Jannaschia seosinensis sp. nov., isolated from hypersaline water of a solar saltern in Korea

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A slightly halophilic alphaproteobacterium, designated CL-SP26^T, was isolated from hypersaline water of a solar saltern located in Seosin, Korea. 16S rRNA gene sequence analysis revealed an affiliation with the genus *Jannaschia*. Sequence similarities between CL-SP26^T and type strains of members of the genus *Jannaschia* were 94.9–97.2%. Cells were rod-shaped and motile with one or more monopolar flagella. Strain CL-SP26^T grew on solid media as circular red colonies. It was able to grow in 3–10% sea salt; however, no growth occurred in media containing NaCl as the only salt. Strain CL-SP26^T grew at 5–35 °C and pH 7–10. The major fatty acids were 18:1 ω 7c (64%) and 18:0 (12.0%). Three fatty acids, 3-OH 14:0/iso 16:1 (3.6%), 18:3 ω 6c (2.2%) and 10-methyl 19:0 (1.9%), found in minor quantities in CL-SP26^T, are unique among *Jannaschia* species. The DNA G + C content was 63 mol%. According to physiological data, fatty acid composition and 16S rRNA gene sequence analysis, CL-SP26^T was assigned to the genus *Jannaschia*, but could be distinguished from recognized species of the genus. Strain CL-SP26^T (=KCCM 42114^T = JCM 13035^T) therefore represents a novel species, for which the name *Jannaschia seosinensis* sp. nov. is proposed.

The genus *Jannaschia* was first described by Wagner-Döbler *et al.* (2003). To date, three species, *Jannaschia helgolandensis* (type species; Wagner-Döbler *et al.*, 2003), *Jannaschia cystaugens* (Adachi *et al.*, 2004) and *Jannaschia rubra* (Macián *et al.*, 2005), have been isolated, from sea water collected from the North Sea, Hiroshima Bay in Japan and the Mediterranean Sea on the Spanish east coast, respectively. Recently, *J. cystaugens* has been transferred to the genus *Thalassobacter* (Pujalte *et al.*, 2005).

A hypersaline water sample [salinity of 318 practical salinity units (p.s.u.)] from a solar saltern in Seosin, Korea, was spread on a plate containing marine agar 2216 (MA; Difco) and the plate was incubated at 30 °C for 2 weeks. A reddish colony, strain CL-SP26^T, was isolated on the plate and subsequently purified four times on MA at 30 °C. The strain was maintained both on MA at 4 °C and in marine broth (MB; Difco) supplemented with 30% (v/v) glycerol at –80 °C.

Morphological and physiological tests were performed. Gram-staining was performed as described by Smibert & Krieg (1994). Cell morphology and motility were examined by phase-contrast microscopy with cells grown at 30 °C in MB. The presence of flagella was observed using TEM (JEOL EX2) after cells had been negatively stained with uranyl acetate (2%). Anaerobic growth was checked on MA using the GasPak anaerobic system (BBL). Bacteriochlorophyll production was determined in acetone extracts using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech) for cells that had been grown in the dark. Catalase and oxidase activities were determined according to the protocols described by Smibert & Krieg (1994). Gelatinase, amylase, DNase, nitrate reductase and degradation of Tween 80 were examined as described by Hansen & Sørheim (1991). Cells were rod-shaped and motile by one or more monopolar flagella (Fig. 1). Chain formation was rarely observed in CL-SP26^T. Bright granules were not seen on wet mounts of cells of different ages. Colonies on MA were red-coloured, circular, smooth and shiny. They could be dispersed well in MB and artificial sea water (ASW), unlike *J. rubra* (Macián *et al.*, 2005). Other morphological characteristics are shown in Table 1.

The temperature range for growth was determined on the basis of colony formation on MA plates that were incubated at 5–45 °C. The pH range for growth (tested at pH 5–11) was determined by changes in OD₆₀₀ with time in MB. The final

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Abbreviations: ASW, artificial sea water; p.s.u., practical salinity units.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-SP26^T is AY906862.

A table showing phenotypic characteristics of *Jannaschia* species is available as supplementary material in IJSEM Online.

