Hoeflea suaedae sp. nov., an endophytic bacterium isolated from the root of the halophyte *Suaeda maritima*

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A Gram-negative, aerobic, short rod-shaped bacterium, designated strain YC6898^T, was isolated from the surface-sterilized root of a halophyte (*Suaeda maritima*) inhabiting tidal flat of Namhae Island, Korea. Strain YC6898^T grew optimally at 30–37 °C and pH 6.5–7.5. The strain inhibited mycelial growth of *Pythium ultimum* and *Phytophthora capsici*. Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain YC6898^T belongs to the genus *Hoeflea* in the family *Phyllobacteriaceae*. Its closest relatives were *Hoeflea alexandrii* AM1V30^T (96.7 % 16S rRNA gene sequence similarity), *Hoeflea anabaenae* WH2K^T (95.7 %), *Hoeflea phototrophica* DFL-43^T (95.5 %) and *Hoeflea marina* LMG 128^T (94.8 %). Strain YC6898^T contained Q-10 as the major ubiquinone. The major fatty acids of strain YC6898^T were $C_{18:1}\omega7c$ (61.1 %), $C_{16:0}$ (11.9 %), 11-methyl $C_{18:1}\omega7c$ (9.6 %) and $C_{19:0}$ cyclo $\omega8c$ (8.0 %). The polar lipids were phosphatidylcholine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylglycerol, unknown lipids and an unknown glycolipid. The total genomic DNA G+C content was 53.7 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic analysis, strain YC6898^T represents a novel species of the genus *Hoeflea*, for which the name *Hoeflea suaedae* sp. nov. is proposed. The type strain is YC6898^T (=KACC 14911^T=NBRC 107700^T).

The genus *Hoeflea* was described with *Hoeflea marina* as the type species in the family *Phyllobacteriaceae* within the order *Rhizobiales* of the class *Alphaproteobacteria*, with the reclassification of *Agrobacterium ferrugineum* LMG 128 (Peix *et al.*, 2005). Members of the genus *Hoeflea* are Gram-negative, aerobic, non-spore-forming and halotolerant, being isolated from marine sources. At the time of writing, the genus comprises four recognized species: *H. marina* (Peix *et al.*, 2005), *Hoeflea phototrophica* (Biebl *et al.*, 2006), *Hoeflea alexandrii* (Palacios *et al.*, 2006) and *Hoeflea anabaenae* (Stevenson *et al.*, 2011).

During a screening of endophytic bacteria with antifungal activity, one strain, YC6898^T, with strong growth inhibition of the oomycete plant pathogens *Phytophthora capsici* and *Pythium ultimum*, were isolated from the root of a tidal flat plant, *Suaeda maritima*, inhabiting Namhae Island, Korea (Bibi *et al.*, 2012). Endophytic bacteria residing in the tissue of plants may have beneficial effects on host plants and, in particular, antagonistic endophytic bacteria against plant pathogens in tidal flat plants may be novel biocontrol agents of oomycete plant pathogens (Hallmann *et al.*, 1997; Downing & Thomson, 2000; Bibi *et al.*, 2012). Here we report the taxonomic characterization of strain YC6898^T, and show that this represents a novel species of the genus *Hoeflea*.

Cell morphology and the presence of flagellum were investigated under a Nikon light microscope (1000× magnification) and a transmission electron microscope (Hitachi, model H-600) using cells grown in marine broth 2216 (MB; Difco) at 30 °C for 48 h. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Catalase and oxidase tests were performed by the procedures as outlined in Cappuccino & Sherman (2002). The physiological properties of strain YC6898^T and related type strains, including *H. marina* KACC 12993^T, *H. alexandrii* KACC 12994^T and *H. phototrophica* KACC 12992^T, were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YC6898^T is HM800935.

