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# *Hoeflea alexandrii* sp. nov., isolated from the toxic dinoflagellate *Alexandrium minutum* AL1V

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A Gram-negative, aerobic, non-symbiotic bacterium (AM1V30$^T$) was isolated from the toxic dinoflagellate *Alexandrium minutum* AL1V. On the basis of 16S rRNA gene sequence similarity, strain AM1V30$^T$ was most closely related (97·4 % similarity) to the type strain of *Hoeflea marina*, which belongs to the family *Phyllobacteriaceae* within the order *Rhizobiales* of the class *Alphaproteobacteria*. A polyphasic approach was used to clarify the taxonomic position of strain AM1V30$^T$. During the course of this study, a second species was described by others as belonging to the genus *Hoeflea*, namely *Hoeflea phototrophica*; it showed a somewhat higher level of 16S rRNA gene sequence similarity with respect to strain AM1V30$^T$ (98·2 %) and was also taken into account. The fatty acid profiles, physiological and biochemical data and DNA G+C content (59·7 mol%) support the classification of strain AM1V30$^T$ as a member of the genus *Hoeflea*. The characteristics of the novel strain were sufficiently distinct to indicate that it represents a separate species. To confirm this conclusion, DNA–DNA hybridizations were performed: low values (between 15·8 and 29·8 %) were obtained in all cases. Thus, AM1V30$^T$ represents a novel species within the genus *Hoeflea*, for which the name *Hoeflea alexandrii* sp. nov. is proposed. Strain AM1V30$^T$ (=CECT 5682$^T$=DSM 16655$^T$) is the type strain.

The *Taxonomic Outline of the Prokaryotes* (Garrity *et al.*, 2004) indicates that the family *Phyllobacteriaceae* consists of eight genera representing various origins and lifestyles: *Phyllobacterium* (Knösel, 1984), *Aminobacter* (Urakami *et al.*, 1992), *Aquamicrobium* (Bambauer *et al.*, 1998), *Defluvibacter* (Fritsche *et al.*, 1999), '*Candidatus* Liberobacter' (Jagoueix *et al.*, 1994), *Mesorhizobium* (Jarvis *et al.*, 1997), *Nitratireductor* (Labbé *et al.*, 2004) and *Pseudaminobacter* (Kämpfer *et al.*, 1999). Two other genera, *Parvibaculum* (Schleheck *et al.*, 2004) and *Hoeflea* (Peix *et al.*, 2005), have since been proposed for consideration as members of this family, on the basis of 16S rRNA gene sequence data. The genus *Hoeflea* was created to accommodate a single species, *Hoeflea marina*, based on a strain of marine origin (from the Baltic Sea, off the coast of Germany) previously assigned to the species *Agrobacterium ferrugineum* (Peix *et al.*, 2005). More recently, a second species within this genus was proposed, *Hoeflea phototrophica* (Biebl *et al.*, 2006), for isolates from cultures of marine dinoflagellates (three strains from *Alexandrium lusitanicum* and two from *Prorocentrum lima*). This study deals with the characterization of a novel isolate, strain AM1V30$^T$, and the proposal of a novel species of the genus *Hoeflea*.

Strain AM1V30$^T$ was isolated from a culture of the toxin-producing marine dinoflagellate *Alexandrium minutum* AL1V (Instituto Español de Oceanografia, Vigo, Spain). *Alexandrium minutum* is a planktonic species associated with shellfish poisoning events (toxic, paralytic) in coastal regions around the world (Lilly *et al.*, 2005). The specific relationship between bacteria and dinoflagellates is not well understood, but some authors consider that some of them might be symbiotic, as bacteria have been detected inside dinoflagellate cells (Cavanaugh, 1994; Lewis *et al.*, 2001). Strain AM1V30$^T$ is able to grow axenically *in vitro* and it also yields blooms in cultures of the microalga from which it was isolated and of another closely related clone (AL4V).

For isolation of bacteria, samples of the harvested dinoflagellate culture were taken under sterile conditions, washed in 1 × PBS and then collected again by centrifugation. Cells were sonicated (Branson 2200E-1; Branson Ultrasonic) for

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AM1V30$^T$ is AJ786600.

