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Hoeflea phototrophica, nov. sp., a new marine aerobic *Alphaproteobacterium* that forms bacteriochlorophyll *a*

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Running title: *Hoeflea phototrophica*, sp. nov.

Subject category: New taxa (*Proteobacteria*)

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Summary

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 phenotypically characterized. The 16S rDNA gene sequence data suggested that the strains belonged to the order *Rhizobiales* closest to the recently described species *Hoeflea marina* (98.5 % similarity). The cells contained bacteriochlorophyll *a* and a carotenoid, presumably spheroidenone, in small to medium amounts. They were small rods and motile by means of single polarly inserted flagella. Good growth occurred in complex media with 0.5 to 7 % sea salts, 25 to 35°C (optimum 31°C) and in a pH range of 6 to 9. With the exception of acetate and malate, the organic carbon sources tested supported poor or no growth. Growth factors were required; they were provided by small amounts of yeast extract, but not by standard vitamin solutions. Growth occurred under aerobic to microaerobic atmospheres but not under anaerobic conditions, neither in the dark nor in the light. Nitrate was not reduced. Photosynthetic pigments were formed at low to medium salt concentration but not at seawater concentration (3.5 %).

Due to a smaller cell size, different substrate utilization and photosynthetic pigment content it is proposed to classify the strains as a second species of *Hoeflea, Hoeflea phototrophica* sp. nov.. The type strain of *Hoeflea phototrophica* is DFL-43^T(= DSM 17068^T = NCIMB 14078^T).

Main text

Bacterial photosynthesis appears to contribute to some extent to the energy generation of heterotrophs in the open oceans. Kolber et al. (2001) have 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 epifluorescence microscopy and quantitative PCR and found them to constitute 1 - 2 % of total bacteria in the euphotic zone off the coast of Southern California. Only in estuarine waters estimates >10% were observed. We have recently isolated a large number of pigmented strains from different habitats of the North Sea and checked them for genes of the photosynthetic apparatus, namely the *puf*L and the *puf*M 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 using 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 subclass of the *Proteobacteria* and initially showed closest 16S rRNA sequence relationship with *Ahrensia kieliensis* (Ahrens, 1968; Uchino et al., 1998). After the completion of their phenotypical characterization, *Hoeflea marina* (Peix et al., 2005) was described that was more closely related according to 16S rRNA gene sequence. Both *Ahrensia kieliensis* and *Hoeflea marina* were originally described by Ahrens (1968) as marine *Agrobacterium spp.* forming star-shaped aggregates and later reclassified (Rüger and Höfle, 1992; Uchino et al., 1998; Peix et al., 2005). In contrast to the related genera and

73 species, the strains to be treated here were able to form photosynthetic pigments, in
74 particular bacteriochlorophyll *a*, if appropriate conditions were provided.
75
76 The isolates were obtained from cultures of marine dinoflagellates, three strains (DFL-
77 13, DFL-33 and DFL-44) from *Alexandrium lusitanicum* ME207 and two strains (DFL-42
78 and DFL-43^T) from *Prorocentrum lima* ME130. Both cultures were maintained in the
79 dinoflagellate collection of the Biological Institute of the island of Helgoland (German
80 Bight). Single algal cells were washed and plated onto agar plates prepared with 10 fold
81 diluted Difco Marine Broth 2216. DFL-43^T and DFL-44 were selected for further charac-
82 terization.
83
84 The colonies of the surface cultures were weakly beige on full-strength Marine Broth
85 2216 and wine-red on tenfold diluted Marine Broth. They were of a smooth consistency,
86 relatively flat and exhibited an opaque center and a translucent halo. Cells were small
87 short rods of 0.3-0.5 x 0.7-2.0 µm (Fig.1, lower insert) and showed rapid movement.
88 Electron micrographs of shadow-cast cells of DFL-43^T showed monotrichous flagellation
89 at one pole or both (Fig. 1). In strain DFL-43^T distinct capsules were visible around the
90 cells. Ultrathin sections revealed a typical gram-negative cell-wall structure (Fig. 1, up-
91 per insert).
92
93 For most of the physiological tests a complex medium was used consisting of 20 g sea
94 salts, 3 g peptone and 0.5 g yeast extract per liter. If required it was replaced by a min-
95 eral medium with Na-acetate as carbon source (Biebl et al., 2005). The temperature
96 range for growth was determined by use of a temperature gradient shaking incubator

(Toyo Kogaku Sangyo Co. Ltd., Tokyo) that allowed growth to be followed between 15 and 45 °C at intervals of 3 °C by 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 1/5 of the maximum rate, beyond 33 °C no growth occurred. The 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 equal. Sea salts were required at a concentration of at least 0.5 %; up to 7 % were tolerated.

