Larsenimonas suaedae sp. nov., a moderately halophilic, endophytic bacterium isolated from the euhalophyte Suaeda salsa

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A moderately halophilic, Gram-stain-negative, non-endospore-forming endophytic bacterium designated strain ST307T was isolated from the euhalophyte Suaeda salsa in Dongying, China. Strain ST307T was aerobic, rod-shaped, motile and orange-yellow-pigmented. The organism grew at NaCl concentrations of 0.6–20% (w/v) (optimum 5–6%, w/v), at temperatures of 5–45 °C (optimum 35 °C) and at pH 5–9 (optimum pH 7–8). It accumulated poly-β-hydroxybutyric acid and produced exopolysaccharides. The major fatty acids were C₁₅:₀-7/C₁₅:₁-6c, C₁₆:₀ and C₁₆:₁-7/C₁₆:₁-6c. The predominant lipoquinone was ubiquinone Q-9. The polar lipids consisted of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, a glycoaminolipid and a phosphoglycoaminolipid. The DNA G+C content was 60.5 mol%. Phylogenetic analyses of 16S rRNA gene sequences and concatenated atpA, rpoD and secA gene sequences revealed that the strain represents a member of the genus Larsenimonas. The closest related type strain was Larsenimonas salina M1-18T. Mean DNA–DNA relatedness values between strain ST307T and the related species L. salina M1-18T, Chromohalobacter beijerinckii DSM 7218T, C. canadensis DSM 6769T, C. israelensis DSM 6768T, C. marismortui CGMCC 1.2321T, C. nigrandesensis DSM 14323T, C. salexigens DSM 3043T and C. sarecensis DSM 15547T were 15±2–45±1%. On the basis of phenotypic, chemotaxonomic and molecular features, strain ST307T clearly represents a novel species of the genus Larsenimonas. The name Larsenimonas suaedae sp. nov. is proposed, with ST307T (=CGMCC 1.8902T=DSM 22428T) as the type strain.

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA, atpA, gnrB, rpoD and secA gene sequences of strain ST307T are FJ463811, KU318994, KP297867, KP297871 and KU318995, respectively.

Three supplementary figures are available with the online Supplementary Material.

Moderately halophilic micro-organisms that grow optimally in media at NaCl concentrations of 3–15% represent a considerable heterogeneous group (Ventosa et al., 1998). Among the domain Bacteria, the family Halomonadaceae comprises mostly moderately halophilic species. These species are distributed in saline habitats such as salt lakes, marine environments, saltern areas and saline soils (Oren 2002; de la Haba et al., 2011, 2014). In 2007, the minimal standards for describing new taxa of the family Halomonadaceae were published by the International Committee on Systematics of Prokaryotes – Subcommittee on the Taxonomy of Halomonadaceae (Arachal et al., 2007). Later, multilocus sequence analysis (MSLA) was used to provide a deep phylogenetic analysis (de la Haba et al., 2012; Oren et al., 2013). At the time of writing, the family Halomonadaceae comprised ten genera with validly published names, the most numerous being the genera Halomonas and Chromohalobacter (de la Haba et al., 2014; Oren et al., 2014). León et al. (2014) described the new genus Larsenimonas, which currently consists of a single species, Larsenimonas salina (León et al., 2015). The type strain of this species, M1-18T, isolated from a saltern in South Spain, was a Gram-stain-negative, moderately halophilic bacterium.

As a result of investigating the biodiversity of endophytic bacteria in leaves and stems of the euhalophyte Suaeda salsa in Dongying, China, 15 moderately halophilic bacterial strains were obtained and identified by using a previously
described procedure (Cui et al., 2010). Among these strains, endophytic strain ST307T showed highest 16S rRNA gene sequence similarity to L. salina M1-18T (99.5 %) and 95.4 % or lower to all the other described species. In this study, we performed a polyphasic taxonomic investigation of strain ST307T, and propose that it represents a novel species of the genus Larsenimonas, with the name Larsenimonas suaedae sp. nov.

MH medium with 5 % (w/v) NaCl was used as the isolation and maintenance medium for halophilic strain ST307T at 35 °C (Ventosa et al., 1982). When necessary, the medium was solidified with 15 g agar (Difco) 1−1. L. salina M1-18T was a kind gift from Prof. Antonio Ventosa, and Chromohalobacter canadensis DSM 6769T, Chromohalobacter beijerinckii DSM 7218T, Chromohalobacter israelensis DSM 6768T, Chromohalobacter saxexiguus DSM 3043T, Chromohalobacter saceccis DSM 15547T and Chromohalobacter nigrandesensis DSM 14323T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Brunschweig, Germany (DSMZ). Chromohalobacter marismortui CGMCC 1.2321T was obtained from China General Microbiological Culture Collection Center (CGMCC). All strains were inoculated on MH, SW10, CAS or 10 % MH medium under their optimum conditions, as described in the literature (Ventosa et al., 1989; Arahal et al., 2001a, b; Quillaguamán et al., 2004; Prado et al., 2006; Peñon et al., 2006; León et al., 2014), except where indicated otherwise.

