Reclassification of *Agrobacterium ferrugineum* LMG 128 as *Hoeflea marina* gen. nov., sp. nov.

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Members of the species *Agrobacterium ferrugineum* were isolated from marine environments. The type strain of this species (=LMG 22047^T=ATCC 25652^T) was recently reclassified in the new genus *Pseudorhodobacter*, in the order '*Rhodobacterales*' of the class '*Alphaproteobacteria*'. Strain LMG 128 (=ATCC 25654) was also initially classified as belonging to the species *Agrobacterium ferrugineum*; however, the nearly complete 16S rRNA gene sequence of this strain indicated that it does not belong within the genus *Agrobacterium* or within the genus *Pseudorhodobacter*. The closest related organism, with 95·5 % 16S rRNA gene similarity, was *Aquamicrobium defluvii* from the family '*Phyllobacteriaceae*' in the order '*Rhizobiales*'. The remaining genera from this order had 16S rRNA gene sequence similarities that were lower than 95·1 % with respect to strain LMG 128. These phylogenetic distances suggested that strain LMG 128 belonged to a different genus. The major fatty acid present in strain LMG 128 was mono-unsaturated straight chain 18 : 1ω7c. The G+C content of the DNA was 53·1 mol%. Strain LMG 128 grew at 4 °C but not at 40 °C, and tolerated up to 5 % NaCl. The pH range for growth was 6–8. It produced urease and β-galactosidase, and hydrolysed aesculin. Denitrification was negative. Growth was observed with many carbohydrates as the only carbon source. The data from this polyphasic study indicate that this strain belongs to a new genus of the family '*Phyllobacteriaceae*', and therefore it is proposed that strain LMG 128^T should be reclassified as representing a novel species within the new genus *Hoeflea* gen. nov., for which the name *Hoeflea marina* sp. nov. is proposed.

The species *Agrobacterium ferrugineum* was originally described by Ahrens and co-workers (Ahrens, 1968; Ahrens & Rheinheimer, 1967) for star-shaped *Agrobacterium*-like organisms. In the 8th edition of *Bergey's Manual of Determinative Bacteriology*, Ahrens withdrew her proposal of classifying these organisms as *Agrobacterium ferrugineum* (Allen & Holding, 1974) and the species was not included in the Approved Lists (Skerman *et al.*, 1980). However, Rüger & Höfle (1992) revived the name *Agrobacterium ferrugineum* when they described marine, star-shaped, aggregate-forming bacteria. The type strain (=Ahrens A7^T=ATCC 25652^T) of this species has been recently reclassified as belonging to the new genus *Pseudorhodobacter* (Uchino *et al.*, 2002). Strain LMG 128 (=Ahrens A43=ATCC 25654) was initially included in the species *Agrobacterium ferrugineum* (Ahrens, 1968), but this strain was not studied by Rüger & Höfle (1992), who only took into consideration the type strain in their study (=Ahrens A7^T=ATCC 25652^T). The nearly complete sequence of the 16S rRNA-encoding gene of LMG 128 indicates that it does not belong to the genus *Agrobacterium*, or to the genus *Pseudorhodobacter*, but to a new genus within the family '*Phyllobacteriaceae*'. In the present study we describe the phylogenetic, morphological, chemotaxonomic and physiological characteristics of this organism. On the basis of these data, we propose to place strain LMG 128^T within a new genus, *Hoeflea*, as *Hoeflea marina* gen. nov., sp. nov.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LMG 128^T is AY598817.

A neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Hoeflea marina* LMG 128^T and related organisms of the family '*Phyllobacteriaceae*' is available as supplementary material in IJSEM Online.

DNA extraction, PCR of the 16S rRNA gene and sequencing of the PCR products were performed as previously described (Rivas *et al.*, 2002). A nearly complete 16S rRNA gene sequence (1477 nucleotides) was obtained and was compared with those deposited in the databases. Sequences were aligned using the CLUSTAL_X software (Thompson *et al.*, 1997). Distances were calculated according to Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were inferred by using the neighbour-joining method (Saitou & Nei, 1987). The bootstrap analysis was based on 1000 resamplings. The MEGA 2 package (Kumar *et al.*, 2001) was used for all analyses. Based on the 16S rRNA gene sequence analysis, strain LMG 128^T formed a new branch within the family '*Phyllobacteriaceae*' (Fig. 1), and its closest relatives were *Aquamicrobium defluvii* (Bambauer *et al.*, 1998) and *Defluvibacter lusatiensis* (Fritsche *et al.*, 1999), with 16S rRNA gene sequence similarities of 95·5 and 95·1 %, respectively (a more complete phylogenetic tree is available as supplementary material in IJSEM Online).

Strain LMG 128^T was grown on nutrient agar for 48 h at 22 °C, to check for motility by phase-contrast microscopy. The cells were also stained according to the classical Gram procedure described by Doetsch (1981). The morphological characteristics of strain LMG 128^T are presented in the species description.