Utilization of carbon sources was checked in a mineral seawater medium containing 0.1 g/l of yeast extract to provide required growth factors. The following carbon sources were tested at a concentration of 1 g/l (acids as sodium salts): Acetate, butyrate, succinate, fumarate, malate, lactate, citrate, glutamate, pyruvate, glucose, fructose, ethanol, methanol, glycerol and yeast extract. Fair growth was only obtained with yeast extract and with acetate and malate. The other substrates allowed only limited growth with optical densities ranging from 20 (glucose) to 60 % (fumarate, citrate) of the acetate culture; methanol and ethanol did not enable growth at all. In this respect the strains resemble the type strain of *Ahrensia kieliensis* which did not use any of the carbon sources offered (Rüger and Höfle, 1992), while *Hoeflea marina* was found to use a series of sugars and sugar alcohols including glucose (Peix et al., 2005). Experiments to culture the cells with acetate as substrate and several additives, e.g. 0.1 g/l yeast extract, 0.25 g/l vitamin-free and vitamin-containing casaminoacids (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. They were provided by yeast extract but not by any of the administered vitamins and

amino acids.

The 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, did not form indole from tryptophane and were unable to form nitrite and nitrogen from nitrate under exclusion of air. Antibiotic inhibition was observed with penicillin G, tetracyclin and chloramphenicol, but not with polymyxin B. Anaerobic growth by 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. In several aerobic phototrophic bacteria (exclusively freshwater organisms), reduction of toxic potassium tellurite to inert elemental tellurium that is deposited in the cytoplasm has been found (Yurkov et al., 1996). Strains DFL-43^T and DFL-44 also possessed this capability. After four days of growth in peptone medium cultures amended with potassium tellurite in concentrations between 0.05 and 1 g/l turned jet-black, and refractive inclusions were visible in the cells.

Cellular fatty acids were determined as described by Labrenz et al. (1998). The percentage of the acids found in DFL-43^T as well as in *Hoeflea marina* and *Ahrensia kieliensis* is shown in table 1. DFL-44 gave almost the same values as DFL-43^T. As usually found in *Alphaproteobacteria* the mono-unsaturated straight chain acid 18:1ω7 was the major component (63 to 85 %) replaced in part by the methylated form, particularly in strain DFL-43^T (21 %). Common to all three strains, but in minor amounts, were also the saturated acids 16:0 and 18:0, whereas 16:1ω7 and 19:1 cyclo were only

found in DFL-43^T and in *Hoeflea marina*. The *Ahrensia kieliensis* strain contained a hydroxylated 12:0 and a 20:0 acid and was thus more different from the other two strains. Polar lipids were extracted and separated by thin layer chromatography according to Tindall (1990). In DFL-43^T extracts phosphatidylglycerol, phosphatidylethanolamine and phosphatidylmonomethylethanolamine were present at highest levels, phosphatidylcholine and sulfoquinovosyldiacetylglycerol at intermediate levels and diphosphatidylglycerol and an unknown amino lipid in minor amounts. Interestingly, *Ahrensia kieliensis* contained the same lipids although in somewhat different proportions; in particular the amount of phosphatidylethanolamine was much lower. *Hoeflea marina* differed in lacking phosphatidylcholine.

