Halomonas stenophila sp. nov., a halophilic bacterium that produces sulphate exopolysaccharides with biological activity

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We have undertaken a polyphasic taxonomic study of two halophilic, Gram-negative bacterial strains, N12T and B-100, that produce sulphated exopolysaccharides with biological activity. They were isolated from two different saline soil samples. Both strains grow at NaCl concentrations within the range 3–15 % (w/v) (optimum 5–10 % (w/v)), at 15–37 °C (optimum 20–32 °C) and at pH 6–8 (optimum pH 7–8). Their 16S rRNA gene sequences indicate that they belong to the genus Halomonas in the class Gammaproteobacteria. Their closest relative is Halomonas nitroreducens, to which our strains show maximum 16S rRNA gene sequence similarity values of 98.7 % (N12T) and 98.3 % (B-100). Their DNA G+C contents are 61.9 and 63.8 mol%, respectively. The results of DNA–DNA hybridizations showed 43.9 % relatedness between strain N12T and H. nitroreducens CECT 7281T, 30.5 % between N12T and Halomonas ventosae CECT 5797T, 39.2 % between N12T and Halomonas fontilapidosi CECT 7341T, 46.3 % between N12T and Halomonas maura CECT 5298T, 52.9 % between N12T and Halomonas saccharivitis LMG 23976T, 51.3 % between N12T and Halomonas koreensis JCM 12237T and 100 % between strains N12T and B-100. The major fatty acids of strain N12T are C12 : 0 3-OH (5.42 %), C15 : 0 iso 2-OH/C16 : 1 ω7c (17.37 %), C16 : 0 (21.62 %) and C18 : 1 ω7c (49.19 %). The proposed name for the novel species is Halomonas stenophila sp. nov. Strain N12T (= CECT 7744T = LMG 25812T) is the type strain.

Members of the family Halomonadaceae (Franzmann et al., 1988; Dobson & Franzmann, 1996; Garrity et al., 2005) form a monophyletic group within the order Oceanospirillales, belonging to the class Gammaproteobacteria. At the time of writing, the family Halomonadaceae comprises ten genera: Aidingimonas (Wang et al., 2009), Carnimonas (Garriga et al., 1998), Chromohalobacter (Ventosa et al., 1989; Aralah et al., 2001), Cobetia (Aralah et al., 2002), Halomonas (Vreeland et al., 1980; Dobson & Franzmann, 1996), Halotalea (Ntougias et al., 2007), Kushneria (Sánchez-Porro et al., 2009), Medicisalibacter (Ben Ali Gam et al., 2007), Salinicola (Ananina et al., 2007) and Zymobacter (Okamoto et al., 1993). Halomonas is the type genus of the family Halomonadaceae and, at the time of writing, contains >70 species (Euzéby, 2011). Strains belonging to the genus Halomonas characteristically have a respiratory metabolism with oxygen as the terminal acceptor. Nevertheless, the genus is very heterogeneous and includes quite diverse species in terms of their physiology, ecology and nutrition. Our research group has discovered eight species of Halomonas that produce exopolysaccharides (EPSs): H. almeriensis (Martínez-Checa et al., 2005), H. anticariensis (Martínez-Cánovas et al., 2004a), H. cerina (González-Domenech et al., 2008b), H. curihalina (Quesada et al., 1990; Dobson & Franzmann, 1996), H. maura (Bouchotroch et al., 2001), H. nitroreducens (González-Domenech et al., 2008a), H. salina (Valderrama et al., 1991; Dobson & Franzmann, 1996) and H. ventosae (Martínez-Cánovas et al., 2004b). As we have reported previously (Arias et al., 2003; Béjar et al., 1998; Bouchotroch et al., 2000; Calvo et al., 1995, 1998; Llamas et al., 2010; Martínez-Cánovas et al., 2004a; Mata et al., 2006, 2008; Quesada et al., 1993, 2004), EPSs produced by these halophilic bacteria have different chemical compositions and functional properties from those already marketed and used by industry, their high sulphate content

Abbreviation: EPS, exopolysaccharide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains N12T and B-100 are HM242216 and HM357129, respectively.