Transmission electron micrographs of strain AM1V30$^T$ are available as a supplementary figure in IJSEM Online.

5 min with 1 min intervals on ice. Aliquots of the sonicated dinoflagellate cell suspension were streaked on full- and half-strength marine agar 2216 (Difco) for 7 days at 15 °C. Only one type of colony – circular and pigmented light brown – was obtained. For maintenance and further testing, cells were incubated in marine agar or marine broth 2216 (Difco) at 30 °C unless stated otherwise.

Cell morphology of cultures (after 3–4 days incubation) was observed under a Zeiss Axioskop light microscope; phase-contrast observations were included. The size and ultrastructure of the cells were determined by transmission electron microscopy (see Supplementary Fig. S1 available in IJSEM Online). Cells were negatively stained with uranyl acetate (2 % w/v, pH 4·5) and were observed with a JEOL1010 microscope (Centro de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain): monotrichous polar flagellation was observed (Supplementary Fig. S1).

Growth was assessed at 4, 10, 20, 30, 37, 42 and 50 °C and at pH 5, 6, 7, 8, 9 and 10. To assess the salinity requirements/tolerance of strain AM1V30$^T$, 0, 0·8, 1·5, 3, 6 and 10 % NaCl or 2·5 and 5 % sea salts (Sigma) were added to salt-free ZoBell medium, half-strength ZoBell medium and full-strength ZoBell medium (Oppenheimer & ZoBell, 1952). In all cases, both broth and solid media were tested. For comparison purposes, strain AM1V30$^T$ was also inoculated into basal medium agar consisting of 0·3 % yeast extract (Difco), 1 % Bacto-peptone (Becton Dickinson), 0·5 % NaCl (Merck) and 1·5 % agar (Pronadisa). The optimal temperature for growth was 30 °C. Strain AM1V30$^T$ was unable to grow at 4 or 50 °C. This micro-organism required pH values within the range 6–9, optimal growth occurring at pH 7. Regarding salinity, growth was supported at 0 (salt-free ZoBell medium without added salts) to 11·8 % total salts (half-strength ZoBell medium plus 10 % NaCl). Optimal growth occurred at 0–6·8 % total salts, indicating that strain AM1V30$^T$ must be considered as a marine organism that exhibits moderate halotolerance. No significant difference in the growth rate was observed with media employing only NaCl instead of sea salts (or sea salts plus NaCl). This, together with the fact that salt-free media supported growth, shows that, despite its marine origin, strain AM1V30$^T$ does not have any specific ionic requirement.

The isolate was tested by using standard procedures (Gerhardt *et al.*, 1994) and using the whole test spectrum of the bioMérieux API 20NE kit (for biochemical reactions) and the API ZYM kit (for extracellular enzyme activities). Strips were incubated at 30 °C for 24 h (API ZYM) or 48 h (API 20NE). Carbohydrate metabolism was investigated using API 50 CH strips (bioMérieux) in conjunction with API 50 CHB/E medium and according to the instructions of the manufacturer. API 50 CH strips were read after incubation for 24 and 48 h at 30 °C.

The response of strain AM1V30$^T$ to different antibiotics was tested by using filter-paper discs (Mast Diagnostics) containing antibiotics at the following concentrations: ampicillin, 25 μg; cephalothin, 30 μg; chloramphenicol, 30 μg; erythromycin, 15 μg; nalidixic acid, 30 μg; streptomycin, 15 μg; tetracycline, 30 μg; and trimethoprim, 5 μg. The isolate was resistant to ampicillin, nalidixic acid and trimethoprim and sensitive to streptomycin, tetracycline, cephalothin, erythromycin and chloramphenicol.

Genomic DNA was extracted from bacterial cells and purified using the Fast DNA kit (Qbiogene). The 16S rRNA gene was amplified by a PCR using the primers 27f [5′-AGAGTTTGATC(C/A)TGGCTCAG-3′] and 1492r [5′-TACGG(C/T)TACCTTGTTACGACTT-3′] (Lane, 1991). PCR amplification of the 16S rRNA gene was done as described by Orphan *et al.* (2001) and the products were sequenced directly as described by Moore *et al.* (1999). The *Taq* dideoxy terminator cycle sequencing kit (Perkin Elmer Applied Biosystems) was used to sequence the 16S rRNA gene. The reactions were run on an Applied Biosystems 373S DNA sequencer.

