Haloferula luteola sp. nov., an endophytic bacterium isolated from the root of a halophyte, *Rosa rugosa*, and emended description of the genus *Haloferula*

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A Gram-negative, non-spore-forming, endophytic bacterium, strain YC6886^T, was isolated from the root of a halophyte, *Rosa rugosa*, which inhabits coastal areas of Namhae Island off the southern coast of Korea. Cells were non-motile, obligately aerobic rods and formed pale-yellow colonies. The isolate grew at 4–32 °C (optimum 25–28 °C) and at pH 6.5–9.5 (optimum pH 7.5) and grew optimally with 2–3% (w/v) NaCl, but NaCl was not an absolute requirement for growth. Strain YC6886^T produced yellow carotenoid pigments. Strain YC6886^T exhibited the highest 16S rRNA gene sequence similarity with *Haloferula sargassicola* MN1-1037^T (97.4%). Sequence similarities between strain YC6886^T and other members of the genus *Haloferula* were 93.9–94.7%. DNA–DNA relatedness between strain YC6886^T and *H. sargassicola* KCTC 22202^T and *Haloferula rosea* KCTC 22201^T was 27 and 15%, respectively. The major fatty acids were iso-C_{14:0}, C_{16:0} and C_{16:1}ω9c and minor components were C_{14:0}, C_{18:0} and anteiso-C_{15:0}. The major respiratory quinone was menaquinone 9 and the DNA G+C content was 58.5 mol%. The polar lipid profile was composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unknown phospholipid and an unknown phosphoglycolipid. On the basis of phenotypic, chemotaxonomic, DNA–DNA hybridization and phylogenetic analysis, strain YC6886^T represents a novel species in the genus *Haloferula*, for which the name *Haloferula luteola* sp. nov. is proposed. The type strain is YC6886^T (=KCTC 22447^T =DSM 21608^T). An emended description of the genus *Haloferula* is also presented.

The genus *Haloferula* belongs to subdivision 1 of the phylum *Verrucomicrobia* and currently includes five species with validly published names: *Haloferula rosea*, isolated from sponge, *H. harenae*, from sediment, *H. phyci*, from green algae, *H. helveola*, from seawater, and *H. sargassicola*, from brown algae. Members of the genus *Haloferula* are characteristically Gram-negative, obligately aerobic, non-spore-forming and non-motile rods with menaquinone 9 (MK-9) as the major respiratory quinone, iso-C_{14:0} as the predominant cellular fatty acid and a genomic DNA G+C content of 63–65 mol% (Yoon *et al.*, 2008).

For the analysis of endophytic bacterial diversity of the halophyte *Rosa rugosa* Thunb. ex Murray, 52 strains were isolated from roots and identified on the basis of 16S rRNA gene sequence similarity. They belonged to various groups of bacteria, with members of class *Alphaproteobacteria* predominant. 16S rRNA gene sequence similarities with related genera were 92.0–100%. One novel strain, designated YC6886^T, had the highest 16S rRNA gene sequence similarity (97.4%) with *H. sargassicola* MN1-1037^T and 93.9–94.7% 16S rRNA gene sequence similarity with other type strains of species of the genus *Haloferula* (Yoon *et al.*, 2008).

Strain YC6886^T was isolated from roots of *Rosa rugosa* growing on coastal tidal flats of Namhae Island, located off the southern coast of Korea. Root pieces were washed several times with running tap water and surface sterilized

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YC6886^T is FJ032193.

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

by washing in 70 % ethanol for 5 min, 1.0 % sodium hypochlorite for 10 min, 70 % ethanol for about 10 s and finally sterile distilled water several times. To confirm the success of the sterilization process, washed root segments were placed on R2A agar (Difco) and incubated at 28 °C for 5–6 days. After confirming the surface sterility of the root segments, 1.0 g dried plant root was ground in 9.0 ml autoclaved filtered seawater (AFS) with a sterile mortar and pestle. Serial dilutions were made using AFS and 200 µl of 10^{-3} – 10^{-5} dilutions was spread on one-tenth-strength (1/10) R2A plates and incubated at 25 °C for 1–2 weeks. Strain YC6886^T was maintained on marine agar 2216 (MA; Difco), marine broth (MB; Difco) or half-strength (1/2) R2A agar.