A supplementary table and four supplementary figures are available with the online version of this paper.
being of special interest. Sulphated polysaccharides are known to inhibit the growth of some viruses and tumours (Itoh et al., 1993; Hasui et al., 1995; Hayashi et al., 1996a, b; Riou et al., 1996; Witvrouw & De Clercq, 1997; Matou et al., 2005; Kwon & Nam, 2006; Guo et al., 2007; Zhu et al., 2007; Arena et al., 2009).

In a parallel study (Ruiz-Ruiz et al., 2011), we have screened the anti-tumoral activity of a panel of sulphated EPSs excreted by a group of newly discovered halophilic bacteria and found that strains N12T and B-100 produce a heteropolysaccharide that exerts a potent inhibitory effect on the proliferation of some tumour cell lines. Furthermore, the EPS produced by strain B-100, when over-sulphated (B100S), exercises anti-tumoral activity on T-cell lines derived from acute lymphoblastic leukaemia via the intrinsic apoptotic pathway. The newly discovered B100S is therefore the first bacterial EPS that has been demonstrated to exert a potent and selective pro-apoptotic effect on T leukaemia cells.

In this work we have determined the taxonomic position of strains N12T and B-100 and propose that they belong to *Halomonas stenophila* sp. nov., with N12T as the type strain.

The bacterial strains were isolated from samples of soil taken from the Sabinar saline wetland (strain B-100) and San Pedro del Pinatar saline wetland (strain N12T) in Murcia, south-east Spain. They were maintained and grown routinely in MY medium (Moraine & Rogovin, 1982). The growth conditions were determined by growing the strain in MY medium at 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30 % (w/v) NaCl, and at 4, 15, 20, 25, 32, 37 and 45 °C. The pH range for the isolate was determined in MY medium at 7.5 % (w/v) NaCl adjusted to pH 5, 6, 7, 8, 9 and 10. The classical medium of Koser (1923), as modified by Ventosa et al. (1982), was used to identify the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources. 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. Sensitivity to antimicrobial compounds was assayed according to the conventional Kirby–Bauer method (Bauer et al., 1966).

Characteristics common to both strains are given in the species description. Phenotypic features distinguishing between the two new strains and other related species of the genus *Halomonas* are shown in Table 1.

The DNA G+C content of strains B-100 and N12T was estimated from the midpoint value (\(T_m\)) of their DNA (Marmur & Doty, 1962). \(T_m\) was determined by the graphic method (Ferragut & Leclerc, 1976) and the DNA G+C content was calculated by using the equation of Owen & Hill (1979). The G+C content of reference DNA of *Escherichia coli* NCTC 9001 \(T\) was 50.9 mol % (Owen & Pitcher, 1985). The values were 63.8 mol % for strain B-100 and 61.9 mol % for strain N12T.

Phylogenetic analyses based on the 16S rRNA gene were performed as described elsewhere (Bouchotroch et al., 2001). In this case, PCR products were cloned in the pGEM-T cloning vector (Promega) according to the manufacturer’s recommendations, and expressed in *Escherichia coli* DH5α. The sequences were then compared with reference 16S rRNA gene sequences available in GenBank by using a BLAST search. We also carried out the identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity by using the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). Phylogenetic analyses were made by using the MEGA version 4 (Tamura et al., 2007) and PHYLIP version 3.69 software, after multiple alignments of the data by CLUSTAL W (Thompson et al., 1997). Distances and clustering were determined using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. The stability of clusters was ascertained by performing a bootstrap analysis (1000 replications).