Two supplementary figures are available with the online version of this paper.

determined using tests as described: hydrolysis of casein, aesculin, gelatin, starch, L-tyrosine and urea (Brown, 2007), and Tweens 20 and 80 (Atlas, 1993) and production of cellulase (Teather & Wood, 1982), chitinase (Singh *et al.*, 1999) and protease (Atlas, 1993). The type strain of *H. anabaenae* was not available from the NRRL or CCUG culture collections. All strains were maintained on marine agar 2216 (MA; Difco). Enzyme activities and acid production from different carbohydrates were determined by using API ZYM and API 20E kits at 30 °C, respectively, according to instructions of the manufacturer (bioMérieux). Assimilation of various substrates was determined by using API 20NE and API 50CH kits as recommended by the manufacturer. The production of bacteriochlorophyll *a* was investigated as described by Biebl *et al.* (2006). Growth at different temperatures (4, 10, 15, 20, 25, 30, 33, 37, 40, 42, 45 °C) and various pH values (pH 4.5–10.5 at intervals of 0.5 pH units) was assayed after 4 days incubation. Growth on nutrient agar, Luria–Bertani (LB) agar, Czapek-dox agar, trypticase soy agar (TSA), MA, MacConkey agar and R2A agar was evaluated at 30 °C after 4 days incubation. Antagonistic activity of strain YC6898^T was detected as inhibition of mycelial growth of *Py. ultimum* and *Ph. capsici* using a confrontation bioassay (Bibi *et al.*, 2012). Growth under anaerobic conditions was determined for 7 days at 30 °C in an anaerobic Gaspak jar containing an atmosphere of CO_2 (Gas-Pack System; Becton Dickinson). Salt tolerance was tested in NaCl-free artificial seawater (ASW) containing 5 g peptone and 1 g yeast extract supplemented with 0–12 % (w/v, at 0.5 % intervals) NaCl after 2 days incubation at 30 °C (Kim *et al.*, 2007). Duplicate antibiotic-sensitivity tests were done using filter-paper discs containing 30 μg tetracycline, 30 μg kanamycin, 10 μg penicillin, 10 μg streptomycin, 30 μg rifampicin, 30 μg chloramphenicol, 10 μg gentamicin, 10 μg ampicillin or 30 μg vancomycin.

Cells of strain YC6898^T were Gram-negative, motile, aerobic and rod-shaped. Strain YC6898^T exhibited positive reactions for oxidase and catalase activities. The strain grew well on LB agar, MA, R2A agar, TSA, Czapek-dox agar and nutrient agar media, but did not grow on MacConkey agar medium. Strain YC6898^T inhibited mycelial growth of *Py. ultimum* and *Ph. capsici*. Other reference strains had no inhibitory activity against either pathogen. Strain YC6898^T had cellulase activity, but no chitinase or protease activity. The physiological and biochemical characteristics of strain YC6898^T are summarized in the species description and a comparison of selective characteristics with related type strains is given in Table 1.

For analysis of cellular fatty acids, strain YC6898^T and the related type strains were cultivated in MB at 30 °C and cells were harvested at the mid-exponential growth phase (OD_{600} of 0.4–0.5). The analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.). Extracts were analysed by GC (Agilent 6890) and identified by comparing the fatty acid profiles with the TSBA 40 database provided with the Sherlock Software 4.0.

Table 1. Differential phenotypic characteristics of strain YC6898^T and members of related taxa

Strains: 1, YC6898^T; 2, *H. alexandrii* KACC 12994^T; 3, *H. anabaenae* WH2K^T; 4, *H. phototrophica* KACC 12992^T; 5, *H. marina* KACC 12993^T. Data for *H. anabaenae* are from Stevenson *et al.* (2011). +, Positive; −, negative; W, weakly positive; ND, not determined. Data for reference strains are from this study unless otherwise indicated.