The phenotypic characterization of strain ST307T included the recommendations of the proposed minimal standards for describing novel taxa of the family Halomonadaceae (Arahal et al., 2007). Morphological, biochemical, nutritional and antimicrobial susceptibility tests were performed according to Mata et al. (2002) with the following modifications. Oxidase was determined by the Gaby–Hadley method (Gaby & Hadley, 1957). The test for oxidation/fermentation of D-glucose used modified Hugh & Leifson medium by adding 5 % (w/v) NaCl (Hugh & Leifson, 1953). For nutritional tests, strain ST307T was suspended in a sterile solution of 5 % (w/v) NaCl when performed with the Biolog GN2 system. The NaCl concentrations required for growth were studied by growing strain ST307T and the reference strains in MH medium supplemented with: 0, 0.5, 0.6, 0.7, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 15, 17, 20, 25 and 30 % (w/v) NaCl at 35 °C. For determination of the pH range and optimum for growth, MH medium was adjusted to pH 4, 5, 6, 7, 8, 9 and 10 by adding different buffers (0.1 M acetate buffer for pH 4–6, 0.1 M Tris/HCl for pH 7–9 and 0.05 M sodium carbonate/bicarbonate buffer for pH 10). To determine the temperature range and optimum for growth, temperatures were varied at 0, 5, 15, 25, 28, 30, 32, 35, 37, 40, 45 and 48 °C. Cells were cultured with constant agitation (180 r.p.m.) and growth was monitored by measuring the optical density at 600 nm. Scanning electron microscopy was used for morphological studies of cells from a 90 h culture of strain ST307T grown on the surface of MH agar plates. Samples were double fixed in 2 % (v/v) glutaraldehyde solution (pH 7.2) and 1 % (w/v) osmium tetroxide, dehydrated in an acetone series, displaced with isoamyl acetate, critical-point-dried, coated with platinum and scanned in a Hitachi S-570 scanning electron microscope. Bacterial flagella were observed by using an H-800 transmission electron microscope (Hitachi).

Cells of strain ST307T were Gram-stain-negative, non-endospore-forming rods, 0.8–1.5×0.4–0.6 μm in size. They were motile by means of flagella (Fig. S1, available in the online Supplementary Material). Colonies of strain ST307T on MH medium containing 5 % (w/v) NaCl were round, entire, convex, opaque and orange–yellow-pigmented. The isolate was able to grow at 35 °C with 0.6–20 % (w/v) NaCl and grew best with 5–6 % (w/v) NaCl. Table 1 provides a comparison of the taxonomic features of strain ST307T with the type strains of L. salina and Chromohalobacter species. In Biolog GN2 microplates, L-arabinose, L-arabinose, D-fructose, D-galactose, α-D-glucose, D-mannose, sucrose, trehalose, turanose, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-gluconic acid, p-hydroxyphenylacetic acid, L-lactic acid, succinic acid, bromosuccinic acid, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, γ-aminobutyric acid, uridine, phenylethylamine and glycerol were oxidized. Strain ST307T was resistant to (μg ml−1, unless specified otherwise): chloramphenicol (30), erythromycin (15), kanamycin (30), nalidixic acid (30), streptomycin (10) and tobramycin (10) but sensitive to ampicillin (20), carbenicillin (100), penicillin G (20 U) and rifampycin (30).

DNA was isolated and purified by the method of Lind & Ussing (1986). The 16S rRNA gene was amplified with universal bacterial primers eub27F and 1492R (DeLong 1992) and compared with reference sequences available in the EzTaxon-e database (Kim et al., 2012). Four additional housekeeping genes, atpA, gyrB, rpoD and secA, were also amplified and analysed by the method of de la Haba et al. (2012). The obtained atpA, gyrB, rpoD and secA gene sequences were compared with the reference sequences available in the GenBank database using the BLAST program. Multiple alignments were performed using CLUSTAL W 2.0 software (Larkin et al., 2007). Phylogenetic trees were reconstructed with the aid of the MEGA version 6.0 software package using the neighbour-joining and maximum-likelihood methods. A distance matrix was generated using Kimura’s two-parameter model (Kimura 1980). The stability of clusters was ascertained by performing a bootstrap analysis (1000 replications).