Distinct pigmentation was observed on tenfold diluted marine agar 2216 (Difco), but not on the original medium containing 5 g peptone, 1 g yeast extract and about 30 g of salts per liter. Liquid cultures with 3 g peptone and 0.5 g yeast extract per liter and varied amounts of sea salts revealed that pigment production was dependent on the salt concentration. Cultures at 3 g, 6 g and 9 g/l appeared strongly pink, while cultures at the concentration of natural seawater salt were colourless. The cell mass from 30 ml of a culture grown at 6 g sea salts per liter was extracted with 3 ml acetone-methanol (7:2), and the absorption spectrum was recorded from the extract (Fig. 2). It showed the typical maxima of bacteriochlorophyll *a* at 367 and 775 nm and a high peak at 482 nm which indicates the presence of a carotenoid. Table 2 shows the specific bacteriochlorophyll *a* content for both strains at the sea salt concentrations indicated. The in-vivo absorption spectrum obtained by suspension of cells in 75% glycerol showed only weak absorption in the infrared region due to the low pigment content, but peaks

around 800 and 865 nm were recognizable (not shown). It can be inferred that the carotenoid peak of the solvent extract at 482 nm originates from spheroidenone. It is almost identical to that of *Dinoroseobacter shibae*, in which the carotenoid has been identified (Biebl et al., 2005).

For determination of the nearest phylogenetic neighbors to DFL-43^T, 16S rRNA sequences were manually aligned and compared with published sequences from the DSMZ 16S rDNA database, including sequences available from the Ribosomal Database Project (Maidak et al., 2001) and EMBL. Sequences were aligned in the BioEdit programm (Hall, 1999). A phylogenetic dendrogram was inferred using DNADIST and the neighbor joining method of the Phylip package (Felsenstein, 1993). Bootstrap analysis was based on 1000 resamplings.

Even though the first variable region of the 16S rDNA sequence from strains DFL-43^T and DFL-44 was more closely related to that of *Ahrensia kieliense*, the nearest phylogenetic neighbor of the strains was identified as *Hoeflea marina*, based on analysis of the nearly complete 16S rDNA molecule. The phylogenetic position of *Hoeflea phototrophica* sp. nov. DFL-43^T as a new member within the family "*Phyllobacteriaceae*" is shown in Fig. 3. The Fig. A in supplementary information shows its phylogenetic position among the α-2 subdivision of the *Alphaproteobacteria*.

The major phenotypic differences of the new species to the species with greatest similarity in the 16S rRNA gene sequence, *Hoeflea marina* and *Ahrensia kieliensis*, are listed in table 3. Although according to the nucleotide base differences the new species is very close to *H. marina* (98.4 %) and more distant to *A. kieliensis* (94.4 %) there are

marked phenotypical differences to both of these species which are also reflected by the wide range of the guanine + cytosine content. DFL-43^T and DFL-44 are distinguished by possessing bacteriochlorophyll *a* and carotenoids possibly enabling them to generate additional energy from light. In addition they differ from *H. marina* by smaller cell size, by the inability to grow well in mineral media with defined organic compounds and by the presence of phosphatidylcholine. In contrast to *A. kieliensis* which has peritrichous flagella, the strains described here show monotrichous flagellation, but with respect to cell size, substrate utilization and polar lipids they correspond more to *A. kieliensis* than to *H. marina*. On the other hand the cellular fatty acid composition is consistent with a closer relationship of DFL-43^T and DFL-44 strains with *Hoeflea marina* than with *A. kieliensis*. In summary the molecular data strongly suggest an affiliation of the investigated strains to the new genus *Hoeflea*. The clear morphological and physiological differences as well as the presence of photosynthesis reaction center genes and pigments require classification in a separate species.

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. p*hototrophica,* referring to the likely ability to use light for energy generation). Cells are small rods, 0.3 -0.5 x 0.7-2.0 µm and motile by means of single, polarly inserted flagella. Colonies grown on Marine Agar 2216 (Difco) are smooth, flat and may form an opaque center 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 sea salt concentrations from 0.5 to 7 %, at temperatures up to 36°C (optimum 31°) and at a pH from 6 to 9. Acetate and malate are good growth substrates, 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 tryptophane. Cells contain bacteriochlorophyll *a* and a carotenoid, probably spheroidenone, in small to medium amounts. The G+C content of the type strain is 59.3 %. The type strain is DFL-43^T and was deposited with the German Collection of Microorganisms and Cell Cultures DSMZ as DSM 17068 and with the National Collection of Industrial, Marine and Food Bacteria as NCIMB 14078. It was isolated from a culture of *Prorocentrum lima* (dinoflagellates).