Characteristic	1	2	3	4	5
Temperature range (°C)	10–42	4–42	ND	15–33	10–37
NaCl (%, w/v) range	0–9.5	0–11.5	ND	0–7.0	0–5.0
pH range	5.0–10.0	6.0–9.0	ND	5.5–9.0	5.5–8.0
Hydrolysis of:					
Tween 20	−	+	ND	−	+
Tween 80	−	+	ND	+	+
Reduction of nitrate to nitrite	+	−	ND	−	−
β-Galactosidase	−	+	+	+	+
Assimilation of:					
N-Acetylglucosamine	+	−	−	−	−
Esterase lipase (C8)	−	+	−	−	−
Trypsin	+	−	+	+	−
α-Glucosidase	+	−	−	+	−
β-Glucosidase	−	+	+	−	−
Utilization of					
Erythritol	+	−	ND	−	−
D-Arabinose	+	−	+	−	−
D-Xylose	+	−	ND	−	−
Salicin	+	−	ND	−	−
L-Fucose	+	−	ND	−	−
Bacteriochlorophyll *a*	−	−	−*	+	−
DNA G +C content (mol%)	53.7	59.7†	58.1*	59.3‡	53.1§

*Data obtained from Stevenson *et al.* (2011).
†Data obtained from Palacios *et al.* (2006).
‡Data obtained from Biebl *et al.* (2006).
§Data obtained from Peix *et al.* (2005).

Isoprenoid quinones were extracted and analysed using reversed-phase HPLC according to the method described by Komagata & Suzuki (1987). For measurement of the G+C content of the chromosomal DNA, the genomic DNA of strain YC6898^T was extracted and purified as described (Ausubel *et al.*, 1995). It was then enzymically degraded into nucleosides and G+C content was determined with a reversed-phase C18 column (Mesbah *et al.*, 1989). Polar lipids were extracted according to the modified method of Minnikin *et al.* (1984) and separated by TLC on a Merck Kieselgel 60-HPTLC system. Aminolipids were detected by spraying the plate with 0.2 % (w/v) solution of ninhydrin in butanol saturated with water followed by heating at 105 °C for 10 min (Ross *et al.*, 1985). Phospholipids were detected by spraying the plate with Zinzadze reagent of Dittmer & Lester (1964). Glycolipids were detected with 1-naphthol spray reagent by heating at 100 °C for 3–5 min (Jacin & Mishkin, 1965).

The presence of phosphatidylcholine was detected with Dragendorff reagent (Sigma-Aldrich). Total lipid profiles were detected by spraying with phosphomolybdic acid solution (Sigma-Aldrich) followed by heating at 150 °C for 10 min.

The cellular fatty acid profiles of strain YC6898^T and the type strains of related *Hoeflea* species are shown in Table 2. The predominant cellular fatty acid was $C_{18:1}\omega7c$ (61.1 %), which was shared with other closely related members of the genus *Hoeflea*. $C_{16:0}$ (11.9 %), 11-methyl $C_{18:1}\omega7c$ (9.6 %), $C_{19:0}$ cyclo $\omega8c$ (8.0 %), $C_{16:1}$ $\omega7c$ and/or iso-$C_{15:0}$ 2-OH (4.2 %), $C_{18:0}$ (2.3 %), $C_{12:0}$ 3-OH (1.3 %) and $C_{19:1}$ $\omega6c$ and/or ECL 18.846 (1.7 %) were found in strain YC6898^T. The major isoprenoid quinone of strain YC6898^T was ubiquinone Q-10. The genomic DNA G+C content of strain YC6898^T was 53.7 mol%. Strain YC6898^T exhibited a polar lipid profile consisting of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylmonomethylethanolamine (PME), sulfoquinovosyl diacylglycerol (SQDG), an unknown glycolipid (GL2) and unknown lipids (L1, 2, 4) (Fig. S1 available in IJSEM Online). PE, PME, PG, PC, SQDG and L1 were common to strain YC6898^T, *H. marina* LMG 128^T and *H. alexandrii* AM1V30^T, but diphosphatidylglycerol (DPG) was detected only in *H. alexandrii* AM1V30^T.

Table 2. Cellular fatty acid composition (%) of strain YC6898^T and the type strains of related taxa

Strains: 1, YC6898^T; 2, *H. alexandrii* KACC 12994^T; 3, *H. anabaenae* WH2K^T; 4, *H. phototrophica* KACC 12992^T; 5, *H. marina* KACC 12993^T. Data for *H. anabaenae* are from Stevenson *et al.* (2011). −, Not detected.

