, H. karajensis DSM 14948T, H. trueperi DSM 10404T and H. halophilus DSM 2266T, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ), were grown in the media suggested by the culture collection [DSMZ medium no. 514 plus 3% (w/v) NaCl at 30 °C for H. yeomjeoni; DSMZ medium no. 1 plus 10% (w/v) NaCl at 38 °C for H. karajensis; DSMZ medium no. 755 at 30 °C for H. trueperi; DSMZ medium no. 123 at 30 °C for H. halophilus].

Cell morphology was determined by phase-contrast microscopy (Zeiss) and by scanning electron microscopy. For scanning electron microscopy analysis, the samples were fixed for 24 h in 2.5% glutaraldehyde. Samples were dehydrated in ethanol, critical-point-dried, sputter-coated with gold (SEM BALTECMED 020) and observed by use of a Philips XL 20 environmental scanning electron microscope. Colony morphology was analysed on solid medium via a Leica M8 stereomicroscope. Tolerance of NaCl and growth at various temperatures and pH were tested in medium A. Growth tests were performed at the optimal growth temperature (37 °C) for 2 days. The pH range for growth was determined by adjusting the pH to 4.0–11.0 with HCl and NaOH. The temperature range for growth was determined by incubation in liquid medium at temperatures between 20 and 60 °C. Sensitivity of the strain to antibiotics was tested by using medium A with agar (1.8%, w/v) and Sensi discs (6 mm; Oxoid) (Romano et al., 1993). Motility was assessed by using test tubes containing medium A with agar (0.5%, w/v). Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa within the order Halobacteriales (Oren et al., 1997). Gelatin hydrolysis was determined as described by Oren et al. (2002). Hydrolysis of casein and activities of oxidase, tyrosinase, aminopeptidase (Bactident-Merck) and catalase were tested in medium A as described by Oren et al. (1997). For tests of nitrate reduction, medium A plus 0.1% (w/v) KNO3 was employed. Hydrolysis of hippurate was assessed in medium A plus hippurate (1%, w/v) (Poli et al., 2006). To test for the presence of indole, strain FPST was grown at pH 8.0 in medium A. Gram-staining was performed according to Dussault (1955). The KOH test was performed according to Halebian et al. (1981). Tests for the hydrolysis of N-benzoyl-arginine-p-nitroanilide stereoisomers were performed according to Oren & Galinski (1994). Cell mass for quinone and lipid analysis was obtained from cultures of test strains grown under their optimal growth conditions for 24 h. Lipid analysis, lipid hydrolysis and identification of core lipids were performed as reported by Nicolaus et al. (2001). For quantitative determination of fatty acid composition, fatty acid methyl ester mixtures were prepared following the instructions of the Microbial Identification System (MIDI). Quinones were analysed by LC/MS on a reversed-phase column and by electron ionization/MS and 1H NMR spectra. Phospholipids and glycolipids were separated by TLC on silica gel plates (10 × 10 cm, 10 × 25 cm, 0.25 mm, F254, Merck) and were analysed according to Nicolaus et al. (2001). Peptidoglycan analyses were performed by using the methods of Schleifer & Kandler (1972), Schleifer (1985), MacKenzie (1987) and Groth et al. (1996).

The DNA G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was isolated by using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). The 16S rRNA gene sequence was determined by direct sequencing of the PCR products. Genomic DNA extraction, amplification of the 16S rRNA gene and purification of the PCR products were carried out as described by Reed et al. (2006) with primers 5' - ATTGTA-ACTCCTGGGTTCAC-3' and 5' - AGAAGGAGGTAC-A TCAGCC-3'. Purified PCR products were sequenced at the DSMZ by using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's protocol. Sequence reaction products were electromorphosed by using an Applied Biosystems 373A DNA sequencer. Sequencing of the 16S rRNA gene and phylogenetic analysis were performed as described by Romano et al. (2007). DNA–DNA hybridization experiments were performed as described by De Ley et al. (1970) with the modifications given by Huß et al. (1983) by using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in-situ temperature probe (Varian). Hybridizations were carried out between strain FPS and the type strains of related species (H. yeomjeoni DSM 17110T, H. karajensis DSM 14948T, H. trueperi DSM 10404T and H. halophilus DSM 2266T).

Cells of strain FPS were Gram-positive, spore-forming cocci, occurring singly or in bunches, and measured 1.57 μm in diameter (see Supplementary Fig. S1 in IJSEM Online). The coccolid morphology of the cells remained the same in all phases of growth. After incubation for 2 days, colonies of strain FPS were about 1–2 mm in diameter, circular, smooth and pale orange. Strain FPS required Na+ and Mg2+ for growth, but was
also able to grow using K\(^+\) instead of Na\(^+\). Strain FP5\(^T\) grew in media containing 0.5–20\% (w/v) NaCl; optimal growth occurred with 10\% (w/v) NaCl. Strain FP5\(^T\) grew at temperatures of 25–45 °C, with optimum growth at 37 °C. The optimum pH for growth was 9.0. Growth was observed up to pH 10, but no growth occurred below pH 6.0. This optimum alkaline pH for growth was not found for other Halobacillus species (Spring et al., 1996; Claus et al., 1983; Yoon et al., 2003, 2004, 2005; Amoozegar et al., 2003; Liu et al., 2005).