Cell morphology was observed using transmission electron microscopy (model H-600; Hitachi) with cells grown for 1 day at 30 °C in MB. Motility was checked using a hanging-drop method (Schaal, 1986). Growth at pH 5.0–11.0 (at intervals of 0.5 pH units) was tested in MB. Growth at 4–40 °C was tested on MA. Growth with 0–10 % (w/v) NaCl was tested on 1/2 R2A agar. Anaerobic growth was determined at 28 °C after 2 weeks of incubation on MA in the GasPak EZ Anaerobe Pouch System (BD), according to the manufacturer's instructions. Catalase and oxidase tests were performed by the procedures outlined by Cappuccino & Sherman (2002). Nitrate reduction was determined according to the method of Lányi (1987). Hydrolysis of casein, gelatin, aesculin, urea, tyrosine, starch and cellulose was investigated using 1/2 R2A agar as the basal medium after 7 days of incubation, according to described methods (Lányi, 1987; Smibert & Krieg, 1994; Hendricks *et al.*, 1995). Cells of strain YC6886^T were rods, 0.8–1.2 µm wide and 1.1–2.5 µm long, and lacked flagella. For the determination of further physiological and biochemical characteristics of strain YC6886^T, the API ZYM, API 20 NE (bioMérieux) and GN2 MicroPlate (Biolog) systems were used, with incubation at 30 °C. API ZYM strips were read after 6 h and API 20 NE strips and GN2 MicroPlates were read after 24 and 48 h, respectively. The physiological characteristics and enzyme activities of strain YC6886^T are listed in the species description and a comparison of selected characteristics with those of members of the genus *Haloferula* is given in Table 1.

Acetone was used to extract the yellow pigment from freeze-dried cells of strain YC6886^T. The absorption spectrum of the extract was determined using a scanning UV/visible spectrophotometer (Optizen 2120UV; Mechasis). The absorption maxima were typical of carotenoids (340, 450 and 490 nm; Supplementary Fig. S1, available in IJSEM Online).

For the measurement of the genomic DNA G + C content of strain YC6886^T, chromosomal DNA was extracted and purified as described by Ausubel *et al.* (1995) and degraded enzymically into nucleosides. The G + C content was analysed using HPLC (Mesbah *et al.*, 1989) with a reversed-phase C18 column. Menaquinones were isolated using the methods of Minnikin *et al.* (1984) and separated

by HPLC (Kroppenstedt, 1982). The G + C content of the genomic DNA was 58.5 mol% and the major respiratory quinone was MK-9.

The 16S rRNA gene of strain YC6886^T was amplified from extracted DNA using primers 27F and 1492R and the purified PCR products were sequenced by GenoTech (Daejeon, Korea) (Lane, 1991). To ascertain the phylogenetic position of the novel strain, the 16S rRNA gene sequence was compared with sequences obtained from NCBI. Multiple sequence alignments were created using CLUSTAL X version 1.83 (Thompson *et al.*, 1997). Gaps were edited using BioEdit (Hall, 1999). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in MEGA version 4 (Tamura *et al.*, 2007) and bootstrap values were calculated using 1000 replications (Felsenstein, 1985). Comparative analysis of 16S rRNA gene sequences revealed that strain YC6886^T belonged to the genus *Haloferula*. Strain YC6886^T exhibited 97.4 % 16S rRNA gene sequence similarity with *H. sargassicola* MN1-1037^T, 94.7 % with *H. rosea* 06SJR1-1^T, 94.4 % with *H. harenae* YM23-227^T, 94.2 % with *H. phyci* AK18-024^T and 93.9 % with *H. helveola* 05IJR53-1^T. In the neighbour-joining phylogenetic tree (Fig. 1), strain YC6886^T formed a tight cluster with *H. sargassicola* MN1-1037^T, which was supported by a high bootstrap value (100 %).

DNA–DNA hybridization was performed using the procedure of Ezaki *et al.* (1989) to evaluate the genomic relatedness between strain YC6886^T and its closest phylogenetic neighbours, *H. sargassicola* KCTC 22202^T and *H. rosea* KCTC 22201^T. Five replicate hybridizations were conducted for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values are quoted as hybridization values. DNA–DNA relatedness between strain YC6886^T and *H. sargassicola* KCTC 22202^T and *H. rosea* KCTC 22201^T was 27 ± 2.4 and 15 ± 1.9 %, respectively; these values (<70 %) indicate a level of DNA–DNA relatedness appropriate for defining a species in current bacterial systematics (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Analysis of cellular fatty acid methyl esters was performed for strain YC6886^T and *H. sargassicola* KCTC 22202^T and *H. rosea* KCTC 22201^T, using cultures grown in MB at pH 7.6 and 25 °C for 24 h. Cellular fatty acids were saponified, methylated and extracted according to Sasser (1990). The fatty acids were analysed by GC (model 6890; Hewlett Packard) and identified using the Microbial Identification software package (Microbial ID). The major cellular fatty acids of strain YC6886^T were iso-C_{14:0} (36.2 %), C_{16:0} (33.3 %) and C_{16:1ω9c} (12.7 %), similar to other members of the genera *Haloferula* and *Roseibacillus*. However, strain YC6886^T could be distinguished by having different proportions of C_{15:0}, C_{16:0}, C_{16:1ω9c}, C_{18:1ω9c}, iso-C_{16:0} and anteiso-C_{17:0} (Table 2).