We determined the 16S rRNA gene sequences of strains N12T (1502 bp) and B-100 (1500 bp). The fragments analysed contained the 15 signature nucleotides defined for the family *Halomonadaceae* and the four defined for the genus *Halomonas* (Dobson & Franzmann, 1996). Phylogenetic analyses based on maximum-likelihood, maximum-parsimony and neighbour-joining methods indicated that strains N12T and B-100 were both included in the cluster comprising *Halomonas* species, and all three methods resulted in highly similar tree topologies (Supplementary Figs S1, S2 and S3, available in IJSEM Online). Fig. 1 shows the tree containing the phylogroup in which our two new strains are included according to the neighbour-joining algorithm. The most closely phylogenetically related species were *H. nitroreducens* CECT 7281T, *H. ventosae* CECT 5797T, *H. fontilapidosi* CECT 7341T, *H. maura* CECT 5298T, *H. saccharivortans* LMG 23976T and *H. koreensis* JCM 12237T, to which strain N12T shows 16S rRNA gene sequence similarity values of 98.70, 98.49, 97.66, 97.62, 97.56 and 97.50 %, respectively. Strain N12T shares a 16S rRNA gene sequence similarity value of 99.2 % with strain B-100.
Table 1. Characteristics that distinguish strain N12T from related type strains of the genus *Halomonas*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>5</th>
<th>6</th>
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<td>Rod</td>
<td>Rod</td>
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<td>Rod</td>
<td>Short rod</td>
<td>Rod</td>
<td>Short rod</td>
<td>Coccus</td>
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<td>Cell width × length (μm)</td>
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<td>0.75 × 3.0</td>
<td>0.5 × 1.5</td>
<td>0.6 × 8.0</td>
<td>0.9 × 1.8</td>
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<td>–</td>
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<td>pH for growth</td>
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<td>5–10</td>
<td>3.5</td>
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<td>8</td>
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<td>Range</td>
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<td>6–8</td>
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<td>6–10</td>
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<td>Optimum</td>
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<tr>
<td>D-Galactose</td>
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<td>L-Lysine</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>–</td>
<td>+</td>
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<td>Erythromycin (15 μg)</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>61.9</td>
<td>63.8</td>
<td>65.37</td>
<td>62.2</td>
<td>65.7</td>
<td>74.3</td>
<td>65.3</td>
<td>65.9</td>
<td>64.2</td>
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</table>

*Data from González-Domenech et al. (2008a, 2009), Martínez-Cánovas et al. (2004b), Lim et al. (2004) and Vreeland et al. (1980).
Evolutionary distances, including a correction factor for reverse mutations (Jukes & Cantor, 1969), were calculated for sequence pairs by using a ‘mask’ (Lane, 1991) for non-homologous or uncertain nucleotide positions.

DNA–DNA hybridization between strains N12 T and B-100 and strain N12 T and \( H. \) nitroreducens CECT 7281 T was undertaken by the Identification Service of the DSMZ bacteria collection (Braunschweig, Germany). Cells were disrupted by using a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite, as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) with the modifications described by Huß et al. (1983) using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an in situ temperature probe (Varian). The rest of the DNA–DNA hybridizations were conducted in our laboratory following the methods of Lind & Ursing (1986) with the modifications of Ziemke et al. (1998) and Bouchotroch et al. (2001). The results demonstrated 43.9 % relatedness between strain N12 \( ^T \) and \( H. \) nitroreducens CECT 7281 \( ^T \), 30.5 % between N12 \( ^T \) and \( H. \) ventosae CECT 5797 \( ^T \), 39.2 % between N12 \( ^T \) and \( H. \) fontalipidiosi CECT 7341 \( ^T \), 46.3 % between N12 \( ^T \) and \( H. \) mauro CECT 5298 \( ^T \), 52.9 % between N12 \( ^T \) and \( \text{Saccharovorans A4(1)} \) \( \text{C37(2)} \), 51.3 % between N12 \( ^T \) and \( H. \) koreensis CICM 12237 \( ^T \) and 100 % between strains N12 \( ^T \) and B-100. These levels of DNA–DNA hybridization classify strains N12 \( ^T \) and B-100 as belonging to a genotypically distinct species (Stackebrandt & Goebel, 1994; Stackebrandt et al., 2002).

The fatty acids of strain N12 \( ^T \) were analysed at the DSMZ by HPLC. To this end, strain N12 \( ^T \) was grown for 24 h at 32 °C in MY medium (Moraine & Rogovin, 1966) containing 7.5 % (w/v) sea salts (Rodrı ´guez-Valera et al., 1981). The strain contained a combination of fatty acids found in other \( H. \) Halomonas species (Dobson & Franzmann, 1996), predominantly C12:0 3-OH (5.42 %), C15:0 iso 2-OH/C16:1 \( \omega 7 \) c (17.37 %), C16:0 (21.62 %) and C18:1 \( \omega 7 \) c (49.19 %) (see Supplementary Table S1, available in IJSEM Online).

A transmission electron micrograph was taken as described by Bouchotroch et al. (2001). Supplementary Fig. S4 (available in IJSEM Online) shows the cell size and morphology of strain N12 T, as well as its EPS.