Fatty acid	1	2	3	4	5
Saturated					
$C_{16:0}$	11.9	8.0	1.8	11.0	12.5
$C_{18:0}$	2.3	−	4.0	−	2.1
Unsaturated					
$C_{18:1}\omega7c$	61.1	68.2	64.0*	51.1	64.4
$C_{18:1}\omega9c$	−	−	6.6	−	2.3
11-methyl $C_{18:1}\omega7c$	9.6	10.8	2.6	11.2	8.7
Hydroxy					
$C_{12:0}$ 3-OH	1.3	−	−	−	−
Cyclopropane					
$C_{19:0}$ cyclo $\omega8c$	8.0	4.4	−	14.3	3.6
Summed features*					
$C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I	−	4.8	−	5.1	4.3
$C_{16:1}$ $\omega7c$ and/or $C_{15:0}$ iso 2-OH	4.2	3.8	−	7.3	−
$C_{19:1}$ $\omega6c$ and/or ECL 18.846	1.7	−	7.5	−	2.3
Unidentified					
ECL 17.603†	−	−	13.5	−	−

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. The values include $C_{18:1}\omega7c/\omega9t/\omega12t$ (Stevenson *et al.*, 2011).
†ECL, equivalent chain-length.

Genomic DNA of strain YC6898^T for 16S rRNA gene amplification was extracted by using a commercial DNA extraction kit (Core Biosystem). The 16S rRNA gene was PCR amplified from the genomic DNA by using primers 27F and 1492R (Lane, 1991). The amplified PCR product was cloned into T&A cloning vector (RBC) and sequenced by GenoTech Inc. 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR) and the sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987) in the program MEGA4 (Tamura *et al.*, 2007) and maximum-parsimony (Fitch, 1971) and maximum-likelihood methods in PHYLIP software, version 3.6 (Felsenstein, 2002), with bootstrap values based on 1000 replications (Felsenstein, 1985). Pairwise sequence similarity values between strain YC6898^T and related taxa were computed by using the EzTaxon-e server (Kim *et al.*, 2012).

The 16S rRNA gene sequence of strain YC6898^T was a continuous stretch of 1449 nt. Based on the phylogenetic analysis of 16S rRNA gene sequences, the closest relatives of strain YC6898^T were *H. alexandrii* AM1V30^T (96.7 % similarity), *H. anabaenae* WH2K^T (95.7 %), *H. phototrophica* DFL-43^T (95.5 %) and *H. marina* LMG 128^T (94.8 %). The phylogenetic tree based on the neighbour-joining algorithm showed that strain YC6898^T formed a cluster with members of the genus *Hoeflea* with 88 % bootstrap support (Fig. 1). Analysis with the maximum-likelihood and maximum-parsimony algorithms also showed that strain YC6898^T formed a phylogenetic cluster with *H. anabaenae* WH2K^T and *H. alexandrii* AM1V30^T within the genus *Hoeflea* (Fig. S2).

The results of phylogenetic analysis (Figs 1 and S2), physiological and biochemical characteristics (Table 1), polar lipid analysis (Fig. S1) and fatty acid profiles (Table 2) supported the affiliation of strain YC6898^T to the genus *Hoeflea* in the family *Phyllobacteriaceae*. We thus suggest that strain YC6898^T represents a novel species of the genus *Hoeflea*, for which the name *Hoeflea suaedae* sp. nov. is proposed.

Description of *Hoeflea suaedae* sp. nov.

Hoeflea suaedae (su.a.e′da.e. N.L. gen. n. *suaedae* of *Suaeda*, isolated from *Suaeda maritima*, referring to the source of isolation of the type strain).

Cells are Gram-negative, aerobic, short rod-shaped (0.3–0.5 μm wide by 1.3–1.4 μm long) and motile by a single polar flagellum. Colonies grown on MA at 30 °C for 2 days are 1.0–2.0 mm in diameter, white-cream, transparent, smooth, flat and circular. The temperature range for growth is 10–42 °C with optimal growth at 30–37 °C. The pH range for growth is 5.0–10.0. The range of NaCl for growth is 0–9.5 % (w/v). Optimal growth occurs with 0–7 % (w/v) NaCl. Catalase and oxidase reactions are