Fig. 1. TEM of negatively stained cells of strain CL-SP26^T. Bars, 0.5 µm.

pH was adjusted using NaOH and HCl solutions. Growth of CL-SP26^T in sea salt was determined using synthetic ZoBell broth (Bacto peptone, 5 g; yeast extract, 1 g; ferric citrate, 0.1 g; distilled water, 1 l) with various concentrations of sea salt (0, 1, 3, 5, 7, 10, 15, 20, 25 %, w/v). Nitrate reductase, indole production, acid production from glucose, arginine dihydrolase, urease, aesculin hydrolysis, gelatinase, β -galactosidase and carbon source utilization were tested using the API 20NE kit (bioMérieux) according to the manufacturer's instructions, except that cell suspensions were prepared using ASW (NaCl, 24 g; MgCl₂, 5.1 g; Na₂SO₄, 4 g; CaCl₂, 1.1 g; KCl, 0.7 g; NaHCO₃, 0.2 g; KBr, 0.1 g; H₃BO₃, 0.027 g; SrCl₂, 0.024 g; NaF, 0.003 g;

distilled water, 1 l; Lyman & Fleming, 1940) as a suspension medium and cell suspensions for carbon assimilation were prepared using an AUX medium containing 2.8 ml 10 % sea salt. Other enzyme activities were assayed using the API ZYM kit (bioMérieux) and ASW as a suspension medium. Carbon utilization by CL-SP26^T was tested on basal medium agar [BMA: Tris/HCl, 50 mM, pH 7.5; NH₄Cl, 190 mM; K₂HPO₄·3H₂O, 0.33 mM; FeSO₄·7H₂O, 0.1 mM; and 1.5 % Bacto agar (Difco) in half-strength ASW; Baumann & Baumann, 1981] containing 0.2 % carbon source. BMA was supplemented with 0.01 % yeast extract. In addition, carbon utilization was investigated using Biolog GN plates. Four kinds of suspension media, ASW, basal medium (BM;

Table 1. Selected phenotypic characteristics of *Jannaschia seosinensis* sp. nov. and other *Jannaschia* species

Strains: 1, *J. seosinensis* CL-SP26^T (data from this study); 2, *J. helgolandensis* Hel 10^T (Wagner-Döbler *et al.*, 2003), 3, *J. rubra* CECT 5088^T (Macián *et al.*, 2005). ND, No data available. All strains are Gram-negative.

Characteristic	1	2	3
Source	Hypersaline water	Sea water	Sea water
Cell shape	Rods, rarely form chains	Irregular rods, frequently form chains	Rods, tend to form tight aggregates
Cell length (µm)	1.1–2.3	1.9–3.2	1.0–2.0
Cell diameter (µm)	0.7–1.2	0.7–1.1	0.5
Motility	Motile (one or more flagella)	Non-motile	Motile (3–5 monopolar flagella)
White inclusion body	Absent	Present	Absent
Colony:			
Colour	Dark red	Whitish	Dark red
Shape	Circular, entire, convex, shining	Shiny	Shiny, smooth
Salt range for growth (% w/v)	3–10	0.68–8.0*	0.34–9
Salt optimum for growth (% w/v)	3–5	ND	ND
Temperature range for growth (°C)	5–35	15–30	4–25
Optimum temperature for growth (°C)	30–35	25–30	ND
pH range for growth	7–10	7–8	7–9†
Isoprenoid quinone	UQ10	UQ10	ND
DNA G + C content (mol%)	63	63.0–63.1	64.6

*From Macián *et al.* (2005).

†Obtained in this study.

Table 2. Selected phenotypic characteristics that differentiate *J. seosinensis* sp. nov. from other *Jannaschia* species

Strains: 1, *J. seosinensis* CL-SP26^T (data from this study); 2, *J. helgolandensis* Hel 10^T (Wagner-Döbler *et al.*, 2003), 3, *J. rubra* CECT 5088^T (Macián *et al.*, 2005). ND, No data available; +, positive; –, negative; w, weak. API 20NE and API ZYM data were obtained in this study. All species are positive for catalase.

Characteristic	1	2	3
Nitrate reduction	+	–	–
Oxidase	–	w	+
Hydrolysis of:			
DNA	–	–*	+
Gelatin	+	–	–
Starch	+	–	–
API 20NE			
Nitrate reduction	–	–	+
Aesculin hydrolysis	+	–	+
<p>-Nitrophenyl α-D-glucopyranoside</p>	+	w	w
API ZYM			
Esterase lipase (C8)	+	w	w
Valine arylamidase	+	–	+
Acid phosphatase	–	w	+
Naphthol-AS-BI-phosphohydrolase	–	–	+
α -Glucosidase	+	–	+
Carbon source utilization:			
Acetone	–	–	+
Citrate	–	+	w
D-Cellobiose	–	+	–
D-Fructose	–	+	+
D-Mannitol	–	+	+
D-Mannose	–	+	+
D-Ribose	–	+	–
D-Salicin	+	–	ND
D-Sorbitol	–	+	+
D-Xylose	–	+	+
Glycerol	–	ND	+
myo-Inositol	–	+	w
L-Arginine	–	ND	w
L-Ornithine	–	ND	+
L-Rhamnose	–	+	–
L-Leucine	–	ND	+
Acetate	–	ND	+

*From Macián *et al.* (2005).

