Hoeflea phototrophica sp. nov., a novel marine aerobic alphaproteobacterium that forms bacteriochlorophyll *a*

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Within a collection of marine strains that were shown to contain the photosynthesis reaction-centre genes *pufL* and *pufM*, a novel group of alphaproteobacteria was found and was characterized phenotypically. The 16S rRNA gene sequence data suggested that the strains belonged to the order *Rhizobiales* and were closest (98·5 % sequence similarity) to the recently described species *Hoeflea marina*. The cells contained bacteriochlorophyll *a* and a carotenoid, presumably spheroidenone, in small to medium amounts. Cells of the novel strains were small rods and were motile by means of single polarly inserted flagella. Good growth occurred in complex media with 0·5–7·0 % sea salts, at 25–33 °C (optimum, 31 °C) and at pH values in the range 6–9. With the exception of acetate and malate, organic carbon sources tested supported poor growth or no growth at all. Growth factors were required; these were provided by small amounts of yeast extract, but not by standard vitamin solutions. Growth occurred under aerobic to microaerobic conditions, but not under anaerobic conditions, either in the dark or light. Nitrate was not reduced. Photosynthetic pigments were formed at low to medium salt concentrations, but not at the salt concentration of sea water (3·5 %). On the basis of smaller cell size, different substrate utilization profile and photosynthetic pigment content, the novel strains can be classified as representatives of a second species of *Hoeflea*, for which the name *Hoeflea phototrophica* sp. nov. is proposed. The type strain of *Hoeflea phototrophica* sp. nov. is DFL-43^T (=DSM 17068^T=NCIMB 14078^T).

Bacterial photosynthesis appears to contribute, to some extent, to the energy generation of heterotrophs in the open oceans. Kolber *et al.* (2001) calculated, by extrapolation from measurements of bacteriochlorophyll *a* in tropical seas, that up to 10 % of the bacterioplankton were potentially capable of photosynthesis. In a recent survey, Schwalbach & Fuhrman (2005) quantified aerobic anoxygenic phototrophs (AAPs) by means of epifluorescence microscopy and quantitative PCR and found them to constitute 1–2 % of all bacteria in the euphotic zone off the coast of Southern California. In estuarine waters, it has been estimated that AAPs constitute >10 % of total bacteria. We have recently isolated a large number of pigmented strains from different habitats of the North Sea and checked them for the presence of genes for the photosynthetic apparatus, namely the *pufL* and *pufM* genes, which code for proteins of the photosynthetic reaction centre (Allgaier *et al.*, 2003). The 16 strains that were positive for these genes were classified into five phylogenetic groups on the basis of their 16S rRNA gene sequences. None of them could be assigned to an existing species.

Here we describe a group consisting of five strains that belong to the α-2 subgroup of the *Proteobacteria* and which initially showed the greatest level of 16S rRNA gene sequence similarity with *Ahrensia kielensis* (Ahrens, 1968; Uchino *et al.*, 1998). However, after our phenotypic characterization of these novel strains was completed, the first description of the micro-organism *Hoeflea marina* was published by Peix *et al.* (2005). On the basis of 16S rRNA gene sequences, the novel strains were found to be more closely related to *H. marina* than to *A. kielensis*. Both *A. kielensis* and *H. marina* were originally described by Ahrens (1968) as marine *Agrobacterium* species forming star-shaped

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The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain DFL-43^T is AJ582088.

The phylogenetic position of strains DFL-43^T and DFL-44 within the α-2 subgroup of the *Alphaproteobacteria* is shown in a supplementary figure available in IJSEM Online.

aggregates and were subsequently reclassified (Rüger & Höfle, 1992; Uchino *et al.*, 1998; Peix *et al.*, 2005). In contrast to related genera and species, the novel strains in this study were able to form photosynthetic pigments, in particular bacteriochlorophyll *a*, if appropriate conditions were provided.