The comparison based on 16S rRNA gene sequences revealed that the sequence of strain ST307T displayed the highest levels of similarity to the genus Larsenimonas, sharing 99.5 % sequence similarity with L. salina M1-18T, and then to the genus Chromohalobacter, sharing 95.4 % with C. israelensis DSM 6768T and 94.9 % with C. saxexiguus DSM 3043T. For housekeeping gene sequence similarity comparisons, strain ST307T showed 98.3 % atpA gene sequence similarity to the most related strain L. salina M1-18T. The comparison based on rpoD gene sequences revealed that
Table 1. Differential characteristics between strain ST307^T and related species of the genera Larsenimonas and Chromohalobacter

| Strains: 1, ST307^T; 2, L. salina M1-18^T (León et al., 2014, 2015); 3, C. marismortui CGMCC 1.3232^T (Ventosa et al., 1989); 4, C. canadensis DSM 6769^T (Arahal et al., 2001a); 5, C. beijerinckii DSM 7218^T (Peçonek et al., 2006); 6, C. israelensis DSM 6768^T (Arahal et al., 2001a); 7, C. salexigens DSM 3043^T (Arahal et al., 2001b); 8, C. sarecensis DSM 15547^T (Quillagüamán et al., 2004). All data are from this study, except where indicated. +, Positive; −, negative. |

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<td>54.5*</td>
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<td>62*</td>
<td>60.7*</td>
<td>65*</td>
<td>64.2*</td>
<td>56.1*</td>
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*Data from the original literature source.

strain ST307^T was also most closely related to L. salina M1-18^T, sharing 95.1 % sequence similarity. Strain ST307^T showed 96.9 % secA gene sequence similarity to the most closely related strain, L. salina M1-18^T. No gyrB gene sequence of L. salina M1-18^T was available in the GenBank/EMBL/DDBJ databases. The comparison based on gyrB gene sequences showed that strain ST307^T was closely related to Salinicola halophilus CECT 5903^T (84 % sequence similarity), which had been transferred from the genus Chromohalobacter as a different species in the genus Salinicola (de la Haba et al., 2010). The phylogenetic tree based on 16S rRNA gene sequences showed strain ST307^T within the branch of L. salina M1-18^T (Fig. 1). The MLSA phylogenetic tree based on a concatenation of three additional genes (atpA, rpoD and secA) confirmed that strain ST307^T belongs to the genus Larsenimonas (Fig. S2). According to the comprehensive phylogenetic analysis mentioned above, strain ST307^T was considered to be a member of the genus Larsenimonas.

DNA–DNA hybridizations were performed by the thermal denaturation and renaturation method of De Ley et al. (1970) as modified by Huss et al. (1983), using a TU-1810 spectrophotometer equipped with a model CH181-1 thermostatic sample holder (Beijing Purkinje General Instrument). The experiments were carried out in triplicate. The DNA G+C content was determined by reversed-phase HPLC using a Shimadzu LC-2010AHT. Purified DNA after drying was hydrolysed in 50 % perchloric acid at 100 °C for 1 h. The resulting deoxyribonucleosides and standard bases were separated on a Venusil MP C18 column by using 90 % redistilled water and 10 % methanol as the mobile phase at a flow rate of 1 ml min⁻¹. UV detection was set at 260 nm. The data were treated via an LC Solution workstation. The whole-cell composition of fatty acids were determined by
**Fig. 1.** Phylogenetic relationship between strain ST307\(^T\) and closely related species. The tree was reconstructed using the maximum-likelihood algorithm based on 16S rRNA gene sequences. Filled circles indicate branches that were also recovered using the neighbour-joining method. GenBank accession numbers of the sequences used in the phylogenetic analysis are given in parentheses. Only bootstrap values greater than 70\% are shown (1000 replications). Bar, 0.02 substitutions per nucleotide position.
GC (Kämpfer & Kroppenstedt, 1996; Miller 1982) using the Microbial Identification System (MIDI, Microbial ID). Cells were cultured on tripticase soy agar (TSA) medium with 5% NaCl at 30 °C for 48 h. The extraction and analysis of fatty acids were performed according to the recommendations of the MIDI system. Polar lipids and quinones were analysed at the DSMZ.

Mean DNA–DNA relatedness values between strain ST307\textsuperscript{T} and L. salina M1-18\textsuperscript{T}, C. israelensis DSM 6768\textsuperscript{T}, C. salexigens DSM 3043\textsuperscript{T}, C. marismortui CGMCC 1.2321\textsuperscript{T}, C. bieberi-inkii DSM 7218\textsuperscript{T}, C. canadensis DSM 6769\textsuperscript{T}, C. nigrandes-sensis DSM 14323\textsuperscript{T} and C. saecensis DSM 15547\textsuperscript{T} were 45 ±1, 43±1, 32±3, 30±1, 22±1, 20±1, 16±1 and 15±2 %, respectively. These values were distinctly below the recommended threshold of 70 % used for the definition of species (Wayne et al., 1987), indicating that ST307\textsuperscript{T} represents a genotypically novel species within the genus Larsenimonas. The DNA G+C content of strain ST307\textsuperscript{T} was 60.5 mol%.