Sequences were compared with public sequences in the EMBL gene sequence databases by using the BLAST program (National Center for Biotechnology Information; http://ncbi.nlm.nih.gov/). Related sequences were further analysed using the program package ARB (Ludwig *et al.*, 2004; http://www.arb-home.de). Sequence alignments were corrected manually using the sequence editor ARB_EDIT. Phylogenetic analysis using alternative treeing methods (maximum parsimony, maximum likelihood and distance matrix) and data subsets was performed using the appropriate ARB tools (Ludwig *et al.*, 1998). Fig. 1 shows the tree derived by analysis with Jukes–Cantor corrections. Phylogenetic analysis revealed that strain AM1V30$^T$ is a member of the family *Phyllobacteriaceae*, its closest relatives being *H. phototrophica* and *H. marina* (98·2 and 97·4 % sequence similarity to the respective type strains). Levels of similarity to sequences from species of neighbouring genera were significantly lower: *Mesorhizobium*, 96·2–96·8 %; *Phyllobacterium*, 94·4–96·2 %; *Aminobacter*, 95·9–96·1 %; *Pseudaminobacter*, 95·4–96·0 %; *Nitratireductor*, 95·8 %; *Defluvibacter*, 95·7 %; and *Aquamicrobium*, 95·6 %. Moreover, the sequence of strain AM1V30$^T$ consistently aligned with those of the two *Hoeflea* species, regardless of the treeing method, which means that it can be considered as a member of the same genus.

The DNA G+C content (mol%) was determined by HPLC at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) according to the procedure of Mesbah *et al.* (1989). Strain AM1V30$^T$ has a DNA G+C content of 59·7 mol%, which is 6·6 mol% higher than that of *H. marina* LMG 128$^T$ (53·1 mol%) (Peix *et al.*, 2005) but almost identical to that of *H. phototrophica* DFL-43$^T$ (59·3 mol%) (Biebl *et al.*, 2006).

The cellular fatty acid composition of strain AM1V30$^T$ was analysed by GLC at the DSMZ, using a method described previously (Kämpfer & Kroppenstedt, 1996). The profile obtained largely resembled those of the two *Hoeflea* species,