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References

Ahrens, R. (1968). Taxonomische Untersuchungen an sternbildenden *Agrobacterium*-Arten aus der westlichen Ostsee. *Kieler Meeresforsch* **24**, 147-173.

Allgaier, M., Uphoff, H., Felske, A. & Wagner-Döbler, I. (2003). Aerobic anoxygenic photosynthesis in *Roseobacter* clade bacteria from diverse marine habitats. *Appl Environ Microbiol* **69**, 5051-5059.

Biebl, H., Tindall, B. J., Koblizek, M., Lünsdorf, H., Pukall, R. & Wagner-Döbler, I. (2005). *Dinoroseobacter shibae*, gen. nov., sp. nov.., a new aerobic phototrophic bacterium isolated from dinoflagellates. *Int J Syst Evol Microbiol* **55**,1089-1096.

Felsenstein, J. (1993). Phylip (phylogenetic inference package) version 3.5.1., distributed by the author. Department of Genetics, University of Washington, Seattle, WA, USA.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* **41**, 95-98.

Kolber, Z. S., Plumley, F. G., Lang, A. S., Beatty, J. T., Blankenship, R. E., VanDover, C. L., Vetrani, C., Koblizek, M., Rathgeber, Ch. & Falkowski, P. G. (2001). Contribution of aerobic photoheterotrophic bacteria to the carbon cycle in the ocean. *Science* **292**, 2492-2495.

Labrenz, M., Collins, M. D., Lawson, P. A., Tindall, B. J., Schumann, P. & Hirsch, P. (1998). *Antarctobacter heliothermus* gen. nov., sp. nov., a budding bacterium from hypersaline and heliothermal Ekho Lake. *Int J Syst Bacteriol* **48**, 1363-1372.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C.T. Jr., Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M. & Tiedje, J. M. (2001). The RDP-II (Ribosomal Database Project). *Nucl Acids Res* **29**, 173-174.

Peix, A., Rivas, R., Trujillo, M. E., Vancanneyt, M., Velazquez, E. & Willems, A. (2005). Reclassification of *Agrobacterium ferrugineum* LMG 128 as *Hoeflea marina* gen. nov., sp. nov.. *Int J Syst Evol Microbiol* **55**:1163-6.

Rüger, H.-J. & Höfle, M. (1992). Marine star-shaped-aggregate-forming bacteria: *Agrobacterium atlanticum* sp. nov.; *Agrobacterium meteori* sp. nov.; *Agrobacterium ferrugineum* sp. nov., nom rev.; *Agrobacterium gelatinovorum* sp. nov., nom. rev.; and *Agrobacterium stellulatum* sp. nov., nom. rev.. *Int J Syst Bacteriol* **42**, 133-143.

Schwalbach, M. S. & Fuhrman, J. A. (2005). Wide-ranging abundance of aerobic anoxygenic phototrophic bacteria in the world ocean revealed by epifluorescence microscopy and quantitative PCR. *Limnol Oceanogr* **50**, 620-628.

Tindall, B. J. (1990). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199-202.

Uchino, Y., Hirahata, A., Yakota, A. & Sugiyama, J. (1998). Reclassification of marine *Agrobacterium species*: Proposals of *Stappia stellulata* gen. nov., comb. nov., *Stappia aggregata* sp. nov., nom. rev., *Ruegeria atlantica* gen. nov., comb. nov., *Ruegeria ge-*

latinovora comb. nov., *Ruegeria algicola* comb. nov., and *Ahrensia kieliensis* gen. nov., sp. nov., nom. rev.. *J Gen Appl Microbiol* **44**, 201-210.

Yurkov, V., Jappé, J. & Verméglio, A. (1996). Tellurite resistance by obligately aerobic photosynthetic bacteria. *Appl Environ Microbiol* **62**: 4195-4198.