Baumann *et al.*, 1971) and BM supplemented with 0.01 and 0.1 % yeast extract, were used in the Biolog tests.

For API 20NE, API ZYM and Biolog analyses, *J. helgolandensis* Hel 10^T and *J. rubra* CECT 5088^T were used as reference strains. DNA G+C content was determined using the thermal denaturation method (Mandel & Marmur, 1968). CL-SP26^T grew optimally at 30–35 °C and pH 10, differentiating it from other species in the genus *Jannaschia* (Table 1). CL-SP26^T was unable to grow at 1 % (w/v)

salinity, unlike *J. helgolandensis* Hel 10^T and *J. rubra* CECT 5088^T (Table 1). Although CL-SP26^T was isolated from hypersaline water of 318 p.s.u., the upper salinity limit for growth was 10 %, suggesting that the strain might have survived in highly saline water. The strain was positive for nitrate reductase, gelatinase and amylase, but negative for the presence of cytochrome oxidase, whereas other species in genus *Jannaschia* gave contrasting results (Table 2).

CL-SP26^T could grow on BMA plates containing D-glucose, salicin, pyruvic acid, Casamino acids, DL-aspartate, succinate, urea and L-glutamate (see Supplementary Table available in IJSEM Online). However, no visible growth could be observed in Biolog GN plate tests or in carbon assimilation tests in API 20NE. The different results from each of the tests might result from differences in methods used and the composition of suspension media. Other results of biochemical and physiological tests are given in Tables 1 and 2 and the species description.

Isoprenoid quinones were isolated according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The major isoprenoid quinone was UQ-10. Fatty acid methyl esters in whole cells were analysed by GC according to the Microbial Identification System (MIDI) at the Korean Culture Center of Microorganisms, Seoul, Korea. The fatty acid profile for CL-SP26^T was dominated by 18:1 ω 7c (64 %) and 18:0 (12 %), which is a characteristic common to all species in the genus *Jannaschia* (Table 3). However, 3-OH 14:0 and/or iso 16:1 (3.6 %), 18:3 ω 6c (2.2 %) and 10-methyl 19:0 (1.9 %) were found in minor quantities in CL-SP26^T; these fatty acids are unique among *Jannaschia* species. Therefore, the fatty acid pattern of strain CL-SP26^T differs distinctly from those of the other species of *Jannaschia*.

Table 3. Fatty acid compositions (%) of *J. seosinensis* sp. nov. and other *Jannaschia* species

Strains: 1, *J. seosinensis* CL-SP26^T (data from this study); 2, *J. helgolandensis* Hel 10^T (Wagner-Döbler *et al.*, 2003), 3, *J. rubra* CECT 5088^T (Macián *et al.*, 2005). –, Not detected or <1 %.

Fatty acid	1	2	3
3-OH 10:0	4.6	5.6	2.3
12:1	–	4.9	–
Unknown 11:799	4.7	–	2.2
Unknown 15:273	–	–	1.7
3-OH 14:0/iso 16:1	3.6	–	–
17:0	–	1.0	–
18:1 ω 7c	64.0	45.1	79.4
18:3 ω 6c	2.2	–	–
18:0	12.0	10.8	8.1
Methyl 18:1 ω 7c	–	5.7	–
10-Methyl 19:0	1.9	–	–
Cyclo 19:0 ω 8c	–	25.1	5.7

Fig. 2. Neighbour-joining tree showing the relationship between strain CL-SP26^T and members of the genus *Jannaschia* and other related genera belonging to the *Rhodobacteraceae*. Only bootstrap values above 70% are shown (1000 resamplings) at the branching points. Solid circles indicate that the corresponding nodes are also recovered in maximum-likelihood and maximum-parsimony trees. Sequence accession numbers are given in parentheses. *Paracoccus denitrificans* IAM 12479^T (Y17512) was used as an outgroup (not shown). Bar, 0.01 nucleotide substitutions per site.