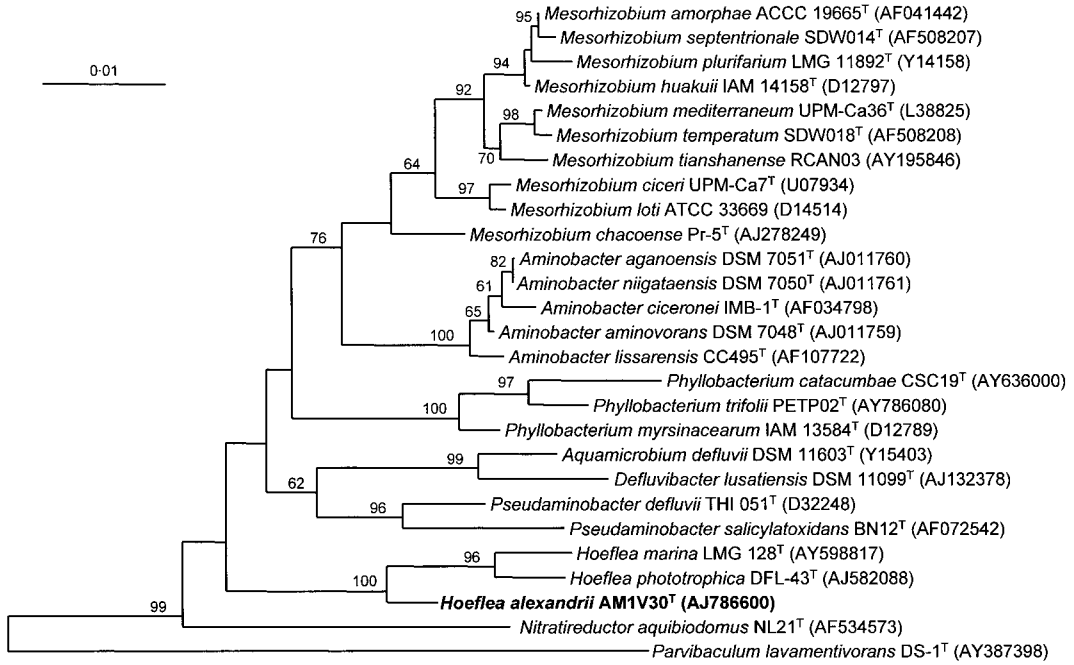


**Fig. 1.** Neighbour-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences of strain AM1V30$^T$ and members of the family *Phyllobacteriaceae*. Bootstrap values (percentages of 1000 resamplings) greater than 60 % are shown at branching points. Sequence accession numbers are given in parentheses. Bar, 1 estimated substitution per 100 base positions.

except with regard to the relative amounts and the presence or absence of some fatty acids in one of the three organisms (Table 1).

The production of bacteriochlorophyll *a* by strain AM1V30$^T$ was investigated as described by Biebl *et al.* (2006) for *H. phototrophica*: the absorption spectrum lacked the peaks of bacteriochlorophyll *a* and a carotenoid that are found in *H. phototrophica*. Thus, in this respect, strain AM1V30$^T$ resembles *H. marina*.

As has been indicated above, strain AM1V30$^T$ belongs to the genus *Hoeflea* on the basis of 16S rRNA gene sequence data. The values for sequence similarity with respect to the type strains of the two species of the genus, *H. phototrophica* and *H. marina* (98·2 and 97·4 %, respectively), are above the limit suggested by Stackebrandt & Goebel (1994) as defining bacterial species. However, given the number of phenotypic traits that differentiate strain AM1V30$^T$ and those species (see Table 2 and comments above), DNA–DNA hybridization was deemed necessary to confirm its status as a separate species.

Whole-genome hybridization assays were performed at the DSMZ. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications of Huß

**Table 1.** Fatty acid methyl ester profiles of strain AM1V30$^T$ and related type strains

Strains: 1, AM1V30$^T$ (data from this study); 2, *H. marina* LMG 128$^T$ (Peix *et al.*, 2005); 3, *H. phototrophica* DFL-43$^T$ (Biebl *et al.*, 2006). Values are mean percentages of total fatty acid methyl esters. tr, Trace amount ($\leqslant$1·0 %); ND, not detected.

| Fatty acid | 1 | 2 | 3 |
|---|---|---|---|
| **Saturated fatty acids** | | | |
| 14 : 0 | tr | ND | ND |
| 16 : 0 | 7·7 | 4·0 | 6·3 |
| 18 : 0 | 1·4 | 1·4 | 1·2 |
| **Unsaturated fatty acids** | | | |
| 16 : 1$\omega$11*c* | tr | ND | ND |
| 17 : 1$\omega$8*c* | ND | tr | ND |
| 18 : 1$\omega$7*c* | 83·1 | 76·0 | 62·8 |
| 18 : 1$\omega$9*c* | tr | ND | ND |
| 20 : 1$\omega$9*t* | ND | tr | ND |
| 11-Methyl 18 : 1$\omega$7*c* | 3·6 | 7·5 | 20·6 |
| **Cyclopropane acids** | | | |
| 19 : 0 cyclo $\omega$8*c* | ND | 5·6 | 1·6 |
| 19 : 1 cyclo | ND | ND | 3·4 |
| **Summed features** | | | |
| 14 : 0 3-OH and/or 16 : 1 iso I | tr | ND | ND |
| 16 : 1$\omega$7*c* and/or 15 : 0 iso 2-OH | 2·3 | 2·6 | 1·6 |
| Unknown | ND | tr | 2·4 |

**Table 2.** Differentiating characteristics for the novel strain and species of the genus *Hoeflea*

Strains: 1, AM1V30$^T$ (data from this study); 2, *H. marina* LMG 128$^T$ (Peix *et al.*, 2005); 3, *H. phototrophica* DFL-43$^T$ (Biebl *et al.*, 2006). ND, Not detected; W, weak reaction.