The isolates were obtained from cultures of marine dinoflagellates. Three strains (DFL-13, DFL-33 and DFL-44) were from *Alexandrium lusitanicum* ME207 and two strains (DFL-42 and DFL-43^T) were from *Prorocentrum lima* ME130. Both cultures were maintained in the dinoflagellate collection of the Biological Institute of the island of Helgoland (German Bight). Single algal cells were washed and plated onto agar plates prepared with 10-fold-diluted Difco marine broth 2216. Strains DFL-43^T and DFL-44 were selected for further characterization.

Colonies of surface cultures were light-beige on full-strength marine broth 2216 (Difco) and wine-red on 10-fold-diluted marine broth. They were of a smooth consistency, relatively flat and exhibited an opaque centre and a translucent halo. The cells were small, short, rods, 0·3–0·5 × 0·7–2·0 μm (Fig. 1c) and showed rapid movement. Electron micrographs of shadow-cast cells of strain DFL-43^T showed monotrichous flagellation at one or both poles (Fig. 1a). In strain DFL-43^T, distinct capsules were visible around the cells. Ultrathin sections revealed a typical Gram-negative cell-wall structure (Fig. 1b).

For most of the physiological tests, a complex medium was used consisting of (l^{-1}) 20 g sea salts, 3 g peptone and 0·5 g yeast extract. If necessary, it was replaced by a mineral medium with sodium acetate as the carbon source (Biebl *et al.*, 2005). The temperature range for growth was determined by using a temperature-gradient shaking incubator (Toyo Kogaku Sangyo) that allowed growth to be followed between 15 and 45 °C (at increments of 3 °C) through periodic measurement of the optical density (600 nm). Good growth was found between 25 and 33 °C, the optimum being at 31 °C. At 15 °C, the growth rate was only 1/5th of the maximum rate. No growth occurred above 35 °C. The novel strains were able to grow at pH values between 5·8 and 9·5. Between pH 6·0 and 9·0, initial culture development was almost the same. Sea salts were required at a concentration of at least 0·5 %; concentrations up to 7 % were tolerated.