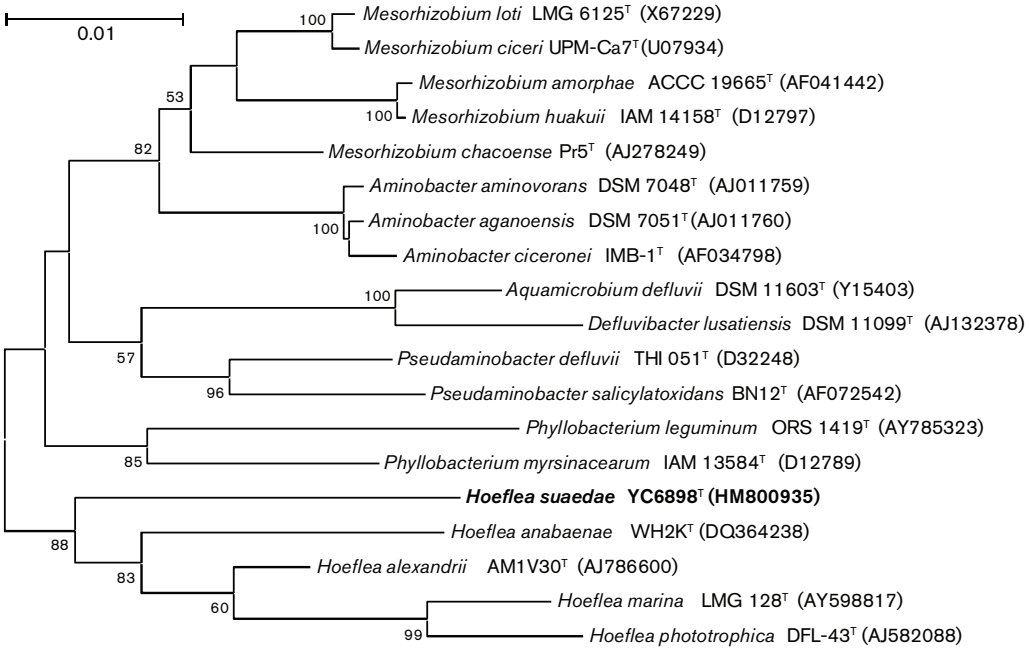

Fig. 1. Phylogenetic tree constructed from the comparative analysis of 16S rRNA gene sequences showing the relationship between strain YC6898^T and related taxa. The phylogenetic tree was constructed by using the neighbour-joining method and Jukes–Cantor evolutionary distance matrix data obtained from aligned nucleotides. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. Bar, 1 substitution per 100 nt positions.

positive. Shows antagonistic activity against plant oomycete pathogens *Py. ultimum* and *Ph. capsici*. Positive for cellulase activity. Sensitive to ampicillin (10 μg), chloramphenicol (30 μg), streptomycin (10 μg), rifampicin (30 μg) and penicillin (10 μg), but resistant to gentamicin (10 μg), tetracycline (30 μg), vancomycin (30 μg) and kanamycin (30 μg). It can hydrolyse aesculin and urea, but not casein, starch, gelatin, Tween 20, Tween 80 or tyrosine. It can use D-glucose, D-ribose, D-xylose, L-xylose, D-fucose, L-fucose, D-arabitol, potassium 5-ketogluconase, D-arabinose, L-arabinose, *N*-acetylglucosamine, malate, salicin and glycerol as a single carbon source available in the API 20NE and API 50CH systems. In the API ZYM kit, it shows enzyme activities of alkaline phosphatase, leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and α-glucosidase, but not of esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-mannosidase or α-fucosidase. Does not produce bacteriochlorophyll *a*. The major isoprenoid quinone is ubiquinone Q-10. The polar lipids are phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, sulfoquinovosyl diacylglycerol, an unknown glycolipid and unknown lipids. The major fatty acids are $C_{18:1}\omega7c$ and $C_{16:0}$.

The type strain, YC6898^T (=KACC 14911^T=NBRC 107700^T), was isolated from the root of *Suaeda maritima* growing on the tidal flat of Namhae Island, Korea. The DNA G+C content of the type strain is 53.7 mol%.

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