Legends to the figures

Fig. 1. Morphological features of strain DFL-43^T. The shadow cast electron microscopical preparation shows a cell with monotrichous monopolar flagellum (fl) insertion. The gram-negative cell wall (upper insert) shows an outer membrane (OM) and peptidoglycan as a thin line (m), cm = cytoplasma membrane, the arrow head marks the shadowing direction. Lower insert: Phase-contrast photomicrograph of cells mounted to an agar surface.

Fig. 2. Absorption spectrum of the acetone-methanol (7:2) extract showing the maxima of bacteriochlorophyll a (367 and 775 nm) and a carotenoid, probably spheroidenone.

Fig. 3 Neighbor joining dendrogram of 16S rRNA gene relatednesss showing the phylogenetic position of *Hoeflea phototrophica* DFL-43^T in the context of related genera in the α2-subclass of the *Proteobacteria*. Bootstrap values greater than 60 % confidence are shown at branching points (percentage of 1000 resamplings). The scale bar represents 10 substitutions per 100 nucleotides. Sequence accession numbers are given in parentheses.

Fig. A (supplementary material). Neighbor joining dendrogram of 16S rRNA gene relatednesss showing the phylogenetic position of *Hoeflea phototrophica* DFL-43^T within the α-subclass of the *Proteobacteria*. Bootstrap values greater than 60 % confidence are shown at branching points (percentage of 1000 resamplings). The scale bar represents 10 substitutions per 100 nucleotides. Sequence accession numbers are given in parentheses.

Table 1. Cellular fatty acids of DFL-43^T in comparison to *Hoeflea marina* DSM 16791 and *Ahrensia kieliensis* DSM 5980

Fatty acid (%)	DFL-43^T	DSM 16791	DSM 5980
12:0 3OH			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 in cultures grown at different sea salt concentrations

Sea salt g/l	OD (600 nm)		Bacteriochlorophyll *a* (nmol/ mg protein)	
	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	trace

353 **Table 3.** Characteristics of DFL-43^T and DFL-44 which differentiate them from the near-
354 est phylogenetic neighbors.
355
356

	Hoeflea phototrophica DFL-43^T, DFL-44 DSM 17068	*Hoeflea marina* A43 DSM 16971[1])	*Ahrensia kieliense* B9 DSM 5890 [2])
Average cell size	0.4 x 1.3	0.8 x 1.2	0.6 x 2.0
Flagellation	monotrichous	n.o.	peritrichous
Bacteriochlorophyll *a*	+	-	-
Sea salts required	+	-	(+)
Use of single organic compounds	(+)	+	-
Use of glucose	-	+	-
Presence of phosphatidylcholine	+	-	+
G+C content (%)	59.3	53.1	48

357
358 (+) means: poor growth, n.o. not observed, [1]) from Peix et al. (2005), [2]) in part from
359 Ahrens (1968) and Rüger & Höfle (1992)