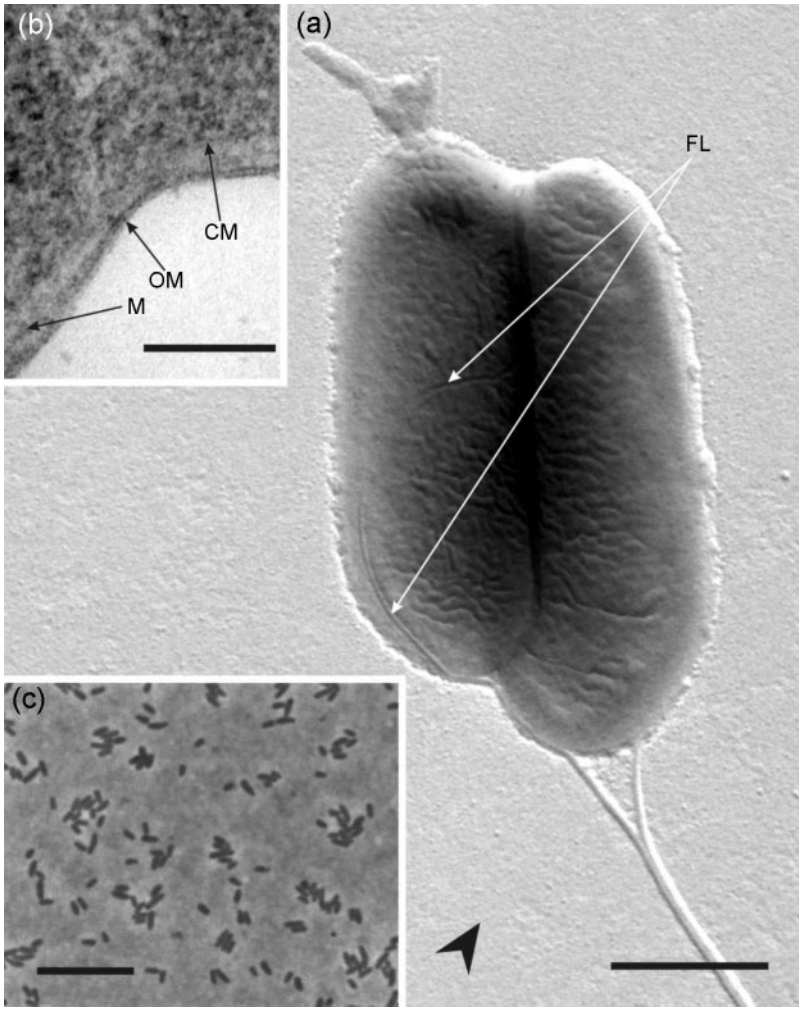

Fig. 1. Morphological features of strain DFL-43^T. (a) Shadow-cast electron microscopic preparation showing a cell with a monotrichous monopolar flagellum (FL) insertion. The arrowhead indicates the shadowing direction. (b) The Gram-negative cell wall shows an outer membrane (OM) and peptidoglycan as a thin line (M). CM, cytoplasmic membrane. (c) Phase-contrast photomicrograph of cells mounted on an agar surface. Bars: 500 nm (a), 100 nm (b) and 10 μm (c).

The utilization of carbon sources was checked in a mineral sea-water medium containing 0·1 g yeast extract l^{-1} to provide the required growth factors. The following carbon sources were tested at a concentration of 1 g l^{-1} (acids as sodium salts): acetate, butyrate, succinate, fumarate, malate, lactate, citrate, glutamate, pyruvate, glucose, fructose, ethanol, methanol, glycerol and yeast extract. Moderate growth was obtained only with yeast extract and with acetate or malate. The other substrates tested allowed only limited growth, with OD_{600} values ranging from 20 (glucose) to 60 % (fumarate, citrate) of that of the acetate culture; methanol and ethanol did not allow any growth at all. In this respect, the novel strains resemble the type strain of *A. kielensis*, which did not use any of the carbon sources tested (Rüger & Höfle, 1992). *H. marina* was found to use a series of sugars and sugar alcohols, including glucose (Peix *et al.*, 2005). Experiments involving culture of the cells with acetate as the substrate and several additives, e.g. yeast extract (0·1 g l^{-1}), vitamin-free and vitamin-containing Casamino acids (0·25 g l^{-1}; Difco) as well as a vitamin solution (consisting of biotin, thiamine, nicotinic acid, pantothenic acid, vitamin B_{12}, pyridoxine and 6-aminobenzoic acid), showed that growth factors were required. These growth factors were provided by yeast extract, but not by any of the administered vitamins and amino acids.

The novel strains were unable to decompose or liquefy any of the following polymers: starch, alginate, gelatin and Tween 80 (for lipase activity). They were positive for catalase and oxidase activity, did not form indole from tryptophan and were unable to form nitrite and nitrogen from nitrate under air-exclusion conditions. Antibiotic inhibition was observed with penicillin G, tetracycline and chloramphenicol, but not with polymyxin B. Anaerobic growth by the fermentation of glucose was not observed, but there was some sensitivity to full oxygen exposure, as the growth zone in agar deep culture was distinctly below the surface. No growth occurred under anaerobic conditions in the light when acetate was the substrate.

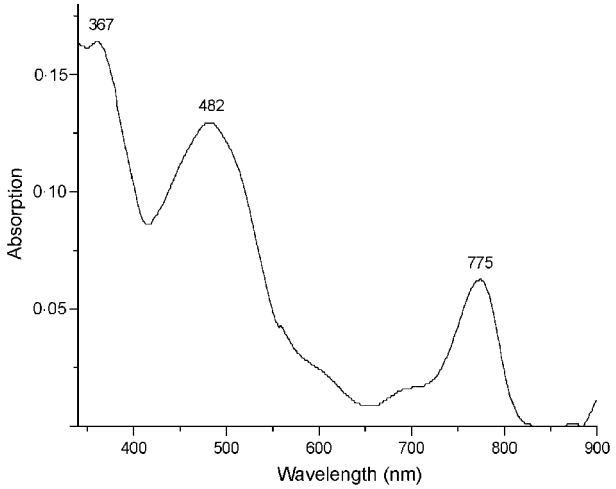

Fig. 2. Absorption spectrum of the acetone/methanol (7 : 2) extract of strain DFL-43^T, showing the maxima for bacteriochlorophyll *a* (367 and 775 nm) and a carotenoid (probably spheroidenone).