On the basis of the data discussed and the full description provided below, we consider that the two EPS-producing strains B-100 and N12 \( ^T \) represent a novel species of the genus \( H. \) Halomonas, for which we propose the name \( H. \) Halomonas stenophila sp. nov.

**Description of \( H. \) Halomonas stenophila sp. nov.**

\( H. \) Halomonas stenophila [ste.no’ phi.ila. Gr. adj. stenos narrow, restricted; N.L. adj. philus -a -um from Gr. adj. philos loving; N.L. fem. adj. stenophila referring to the species’ preference for a narrow range of growth conditions (salt concentration, pH and temperature) compared with other \( H. \) Halomonas species, i.e. having an affinity for restricted environmental conditions].

Cells are straight, Gram-negative rods, 2.5–3.0 × 0.7–0.75 \( \mu \)m, appearing either singly or in pairs. They are
non-motile and do not form endospores. Exopolysaccharides are produced. Colonies on MY medium are circular, convex, cream-coloured and noticeably mucoid after 72 h at 32 °C. The growth pattern is uniform in a liquid medium. Moderately halophilic; capable of growing in NaCl concentrations of 3–15 % (w/v), with optimum growth occurring between 5 and 10 % (w/v) NaCl. Growth occurs at 15–37 °C, with optimum growth at 20–32 °C, and at pH 6–8, with optimum growth at pH 7–8. Chemoorganotrophic. Metabolism is respiratory with oxygen as terminal electron acceptor. No growth occurs anaerobically in the presence of nitrate, nitrite or fumarate. Under aerobic conditions, nitrate is not reduced. Catalase and oxidase are produced. Acid is not produced from adonitol, D-cellobiose, D-fructose, D-galactose, D-glucose, myo-inositol, lactose, maltose, D-mannitol, D-mannose, melezitose, L-rhamnose, sucrose, D-salicin, D-sorbitol, sorbose or trehalose. Indole, methyl red and Voges–Proskauer tests are negative. Tween 20 is hydrolysed, but starch, casein, gelatin, Tween 80, urea and blood are not. Phosphatase is produced, but not phenylalanine deaminase or lecithinase. H₂S is produced after 72 h at 32 °C. Charides are produced. Colonies on MY medium are circular, convex, cream-coloured and noticeably mucoid. Phosphatase is produced, but not phenylalanine deaminase or lecithinase. H₂S is produced from L-cysteine. Gluconate is oxidized. Pyocyanin, fluorescein and pigment are not produced in tyrosine medium. No growth occurs on MacConkey agar or cetrimide agar. ONPG is negative. The following compounds are acceptable as sole carbon and energy sources: acetate, citrate, ethanol, D-glucose, D-galactose, DL-glycerol, D-mannose, mannitol, trehalose, sorbitol and succinate, but lactose and adonitol are not. The following compounds are used as sole carbon, nitrogen and energy sources: L-alanine and L-serine, but not L-cysteine, L-lysine, L-methionine or L-valine. Susceptible to ampicillin (10 μg), amoxicillin (25 μg), kanamycin (30 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), polymyxin B (300 IU), rifampicin (30 μg), sulphamidine (250 μg), trimethoprim/sulphamethoxazole (1.25–23.75 μg) and tobramycin (100 μg). Resistant to erythromycin (15 μg), amoxicillin (25 μg), kanamycin (30 μg), polymyxin B (300 IU), rifampicin (30 μg), sulphamidine (250 μg), trimethoprim/sulphamethoxazole (1.25–23.75 μg) and tobramycin (100 μg). Resistant to erythromycin (15 μg). The type strain does not accumulate PHA. DNA and tyrosine are hydrolysed, but tyrosine pigment is not produced. Aesculin is not hydrolysed. Arabinose, cellobiose, D-fructose, D-glucosan, isoleucine, lactate, maltose, propionate, D-salicin, starch and L-histidine are not used as sole carbon and energy sources. Principal fatty acids are C₁₂:0 3-0H, C₁₃:0 iso 2-OH/C₁₆:1 07c, C₁₆:0 and C₁₈:1 07c. DNA G+C content of the type strain is 61.9 mol% (T₂₅ method).

The type strain, N12ᵀ (=CECT 7744ᵀ =LMG 25812ᵀ), was isolated from a sample of soil taken from the San Pedro del Pinatar saline wetland in Murcia (Spain). Reference strain is B-100.

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References


Halomonas stenophila sp. nov.