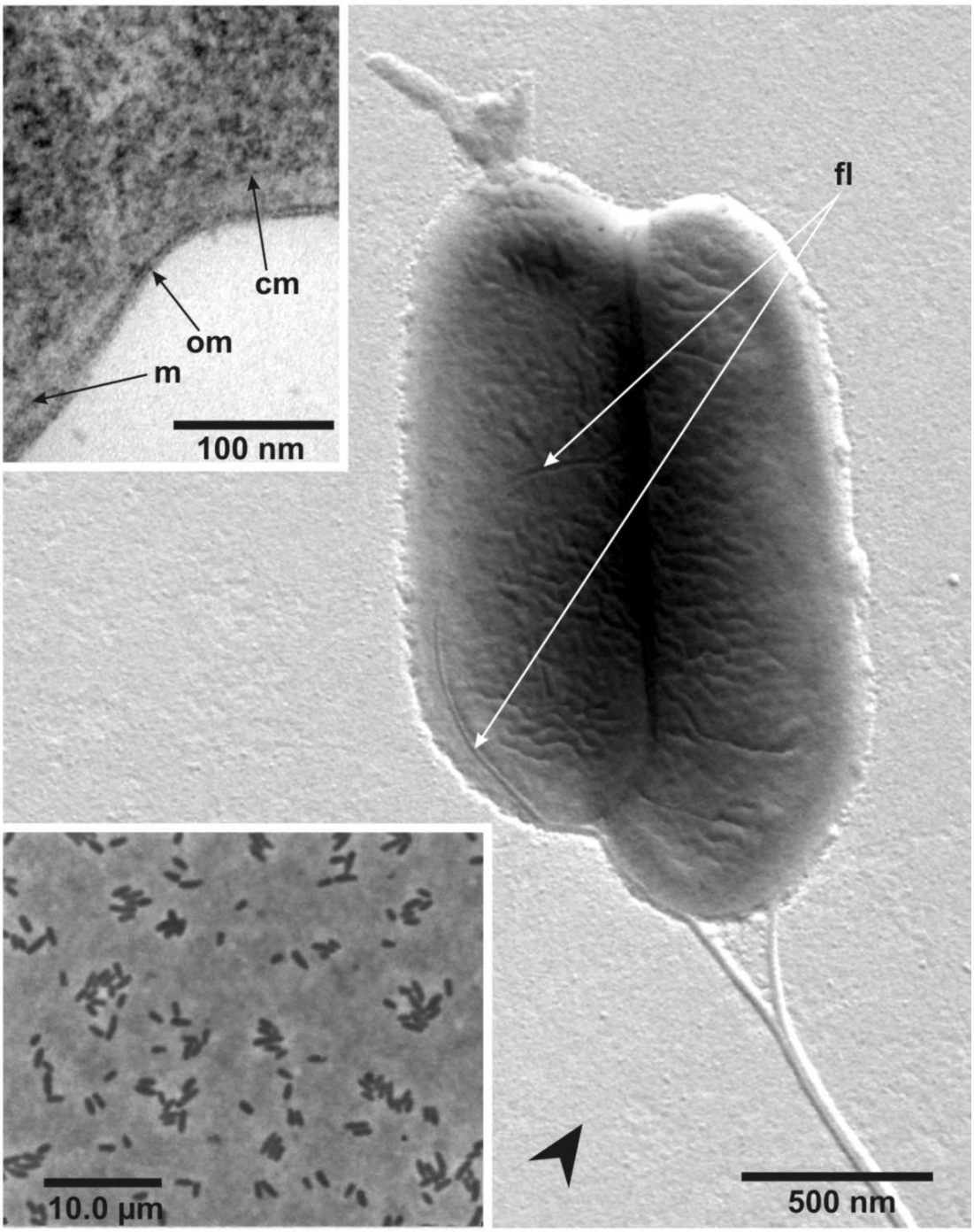


Fig. 1

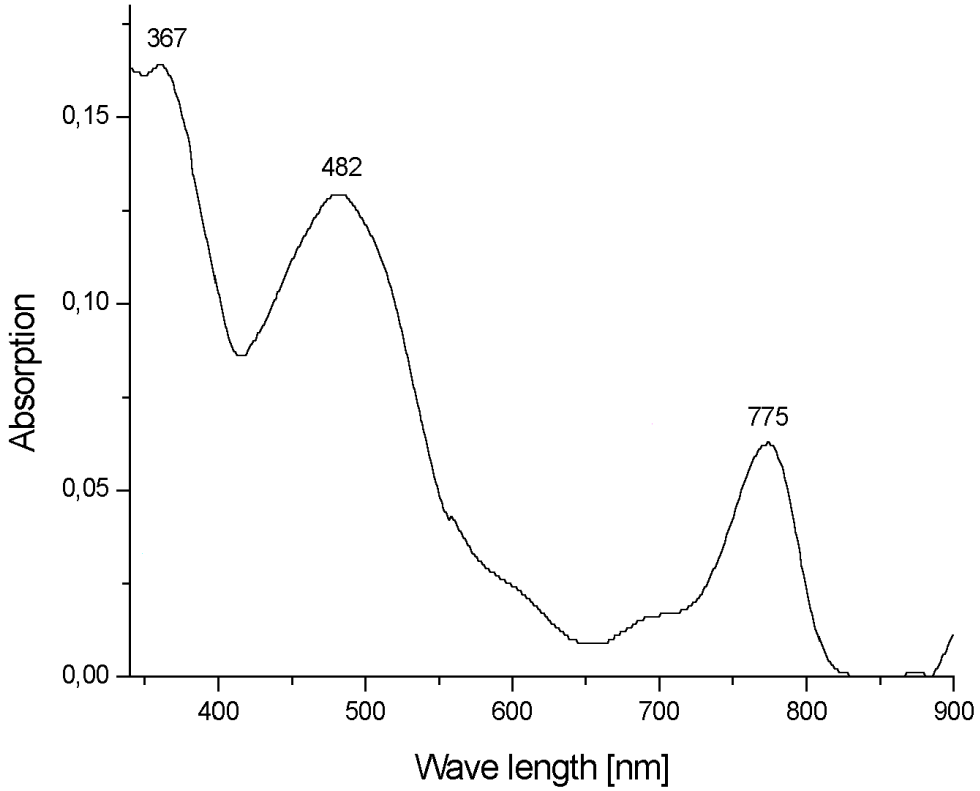
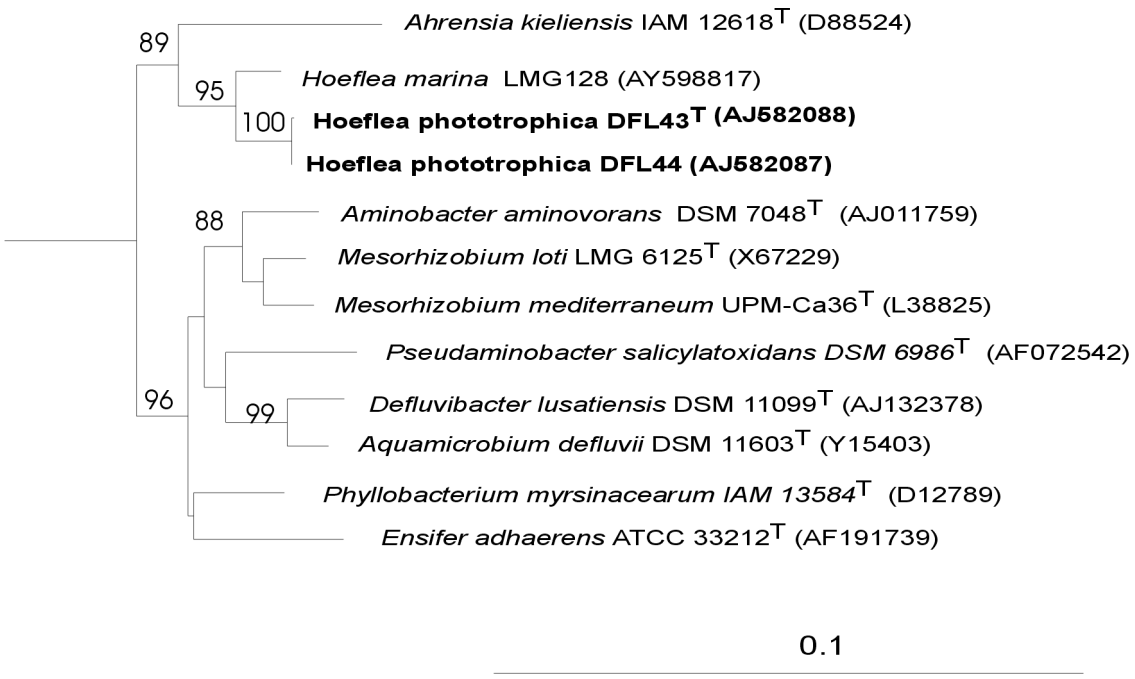

367
482
775
0,15
0,10
0,05
0,00
Absorption
400
500
600
700
800
900
Wave length [nm]

Fig. 2


Ahrensia kieliensis IAM 12618T (D88524)
89
Hoeflea marina LMG128 (AY598817)
95
100
Hoeflea phototrophica DFL43T (AJ582088)
Hoeflea phototrophica DFL44 (AJ582087)
Aminobacter aminovorans DSM 7048T (AJ011759)
88
Mesorhizobium loti LMG 6125T (X67229)
Mesorhizobium mediterraneum UPM-Ca36T (L38825)
Pseudaminobacter salicylatoxidans DSM 6986T (AF072542)
96
99
Defluvibacter lusatiensis DSM 11099T (AJ132378)
Aquamicrobium defluvii DSM 11603T (Y15403)
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Ensifer adhaerens ATCC 33212T (AF191739)
0.1