In several aerobic phototrophic bacteria (exclusively freshwater organisms), reduction of toxic potassium tellurite to inert elemental tellurium (deposited in the cytoplasm) has been observed (Yurkov *et al.*, 1996). Strains DFL-43^T and DFL-44 also possessed this capability. After 4 days growth in peptone medium to which 0·05–1 g l^{-1} potassium tellurite had been added, cultures turned jet-black and refractile inclusions were visible in the cells.

Cellular fatty acids were determined as described by Labrenz *et al.* (1998). The percentages of fatty acids found in strain DFL-43^T and in *H. marina* and *A. kielensis* are shown in

Table 1. Cellular fatty acid content (%) of strain DFL-43^T in comparison with those of *H. marina* DSM 16791^T and *A. kielensis* DSM 5980^T

Fatty acid values of less than 1 % are not shown.

Fatty acid	DFL-43^T	*H. marina* DSM 16791^T	*A. kielensis* DSM 5980^T
12 : 0 3-OH			1·0
16 : 0	6·3	4·0	1·0
16 : 1ω7	1·6	2·6	
18 : 0	1·2	1·4	5·3
18 : 1ω7	62·8	76·0	85·0
18 : 1ω7 11Me	20·6	7·5	3·8
19 : 0 cyclo	1·6	5·6	
19 : 1	3·4		
20 : 0			1·6
Unknown	2·4		

Table 2. Growth and specific bacteriochlorophyll *a* content of strains of *Hoeflea phototrophica* sp. nov. in cultures grown at different concentrations of sea salts

Sea salts (g l^{-1})	OD_{600}		Bacteriochlorophyll *a* (nmol mg protein^{-1})	
	DFL-43^T	DFL-44	DFL-43^T	DFL-44
3	0·31	0·36	0·26	0·24
6	0·65	0·48	0·89	0·61
9	0·66	0·54	0·70	0·53
35	0·53	0·59	0·00	tr

Table 1. Strain DFL-44 had almost the same values as strain DFL-43^T. As is usually the case for the *Alphaproteobacteria*, the mono-unsaturated straight chain acid 18 : 1ω7 was the major component (63–85 %), replaced, in part, by the methylated form, particularly in strain DFL-43^T (21 %). Strain DFL-43^T, *H. marina* and *A. kielensis* all contained the saturated acids 16 : 0 and 18 : 0, albeit in small amounts, whereas the fatty acids 16 : 1ω7 and 19 : 1 cyclo were found only in strain DFL-43^T and in *H. marina*. The type strain of *A. kielensis* contained a hydroxylated 12 : 0 acid and a 20 : 0 acid that were not found in either strain DFL-43^T or *H. marina*. Polar lipids were extracted and separated by TLC according to Tindall (1990). For strain DFL-43^T, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylmonomethylethanolamine were the predominant polar lipids. Moderate amounts of phosphatidylcholine and sulfoquinovosyldiacetylglycerol were present and only minor amounts of diphosphatidylglycerol and an unknown amino lipid were found. Interestingly, *A. kielensis* contained the same lipids, albeit in somewhat different proportions; in particular, the amount of phosphatidylethanolamine was much lower. *H. marina* differed from the other strains in that it lacked phosphatidylcholine.