| Characteristic | 1 | 2 | 3 |
|---|---|---|---|
| Colony pigmentation | Light brown | White–cream | None/beige |
| Bacteriochlorophyll *a* | − | − | + |
| Optimal salinity (% NaCl) | 0–6·8 | 3 | ND |
| Salinity range (% NaCl) | 0–11·8 | 0–5 | 0·5–7 |
| Temperature range (°C) | 10–42 | 4–37 | 15–33 |
| Urease activity | − | + | ND |
| Hydrolysis of gelatin | − | W | − |
| Assimilation of: | | | |
| D-Glucose | − | + | W |
| L-Arabinose | − | + | ND |
| D-Mannose | − | + | ND |
| D-Mannitol | − | + | ND |
| D-Maltose | − | + | ND |
| Malate | − | + | + |
| Gentiobiose | + | − | ND |
| DNA G+C content (mol%) | 59·7 | 53·1 | 59·3 |

*et al.* (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). Experiments were run in duplicate, giving the following results: strain AM1V30$^T$ against *H. marina* DSM 16791$^T$, 15·8 and 17·2 %; strain AM1V30$^T$ against *H. phototrophica* DSM 17068$^T$, 29·8 and 21·3 %. The low values obtained support the definitive conclusion that strain AM1V30$^T$ represents a novel species within the genus *Hoeflea*, for which the name *Hoeflea alexandrii* sp. nov. is proposed.

## Description of *Hoeflea alexandrii* sp. nov.

*Hoeflea alexandrii* (a.lex.an′dri.i. N.L. gen. n. *alexandrii* of *Alexandrium*, the genus name of the dinoflagellate *Alexandrium minutum*, the source of isolation of the type strain).

Cells are Gram-negative, non-spore-forming rods, about 2·5×0·8 μm in size and motile by single polar flagella. Colonies on marine agar 2216 are circular, around 1–2 mm in diameter and pigmented light brown after 2 days incubation at 30 °C. Optimal growth occurs at 30 °C and pH 7. The temperature and pH ranges for growth are 10–42 °C and pH 6–9, respectively. Shows no specific ionic requirement, growing with salts in the range 0–11·8 % (optimally between 0 and 6·8 %). Oxidase-negative and catalase-positive. Does not reduce nitrate to nitrite or nitrogen. The following API 20NE tests give positive results: hydrolysis of aesculin and β-galactosidase activity (PNPG). The following API 20NE tests give negative results: nitrate reduction, indole production, acids from glucose, arginine dihydrolase, urease, hydrolysis of gelatin and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, gluconate, caproate, adipate, malate, citrate and phenylacetate. Positive for the following enzyme activities (API ZYM): alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase. Negative for the following enzyme activities (API ZYM): lipase (C14), trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Ferments the following substrates (API 50 CH): glycerol, L-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, sucrose, D-trehalose, inulin, D-raffinose, starch, glycogen, gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, potassium gluconate and potassium 2-ketogluconate. Does not ferment erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, methyl α-D-mannopyranoside, *N*-acetylglucosamine, D-lactose, D-melibiose, D-melezitose, xylitol, D-tagatose, D-arabitol, L-arabitol or potassium 5-ketogluconate. Growth on artificial seawater with phosphate and traces of minerals and vitamins, supplemented with glycerol or glucose as sole carbon source, is weak. Organic acids do not support growth. Resistant to ampicillin (25 μg), nalidixic acid (30 μg) and trimethoprim (5 μg); sensitive to cephalothin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), streptomycin (15 μg) and tetracycline (30 μg). Fatty acids detected in strain AM1V30$^T$ include (in order of abundance): 18 : 1ω7*c* (83·1 %), 16 : 0 (7·7 %), 11-methyl 18 : 1ω7*c* (3·6 %), summed feature 3 (16 : 1ω7*c* and/or 15 : 0 iso 2-OH; 2·3 %), 18 : 0 (1·4 %) and minor amounts of other fatty acids. The DNA G+C content of the type strain is 59·7 mol%.

The type strain, AM1V30$^T$ (=CECT 5682$^T$=DSM 16655$^T$), was isolated from a culture of the toxin-producing marine dinoflagellate *Alexandrium minutum* AL1V (Instituto Español de Oceanografia, Vigo, Spain).

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