For fatty acid methyl ester (FAME) analysis, strain LMG 128^T was cultivated for 24 h at 28 °C on TSBA plates containing 30 g trypticase soy broth (BBL), supplemented with 15 g Bacto agar (l distilled water)$^{-1}$ (Difco). The cells were saponified, and the fatty acids were methylated to FAMEs and extracted following the Sherlock Microbial Identification System version 3.0 (MIDI, 1999). FAMEs were separated on an Agilent 6890A series gas chromatograph, with 7683 autoinjector and autosampler tray module (Agilent Technologies). Separation of FAMEs was achieved with a fused-silica capillary column (25 m × 0·2 mm), with cross-linked 5 % phenylmethyl silicone (film thickness, 0·33 μm; HP Ultra2). Hydrogen served as the carrier gas. Peak integration and identification were performed using the Hewlett Packard Chemstation software and Sherlock software. The results of the analysis are shown in Table 1. For comparison, the type strains of *Aquamicrobium defluvii* and *Pseudorhodobacter ferrugineus* were also included. As *P. ferrugineus* could not be grown on TSBA, it was grown on marine agar (Medium 12; BCCM/LMG catalogue of strains, http://www.belspo.be/bccm/db/media.htm). All three strains contained as a major component the mono-unsaturated straight-chain 18 : 1ω7*c* (60–80 %). Minor fatty acids were the saturated straight-chain components 16 : 0 and 18 : 0. No significant hydroxy fatty acids were detected in LMG 128^T (summed feature 3, comprising 14 : 0 3-OH and 16 : 1 iso, which could not be separated, was present at less than 1 %), whereas *Aquamicrobium defluvii* and *P. ferrugineus* contained 12 : 0 3-OH and 10 : 0 3-OH, respectively. For *D. lusatiensis*, Fritsche *et al.* (1999) reported that the main component was octadecanoic acid (18 : 1) and that 12 : 0 3-OH was present as a diagnostic component in small amounts. Strain LMG 128^T contained a significant amount (7·5 %) of 18 : 1ω7*c* 11Me, whereas no methylated fatty

Table 1. FAME profiles

Strains: 1, *H. marina* LMG 128^T; 2, *Aquamicrobium defluvii* LMG 22048^T; 3, *P. ferrugineus* LMG 22047^T. Values are mean percentages of total FAMEs. Only fatty acids accounting for more than 1·0 % (mean amount) are indicated. tr, Trace amount (⩽1·0 %); ND, not detected.

FAME	1	2	3
10 : 0 3-OH	ND	ND	4·0–6·4
12 : 0 3-OH	ND	2·5	ND
16 : 0	4·0	2·4	2·6–2·7
17 : 0	ND	2·1	tr
17 : 1ω6*c*	ND	1·5	ND
17 : 1ω8*c*	tr	2·6	ND
18 : 0	1·4	2·7	3·0–4·6
18 : 1ω7*c*	76·0	80·7	61·3–66·5
18 : 1ω9*c*	ND	ND	1·7–2·1
18 : 1ω7*c* 11Me	7·5	ND	ND
Unknown ECL 18·797	ND	1·8	ND
19 : 0 cyclo ω8*c*	5·6	1·2	ND
20 : 1ω9*t*	tr	1·5	ND
Summed feature 4*	2·6	ND	tr
Unknown ECL 11·798	ND	ND	3·2–4·7
Unknown ECL 15·275	ND	ND	3·6–5·2
Unknown ECL 17·606	tr	ND	11·4–13·1

*Summed feature 4 consisted of one or both of the following fatty acids, which could not be separated by the Microbial Identification System: 16 : 1ω7*c* and 15 : 0 iso 2-OH.

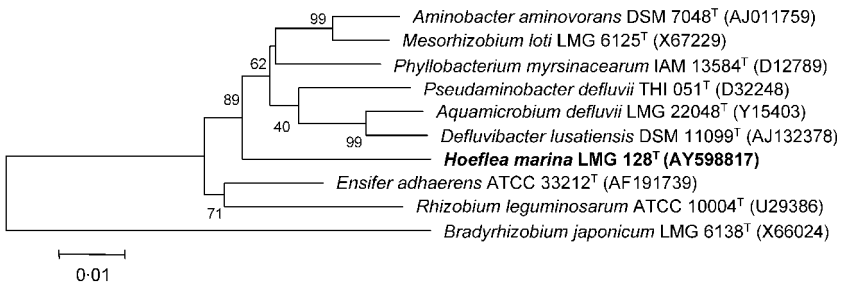

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *H. marina* LMG 128^T and other related organisms of the family '*Phyllobacteriaceae*'. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. Bar, 1 estimated substitution per 100 base positions.

acids were detected in the other strains. To exclude the possibility that the original strain LMG 128^T, deposited by R. Ahrens in 1969, had been contaminated or mislabelled during strain maintenance at the culture collection, we included lyophilized cultures from different batches, from 1973 and 1992. Both had identical FAME patterns.

Quinone and lipid compositions were determined by HPLC (Tindall, 1990a, b). Similar to other related genera from the order '*Rhizobiales*' (Fritsche *et al.*, 1999), the major respiratory lipoquinone in strain LMG128^T was ubiquinone Q-10. The polar lipid pattern of strain LMG 128^T was composed of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and sulphoquinovosyldiacylglyceride. Small amounts of diphosphatidylglycerol, an unidentified phospholipid and an unidentified phosphoglycolipid were also detected.