Distinct pigmentation was observed on 10-fold-diluted marine agar 2216, but not on the original medium containing (l^{-1}) 5 g peptone, 1 g yeast extract and about 30 g salts. Liquid cultures containing (l^{-1}) 3 g peptone and 0·5 g yeast extract and varied amounts of sea salts revealed that pigment production was dependent on the salt concentration. Cultures containing 3, 6 and 9 g sea salts l^{-1} appeared very pink, while cultures at the salt concentration of natural sea water were colourless. The cell mass from 30 ml culture grown at 6 g sea salts l^{-1} was extracted with 3 ml acetone/methanol (7 : 2) and the absorption spectrum of the extract was recorded (Fig. 2). It showed the typical maxima of bacteriochlorophyll *a* at 367 and 775 nm and a high peak at 482 nm, indicating the presence of a carotenoid. Table 2 shows the specific bacteriochlorophyll *a* content for both novel strains at the sea salts concentrations indicated. The *in vivo* absorption spectrum obtained by suspension of cells in 75 % glycerol showed only weak absorption in the infrared region because of the low pigment content, but peaks at around 800 and 865 nm were recognizable (not shown). It can be inferred that the carotenoid peak (at 482 nm) of the solvent extract originates from spheroidenone. The peak was almost identical to that of *Dinoroseobacter shibae* in which the carotenoid has been identified as spheroidenone (Biebl *et al.*, 2005).

To identify the closest phylogenetic neighbours of strain DFL-43^T, 16S rRNA sequences were manually aligned and compared with published sequences from the 16S rRNA gene sequence database of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany), including sequences available from the Ribosomal Database Project (Maidak *et al.*, 2001) and EMBL. Sequences were aligned using the BioEdit program (Hall, 1999). A phylogenetic dendrogram was inferred using DNADIST and the neighbour-joining method of the PHYLIP package (Felsenstein, 1993). Bootstrap analysis was based on 1000 resamplings.

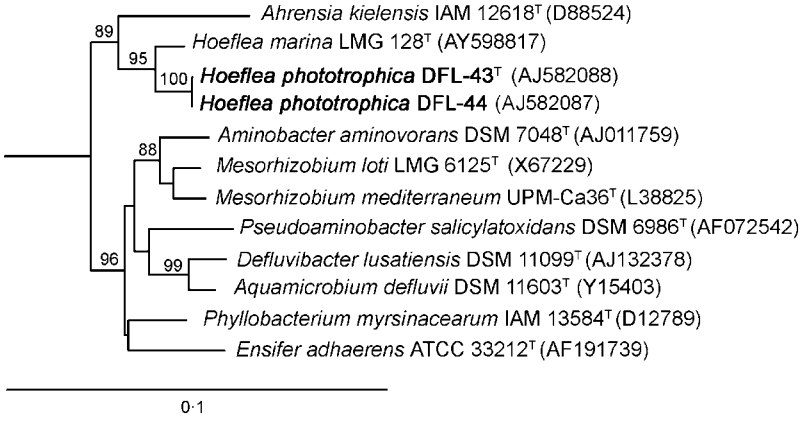

Fig. 3. Neighbour-joining dendrogram based on 16S rRNA gene sequences showing the phylogenetic position of *Hoeflea phototrophica* DFL-43^T in the context of related genera in the α-2 subgroup of the *Proteobacteria*. Bootstrap values with greater than 60 % confidence (percentage of 1000 resamplings) are shown at the branching points. GenBank accession numbers are given in parentheses. Bar, 10 substitutions per 100 nt.

Table 3. Characteristics of *Hoeflea phototrophica* sp. nov. strains DFL-43^T and DFL-44 that differentiate them from their closest phylogenetic neighbours

Species: 1, *H. phototrophica* strains DFL-43^T and DFL-44 (data from this study); 2, *H. marina* DSM 16791^T (Peix *et al.*, 2005); 3, *A. kielensis* DSM 5890^T (partly from Ahrens, 1968 and Rüger & Höfle, 1992). +, Growth; (+), poor growth; −, no growth; NO, not observed.