The 16S rRNA gene was amplified from a single colony by PCR with *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified using the AccuPrep PCR Purification kit (Bioneer) and cloned using the pCR2.1 TOPO TA Cloning kit (Invitrogen). Sequencing of the 16S rRNA gene was performed with an Applied Biosystems automatic sequencer (ABI3730XL) at MacroGen Corporation, Seoul, Korea. The sequence of strain CL-SP26^T was compared with 16S rRNA gene sequences available in GenBank using the BLAST (Altschul *et al.*, 1990) search. The sequence of strain CL-SP26^T was manually aligned with sequences from other species of the genus *Jannaschia* and type species of other genera belonging to the family *Rhodobacteraceae* obtained from GenBank and the Ribosomal Database Project (Cole *et al.*, 2003) using known 16S rRNA secondary structure information. Phylogenetic trees were obtained by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for neighbour-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method. Alignment analysis was carried out using the program jPHYDIT (version 1.0; available at <http://chunlab.snu.ac.kr/jphydit/>) and phylogenetic analysis was carried out using MEGA3 (Kumar *et al.*, 2004) and PAUP* 4.0 (Swofford, 1998). Likelihood parameters were estimated by the hierarchical ratio tests in MODELTEST version 3.04 (Posada & Crandall, 1998). The almost complete 16S rRNA gene sequence of strain CL-SP26^T (1384 bp) was obtained. Sequence similarity analysis indicated that the closest relatives of strain CL-SP26^T were *J. rubra* (97.2%), *J. helgolandensis* (96.9%) and *Thalassobacter stenotrophicus* (94.9%). Phylogenetic analyses based on the 16S rRNA gene

sequence showed that strain CL-SP26^T formed a robust cluster with species of the genus *Jannaschia* (Fig. 2). Thus, it is clear that this isolate belongs to the genus *Jannaschia*. Although the similarities between strain CL-SP26^T and two species (*J. rubra* and *J. helgolandensis*) were close to general guidelines (97%) for species designation at the 16S rRNA gene level (Stackebrandt & Goebel, 1994), a dendrogram of 16S rRNA gene relationships showed that CL-SP26^T is phylogenetically distant from these two species, forming a distinct branch. Furthermore, phenotypic features (salt, temperature and pH ranges for growth, presence of nitrate reductase, amylase and gelatinase activities, absence of oxidase activity) and fatty acid profiles clearly differentiated CL-SP26^T from *J. rubra* and *J. helgolandensis*. In conclusion, physiological features, fatty acid profiles and phylogenetic analyses based on 16S rRNA gene sequences suggest that strain CL-SP26^T represents a novel species of the genus *Jannaschia*, for which the name *Jannaschia seosinensis* sp. nov. is proposed.

Description of *Jannaschia seosinensis* sp. nov.

Jannaschia seosinensis (se.o.sin.en'sis. N.L. fem. adj. *seosinensis* referring to the Seosin region in Korea, where the type strain was found).

Gram-negative, strictly aerobic and straight rods, approximately 0.7–1.2 µm wide and 1.1–2.3 µm long. Cells are motile by one or more monopolar flagella. On MA solid medium, colonies are dark red, entire, circular, convex and shiny. Bacteriochlorophyll is not detected. After 4 days on MA at 30 °C colonies are approximately 0.8–1.5 mm in diameter. Grows at 5–35 °C (optimum of 30–35 °C) and pH 7–10. Growth occurs in sea salt concentrations of 3–10% (w/v) (optimum of 3–5%), but no growth occurs in media containing only NaCl as a salt. Positive for catalase, amylase, gelatinase and nitrate reductase, but negative for

oxidase, DNase and degradation of Tween 80. Major fatty acids are 18:1 ω 7c (64 %) and 18:0 (12.0 %); 3-OH 14:0 and/or iso 16:1 (3.6 %), 18:3 ω 6c (2.2 %) and 10-methyl 19:0 (1.9 %) are found in minor quantities. In API 20NE tests, the type strain is negative for nitrate reductase, gelatinase, arginine dihydrolase, urease, indole production and acid production from glucose, but positive for hydrolysis of aesculin and β -galactosidase. In API ZYM tests, the type strain is positive for alkaline phosphatase, esterase (C4 and C8), leucine arylamidase, valine arylamidase, α -galactosidase, β -galactosidase and α -glucosidase, but negative for lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. The following substrates are utilized: D-glucose, salicin, pyruvic acid, Casamino acids, DL-aspartate, succinate, urea and L-glutamate.

The type strain is CL-SP26^T (=KCCM 42114^T=JCM 13035^T), isolated from a hypersaline water sample from a solar saltern. The DNA G+C content of the type strain is 63 mol%.

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