Table 1. Characteristics that differentiate strain YC6886^T from type strains of species of the genus *Haloferula*

Strains: 1, *Haloferula luteola* sp. nov. YC6886^T; 2, *H. sargassicola* KCTC 22202^T; 3, *H. rosea* KCTC 22201^T; 4, *H. helveola* 05IJR53-1^T; 5, *H. phyci* AK18-024^T; 6, *H. harenae* YM23-227^T. Data in columns 1–3 were obtained in this study, unless otherwise indicated; data in columns 4–6 were taken from Yoon *et al.* (2008). +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6
Colony colour*	PY	PO	PP	Y	PO	P
Catalase	+	+	–	–	–	W
Oxidase	+	–	+	+	+	+
Temperature range (°C)	4–32	4–37	20–37	20–37	20–37	20–37
pH range	6.5–9.5	6.5–9.0	6.5–9.0	6.0–8.5	6.0–9.0	6.0–9.0
Maximum NaCl concentration for growth (%)	4.5	7.0	5.0	6.0	5.0	5.0
NaCl requirement	–	+	+	+	+	+
Nitrate reduction	+	–	–	–	–	–
Glucose fermentation	–	+	+	+	+	+
Hydrolysis of:						
Gelatin	–	+	+	ND	ND	ND
Cellulose	+	–	–	ND	ND	ND
Casein	+	–	–	ND	ND	ND
Enzyme activities						
Esterase (C4)	+	–	–	–	–	–
Valine arylamidase	+	–	–	–	–	–
Trypsin	+	+	+	–	–	–
β-Galactosidase	+	–	–	–	+	+
β-Glucuronidase	+	–	–	–	–	–
N-Acetyl-D-glucosaminidase	+	–	–	–	–	+
α-Mannosidase	+	+	+	–	–	–
Utilization of:						
α-Cyclodextrin	+	+	–	–	–	–
Dextrin	+	+	+	+	–	+
Glycogen	–	+	+	–	–	–
Glycerol	+	+	–	–	–	+
Trehalose	+	+	+	+	–	+
Sucrose	+	+	+	+	–	+
Cellobiose	+	+	+	+	–	+
Acetic acid	+	+	–	–	+	–
Thymidine	–	+	+	–	–	–
Succinamic acid	w	+	–	–	+	–
DNA G + C content (mol%)	58.5	64.7†	63.1†	64.9	64.3	63.8

*O, Orange; P, pink; PO, pale orange; PP, pale pink; PY, pale yellow.

†Data from Yoon *et al.* (2008).

Polar lipids were extracted by the modified method of Minnikin *et al.* (1984) and separated by TLC on Kieselgel 60 HPTLC plates (Merck). Aminolipids were detected by spraying with 0.2 % (w/v) ninhydrin in butanol saturated with water, followed by heating at 105 °C for 10 min (Ross *et al.*, 1985). Phospholipids were detected by spraying with Zinzadze reagent (Dittmer & Lester, 1964). Glycolipids were detected with 0.5 % 1-naphthol in methanol/water (1:1, v/v) and then with sulfuric acid/ethanol (1:1 v/v), followed by heating at 120 °C for 5–10 min (Xin *et al.*, 2000). Total lipid profiles were detected by spraying with phosphomolybdic acid (Sigma-Aldrich), followed by heating at 150 °C for 10 min. Strain YC6886^T exhibited a polar lipid profile consisting of diphosphatidylglycerol,

phosphatidylethanolamine, phosphatidylglycerol, an unknown phospholipid and an unknown phosphoglycolipid (Supplementary Fig. S2). *H. sargassicola* KCTC 22202^T exhibited similar major and minor polar lipids to strain YC6886^T, although different unknown phosphoglycolipids were detected in each strain. A trace amount of an unknown glycolipid was detected in *H. rosea* KCTC 22201^T but not in strain YC6886^T (Supplementary Fig. S2). These differences clearly distinguished strain YC6886^T from its closest phylogenetic neighbours and confirmed strain YC6886^T as a representative of a novel species in the genus *Haloferula*.