DNA from strain LMG 128^T was prepared according to Chun & Goodfellow (1995). The mol% G+C content was determined by using the thermal denaturation method (Mandel & Marmur, 1968). The G+C content of strain LMG 128^T was 53·1 mol%.

Catalase and oxidase activities were determined as described previously (Rivas *et al.*, 2003). Other physiological and biochemical tests were done by using API 20NE (bioMérieux), according to the manufacturer's instructions. *P. ferrugineus* LMG 22047^T and *Aquamicrobium defluvii* LMG 22048^T were also included for comparison. The temperature range for growth was determined by incubating cultures on Yeast Mannitol agar (YMA) medium (Vincent, 1970) at 4–45 °C. The pH range was determined on YMA medium, with a final pH between 4·0 and 9·0. Salt tolerance was studied on YMA medium containing 0–8 % (w/v) NaCl. Differentiating physiological characteristics are listed in Table 2. They clearly demonstrate that strain LMG 128^T is different from strains of neighbouring genera.

In conclusion, the results of the present study, including a low similarity value for the 16S rRNA gene sequence and differences in the chemotaxonomical, morphological and physiological analyses, indicate that isolate LMG 128^T is not related to *P. ferrugineus* and should be reclassified as representing a novel species within a new genus, for which we propose the name *Hoeflea* gen. nov., with *Hoeflea marina* sp. nov. as the type species.

Description of *Hoeflea* gen. nov.

Hoeflea (Hoef.le.a′. N.L. fem. n. *Hoeflea* honouring Manfred Höfle, German microbiologist, in recognition of his contribution to the taxonomy of marine bacteria).

Gram-negative, non-spore-forming, short, regular and motile rod-shaped cells. Strictly aerobic and chemoorganotrophic. Oxidase- and catalase-positive. Grow in the presence of NaCl concentrations up to 5 % (w/v), although salt is not essential for growth. Temperature range for growth is 4–37 °C and the pH range for growth is 6–8. Do not reduce nitrate to nitrite or nitrogen. The major respiratory lipoquinone is ubiquinone Q-10. The polar lipids are phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and sulphoquinovosyldiacylglyceride. Diphosphatidylglycerol, an unidentified phospholipid and an unidentified phosphoglycolipid are present in small amounts. The main fatty acid is the unsaturated straight-chain fatty acid 18 : 1ω7*c*. Other significant fatty acids (>3 %) include 16 : 0, 18 : 1ω7*c* 11Me and 19 : 0 cyclo ω8*c*. Hydroxy fatty acids are present in small amounts. Phylogenetically a member of the family '*Phyllobacteriaceae*'. The DNA G+C content of the type species is 53·1 mol%.

The type species of the genus is *Hoeflea marina*.

Table 2. Differentiating characteristics among the strains from this study

Strains: 1, *H. marina* LMG 128^T; *P. ferrugineus* LMG 22047^T; 3, *Aquamicrobium defluvii* LMG 22048^T; 4, *D. lusatiensis* DSM 11099^T (data from Fritsche *et al.*, 1999). +, Positive; −, negative; W, weak; ND, no data.

Characteristic	1	2	3	4
Cell shape	Short rods	Rods	Rods	Short rods
Motility	+	−	+	+
Growth at/in:				
4 °C	+	ND	−	−
40 °C	−	ND	+	+
pH 9	−	ND	+	+
5 % NaCl	+	−	−	ND
Denitrification	−	−	+	−
Urease	+	−*	−*	−
Aesculin hydrolysis	+	+*	−*	−
β-Galactosidase	+	+*	−*	ND
Assimilation of:				
Mannose	+	−*	+	+
Mannitol	+	−*	+	−
N-Acetylglucosamine	−	−*	+*	+
Gentiobiose	−	W*	+*	−
Citrate	−	W*	+*	−

*Data from this study using the API 20NE system.

Description of *Hoeflea marina* sp. nov.

Hoeflea marina (ma.ri′na. L. fem. adj. *marina* of the sea, marine, referring to the isolation source of this microorganism, sea water).

In addition to the properties listed in the genus description, the following properties are reported. Colonies on nutrient agar are circular, convex, white–cream, opaque and usually 1–3 mm in diameter, within 7 days at 28 °C. Cells are short rods of 0·7–0·9 × 1·1–1·4 μm. Optimal growth occurs at 3 % NaCl. Optimal growth temperature and pH are 28 °C and 7, respectively. Does not produce arginine

dihydrolase, and hydrolysis of gelatin is weak and slow. Utilizes glucose, L-arabinose, mannose, mannitol, maltose and malate, but not *N*-acetylglucosamine, gentiobiose, caproate, adipate, citrate or phenylacetate. Fatty acids are listed in Table 1.

The type strain, and so far the only strain, is LMG 128^T (=ATCC 25654^T), which was isolated from the Baltic Sea, off the coast of Germany. The DNA G+C content is 53·1 mol%.

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