Fig. 3

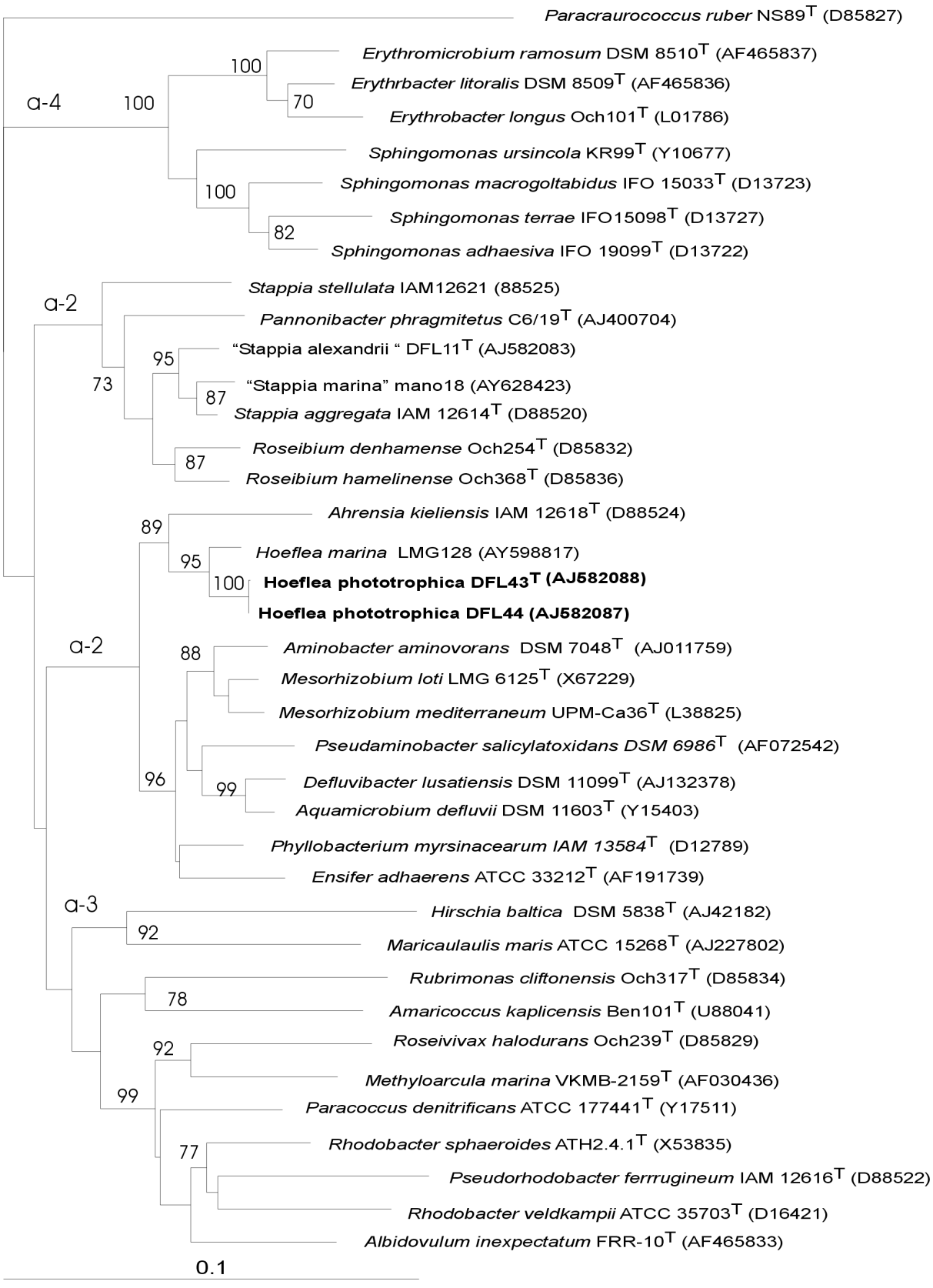

Fig. A Supplementary Material