The characterization of strain YC6886^T and its differentiation from its closest phylogenetic relatives (Table 1)

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing relationships between strain YC6886^T and other members of the genus *Haloferula* and related genera. Percentages at nodes indicate levels of bootstrap support based on 1000 replications. Filled circles indicate that the corresponding nodes were also recovered with the maximum-parsimony method. *Victivallis vadensis* Cello^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

followed recommended standards (Tindall *et al.*, 2010). Although strain YC6886^T could grow with 4.5% (w/v) NaCl, it could grow without NaCl, in contrast to members of closely related species, for which NaCl is an important factor for growth (Yoon *et al.*, 2008). In brief, the ability to grow without NaCl, reduce nitrate, produce indole and

Table 2. Cellular fatty acid contents of strain YC6886^T and type strains of species of the genus *Haloferula*

Strains: 1, *Haloferula luteola* sp. nov. YC6886^T; 2, *H. sargassicola* KCTC 22202^T; 3, *H. rosea* KCTC 22201^T; 4, *H. helveola* 05IJR53-1^T; 5, *H. phyci* AK18-024^T; 6, *H. harenae* YM23-227^T. Data in columns 1–3 were obtained in this study; data in columns 4–6 were taken from Yoon *et al.* (2008). Values are percentages of total fatty acids. tr, <1.0% of total fatty acids; –, not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
C _{14:0}	7.6	4.2	1.3	1.0	tr	tr
C _{15:0}	–	3.2	2.0	7.8	13.2	6.0
C _{16:0}	33.3	21.7	1.7	9.2	11.6	6.1
C _{17:0}	–	–	–	tr	8.7	tr
C _{18:0}	2.5	2.8	tr	tr	tr	–
Unsaturated						
C _{16:1ω7c}	–	tr	–	tr	tr	–
C _{16:1ω9c}	12.7	4.1	–	4.7	4.7	1.7
C _{18:1ω9c}	–	5.7	tr	tr	tr	–
Branched						
iso-C _{14:0}	36.2	40.3	2.2	47.1	37.6	41.2
iso-C _{15:0}	–	–	tr	2.5	tr	tr
iso-C _{16:0}	–	4.7	25.1	8.5	8.8	22.3
anteiso-C _{15:0}	5.5	6.1	48.0	15.3	11.3	20.3
anteiso-C _{17:0}	–	2.2	13.8	tr	tr	tr

hydrolyse cellulose and other characteristics given in Table 1 clearly distinguished strain YC6886^T from other members of the genus *Haloferula*. In addition, it was clear from phylogenetic analysis, DNA–DNA relatedness, polar lipid analysis and other biochemical and physiological analyses that strain YC6886^T represents a novel species within the genus *Haloferula*, for which the name *Haloferula luteola* sp. nov. is proposed. An emended description of the genus *Haloferula* is also required in order to accommodate the properties of strain YC6886^T.

Emended description of the genus *Haloferula* Yoon *et al.* 2008

Characteristics are the same as given in the original description of the genus *Haloferula* (Yoon *et al.*, 2008) with the following amendments. The DNA G + C content is 58.5–65.0 mol%. Nitrate reduction is variable. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol, while traces of an unknown glycolipid are detected in the type strain of the type species.

Description of *Haloferula luteola* sp. nov.

Haloferula luteola (lu.te'ō.la. L. fem. dim. adj. *luteola* yellowish).

Cells are Gram-negative, non-motile and obligately aerobic rods (0.8–1.2 µm wide and 1.1–2.5 µm long) and lack flagella. Colonies on MA are circular, convex and pale yellow. Grows at 4–32 °C (optimum 25–28 °C), but not at 37 °C, and at pH 6.5–9.5. Grows with up to 4.5% (w/v) NaCl, but NaCl is not required for growth. Catalase- and oxidase-positive. Nitrate is reduced to nitrite but not to nitrogen. Carotenoid pigments are found. Solubilizes phosphate, produces siderophores and exhibits plant growth promotion activity. Hydrolyses casein and cellulose, but not starch. Positive for indole production, aesculin hydrolysis and β-galactosidase (*p*-nitrophenyl β-D-galactopyranoside hydrolysis); in API ZYM tests, positive for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase and α-mannosidase. Oxidizes the following carbon compounds: α-cyclodextrin, dextrin, D-arabitol, Tweens 20 and 80, *N*-acetyl-D-glucosamine, L-arabinose, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-sorbitol, sucrose, trehalose, turanose, succinic acid mono-methyl ester, acetic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, succinic acid, bromosuccinic acid, succinamic acid (weakly), L-alaninamide, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-proline,

L-pyroglutamic acid, D-serine, L-serine, urocanic acid, inosine, 2-aminoethanol, 2,3-butanediol, glycerol and D-glucose 6-phosphate. The major respiratory menaquinone is MK-9. The major fatty acids are iso-C_{14:0}, C_{16:0} and C_{16:1ω9c} and minor components are C_{14:0}, C_{18:0} and anteiso-C_{15:0}. The polar lipid profile is composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unknown phospholipid and an unknown phosphoglycolipid. The DNA G+C content of the type strain is 58.5 mol%.

The type strain, YC6886^T (=KCTC 22447^T =DSM 21608^T), was isolated from roots of *Rosa rugosa* Thunb. ex Murray growing on a sand dune of Namhae Island in Korea.

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