Characteristic	1	2	3
Mean cell size (μm)	0·4 × 1·3	0·8 × 1·2	0·6 × 2·0
Flagellation	Monotrichous	NO	Peritrichous
Bacteriochlorophyll *a*	+	−	−
Sea salts required for growth	+	−	(+)
Use of single organic compounds	(+)	+	−
Use of glucose	−	+	−
Presence of phosphatidylcholine	+	−	+
DNA G+C content (mol%)	59·3	53·1	48

Even though the first variable region of the 16S rRNA gene sequences of strains DFL-43^T and DFL-44 was more closely related to that of *A. kielensis*, the almost-complete 16S rRNA gene sequences of the novel strains revealed them to be most closely related to *H. marina*. The phylogenetic position of strain DFL-43^T as a novel member within the family *Phyllobacteriaceae* is shown in Fig. 3. Its phylogenetic position within the α-2 subgroup of the *Alphaproteobacteria* is shown in Supplementary Fig. S1, available in IJSEM Online.

The major phenotypic differences between strains DFL-43^T and DFL-44 and the species with greatest similarity in terms of 16S rRNA gene sequence, i.e. *H. marina* and *A. kielensis*, are listed in Table 3. Although the 16S rRNA gene sequence comparisons indicate that the novel strains are very close to *H. marina* (98·5 % similarity) and more distant to *A. kielensis* (94·4 % similarity), there are marked phenotypic differences between the novel strains and both of these recognized species and these are also reflected in the wide range of DNA G+C contents. Strains DFL-43^T and DFL-44 are distinguished from *H. marina* and *A. kielensis* by possessing bacteriochlorophyll *a* and carotenoids, possibly enabling them to generate additional energy from light. In addition, they differ from *H. marina* by their smaller cell size, by their inability to grow well in mineral media with defined organic compounds and by the presence of phosphatidylcholine. In contrast to *A. kielensis*, which has peritrichous flagella, the novel strains described here show monotrichous flagellation. With respect to cell size, substrate utilization and polar lipids, however, the novel strains are more similar to *A. kielensis* than to *H. marina*. On the other hand, the cellular fatty acid content of strains DFL-43^T and DFL-44 is consistent with a closer relationship to *H. marina* than to *A. kielensis*. In summary, the molecular data strongly suggest an affiliation of the investigated strains to the genus *Hoeflea*. The clear morphological and physiological differences, as well as the presence of photosynthesis reaction-centre genes and pigments, indicate that the novel isolates are representatives of a separate species, for which the name *Hoeflea phototrophica* sp. nov. is proposed. The type strain is DFL-43^T (=DSM 17068^T=NCIMB 14078^T).

Description of *Hoeflea phototrophica* sp. nov.

Hoeflea phototrophica (pho.to′tro.phi.ca. Gr. n. *phos photos* light; Gr. adj. *trophikos* nursing, tending or feeding; N.L. fem. adj. *phototrophica* referring to the likely ability to use light for energy generation).

Cells are small rods, 0·3–0·5 × 0·7–2·0 μm in size and are motile by means of single, polarly inserted flagella. Colonies grown on marine agar 2216 are smooth, flat and may form an opaque centre and a translucent halo. They are colourless to slightly beige if grown in the light. Cultures require microaerobic growth conditions, but do not grow anaerobically. Growth occurs at concentrations of sea salts from 0·5–7·0 %, at temperatures of up to 33 °C (optimum, 31 °C) and at pH values in the range 6–9. Acetate and malate are good growth substrates, whereas succinate, fumarate, lactate, citrate, glutamate, pyruvate, glucose, fructose and glycerol allow only poor growth; ethanol and methanol are not used. Yeast extract is required for growth. Gelatin, starch, alginate and Tween 80 are not decomposed. Nitrate is not reduced to nitrite or nitrogen. Indole is not formed from tryptophan. Cells contain bacteriochlorophyll *a* and a carotenoid, probably spheroidenone, in small to medium amounts. The DNA G+C content of the type strain is 59·3 mol%.

The type strain, DFL-43^T (=DSM 17068^T=NCIMB 14078^T), was isolated from a culture of *Prorocentrum lima* (dinoflagellates).

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