The predominant fatty acids of strain ST307\textsuperscript{T} were summed feature 8 (C\textsubscript{18:1}ω7c/C\textsubscript{18:1}ω6c, 44.2 %), C\textsubscript{16:0} (18.2 %) and summed feature 3 (C\textsubscript{16:1}ω7c/C\textsubscript{16:1}ω6c, 15.5 %), similar to data for L. salina M1-18\textsuperscript{T} (Table 2). The predominant respiratory quinone of strain ST307\textsuperscript{T} was Q\textsubscript{9} (86 %), and Q8 was also detected (14 %). However, only Q9 was detected in L. salina M1-18\textsuperscript{T}. Phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, a glycoaminolipid and a phosphoglycoaminolipid were identified as major polar lipids (Fig. 53), which is generally in accordance with data for L. salina.

On the basis of morphological and physiological characteristics, as well as DNA–DNA hybridization studies and phylogenetic position, it is proposed that strain ST307\textsuperscript{T} should be classified as representing a novel species within the genus Larsenimonas, for which we propose the name Larsenimonas suaedae sp. nov.

**Description of Larsenimonas suaedae sp. nov.**

*Larsenimonas suaedae* sp. nov. (su.ae'dae, N.L. gen. n. suae-dae of *Suaeda*, a genus of halophilic plants from which the type strain was isolated).

Cells are Gram-stain-negative, non-endospore-forming, motile rods that are 0.8–1.5 × 0.4–0.6 μm in size. Colonies are round, entire, convex, opaque and orange–yellow–pigmented. Moderately halophilic, growing at NaCl concentrations in the range 0.6–20 % (w/v), with optimal growth at 5–6 % (w/v) NaCl; no growth occurs in the absence of salt. Growth occurs at temperatures from 5 to 45 °C (optimally at 35 °C) and at pH 5–9 (optimally at 7–8). Strictly aerobic. Exopolysaccharides, poly-β-hydroxybutyric acid, catalase, lysine decarboxylase and ornithine decarboxylase are produced. Negative for oxidase, indole production, H\textsubscript{2}S production, nitrate reduction, the Voges–Proskauer test, phenylalanine deaminase test and hydrolysis of casein, gelatin, starch, DNA and Tween 80. Tween 20 is hydrolysed. Respiration on fumarate, nitrate and nitrite is negative. Positive for the Simmons’ citrate test, urease activity and o-nitrophenyl-β-D-galactopyranosidase activity. Methyl red and oxidation/fermentation of D-glucose tests are positive. Acid is produced from D-fructose, D-galactose, D-lactose, sucrose, maltose, D-mannose and trehalose, but not from D-arabinose, L-erythritol, myo-inositol, D-mannitol, raffinose, L-rhamnose, D-sorbitol or sorbose. The following compounds are used as sole carbon and energy sources: D-fructose, D-galactose, α-D-galactose, maltose, D-mannose, sucrose, trehalose and glycerol. The following compounds are not used as sole carbon and energy sources: formate, methanol, lactose, myo-inositol, raffinose, L-rhamnose, starch, D-sorbitol and D-xylitol. The following compounds are utilized as sole sources of carbon, nitrogen and energy: L-alanine, L-glycine, L-tyrosine and L-valine. The following compounds are not utilized as sole sources of carbon, nitrogen and energy: L-lysine, L-threonine and L-tryptophan. The major fatty acids are C\textsubscript{18:1}ω7c/C\textsubscript{18:1}ω6c, C\textsubscript{16:0} and C\textsubscript{16:1}ω7c/C\textsubscript{16:1}ω6c. The predominant lipoquinone is ubiquinone Q-9. The polar lipids consist of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, a glycoaminolipid and a phosphoglycoaminolipid.

The type strain is ST307\textsuperscript{T} (=CGMCC 1.8902\textsuperscript{T}=DSM 22428\textsuperscript{T}), isolated from the halophyte *Suaeda salsa* in seaside saline soil of Dongying, China. The G+C content of the DNA of the type strain is 60.5 mol% (by HPLC).

**Acknowledgements**

We thank Dr Bernhard Schink for checking the Latin etymology of the species name. This work was supported by the National Natural Science Foundation (Grant No. 31400004), the Natural Science Foundation of Shandong Province (Grant No. ZR2013CQ011) and...
Excellent Young Scholars Research Fund of Shandong Normal University of China.

References