Strain FP5\(^T\) was aerobic and catalase- and oxidase-positive. Acid was produced from glucose, trehalose, maltose, ribose, sucrose, raffinose, fructose and mannose, but not from galactose, cellobiose or xylose. Tyrosine was hydrolyzed, but starch, casein, hippurate, gelatin and urease were not. The strain was negative for nitrate reduction and phylaniline deaminase. Strain FP5\(^T\) was sensitive to (μg per disc) bacitracin (10), tetracycline (30), novobiocin (30), erythromycin (5), ampicillin (25), chloramphenicol (10), fusidic acid (10), penicillin (10 U), lincomycin (10) and vancomycin (30), but resistant to (μg per disc) streptomycin (25), nystatin (100), kanamycin (30) and neomycin (30). Detailed results of morphological analyses and biochemical tests for strain FP5\(^T\) are given in the species description. Differential characteristics between strain FP5\(^T\) and recognized species of the genus Halobacillus are given in Table 1. Of particular note was that only strain FP5\(^T\) was positive for tyrosine hydrolysis and that no growth was observed at <5\% (w/v) NaCl for this strain. This appears to be the first report of an alkalophilic member of the genus Halobacillus. Cell-wall analysis revealed that the peptidoglycan type of strain FP5\(^T\) was A4\(\beta\) based on L-Orn–D-Asp. The predominant menaquinone was MK-7, as reported for H. halophilus, the type species of the genus. The major fatty acids were anteiso-C\(_{15:0}\) (40.4\%), anteiso-C\(_{17:0}\) (31.0\%), iso-C\(_{15:0}\) (9.6\%) and iso-C\(_{16:0}\) (8.3\%). The polar lipid profile of strain FP5\(^T\) was quite similar to the complex lipid patterns reported for recognized Halobacillus species, with the

### Table 1. Differential characteristics between strain FP5\(^T\) and recognized Halobacillus species

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<td>NaCl concentration for growth (% w/v)</td>
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<td>DNA G+C content (mol%)</td>
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<td>42</td>
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<td>41.3</td>
<td>44</td>
<td>41.4</td>
<td>42.2</td>
<td>42.9</td>
<td>43.3</td>
<td>42.1</td>
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*E, Ellipsoidal; s, spherical.
†CW, Cream or white; LOW, light orange–yellow; LY, light yellow; O, orange; POW, pale orange–yellow; PY, pale yellow; YO, yellow–orange.
presence of two phospholipids, phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid as major lipids. By using two-dimensional TLC, eluted with chloroform/methanol/water (65:25:4 by volume) for the first dimension and with chloroform/methanol/acetic acid/water (85:15:12:4 by volume) for the second dimension, the lipid profile of strain FP5\textsuperscript{T} revealed the presence of two minor phospholipids and one minor glycolipid (Supplementary Fig. S2).

The complete 16S rRNA gene sequence of strain FP5\textsuperscript{T} determined in this study comprised 1484 nt. Comparative 16S rRNA gene sequence analyses showed that strain FP5\textsuperscript{T} was phylogenetically most closely affiliated to members of the genus Halobacillus (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain FP5\textsuperscript{T} fell within the radiation of the cluster comprising Halobacillus species (Fig. 1). The 16S rRNA gene sequence of strain FP5\textsuperscript{T} showed similarity levels of 97.0–99.0 % with respect to sequences of the type strains of recognized Halobacillus species (Fig. 1).

The above results indicated that strain FP5\textsuperscript{T} was a member of the genus Halobacillus. However, it could be distinguished from recognized species of the genus Halobacillus on the basis of several phenotypic characteristics (Table 1).

The DNA G+C content of strain FP5\textsuperscript{T} was 43 mol%. Mean levels of DNA–DNA relatedness between strain FP5\textsuperscript{T} and the type strains of recognized Halobacillus species were in the range 4.5–35 % (H. trueperi, 4.5%; H. salinus, 10.5%; H. karajensis, 18.2%; H. yeomjeoni, 22.5%; H. dabanensis, 30.3%; H. halophilus, 35.0%). Therefore, on the basis of the data presented, strain FP5\textsuperscript{T} should be placed in the genus Halobacillus as a member of a novel species, for which the name Halobacillus alkaliphilus sp. nov. is proposed.

**Description of Halobacillus alkaliphilus sp. nov.**

Halobacillus alkaliphilus (al.ka.li.phi’lus. N.L. n. alkali alkal; Gr. adj. philos loving; N.L. masc. adj. alkaliphilus loving alkaline conditions).

Cells are Gram-positive, spore-forming cocci (1.57 \( \mu \)m in diameter). Colonies on agar medium are circular (1–2 mm in diameter), smooth and pale orange. Growth occurs at NaCl concentrations of 0.5–20 % (w/v), with optimal growth at 10 % (w/v), at temperatures of 25–45 \( ^\circ \)C, with optimum growth at 37 \( ^\circ \)C, and at pH 6.0–10.0. Aerobic. Catalase- and oxidase-positive. Acid is produced from xylose, glucose, trehalose, maltose, ribose, sucrose, raffinose, fructose and mannose, but not from galactose or cellobiose. Hydrolyses tyrosine, but not starch, casein, hirupate, gelatin or urease. Negative for nitrate reduction and phenylalanine deaminase. The peptidoglycan type is A4\( _{b} \) based on L-Orn–D-Asp. The major menaquinone is MK-7. The major fatty acids are anteiso-C\(_{15}:0\) (40.4%), anteiso-C\(_{17}:0\) (31.0%), iso-C\(_{15}:0\) (9.6%) and iso-C\(_{16}:0\) (8.3%). Major lipids are two phospholipids (phosphatidylglycerol and diphosphatidylglycerol) and one unidentified glycolipid. The DNA G+C content of the type strain is 43.5 mol% (\( T_{m} \)).

The type strain, FP5\textsuperscript{T} (=DSM 18525\textsuperscript{T} =ATCC BAA-1361\textsuperscript{T}), was isolated from Fuente de Piedra salt lake, southern Spain.
Acknowledgements

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References


