Elstera litoralis gen. nov., sp. nov., isolated from stone biofilms of Lake Constance, Germany

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An alphaproteobacterium, strain Dia-1^T, was isolated from algae-dominated biofilms on stones from the littoral zone of Lake Constance, Germany. This bacterium was isolated after initial enrichment in spent medium obtained after growth of a diatom culture. Numerous sugars and some organic acids and alcohols served as growth substrates. The bacterium grew slowly, was strictly aerobic but microaerophilic, and did not grow in cultures shaken under air. 16S rRNA gene sequence analysis indicated that strain Dia-1^T was distantly related to representatives of the genera *Azospirillum* (90–91 % sequence similarity), *Skermanella* (88–89 %), *Rhodocista* (87–88 %) and *Dongia* (88–89 % sequence similarity). Based on this sequence comparison, on phenotypic characterization including substrate utilization patterns, and comparison of cellular fatty acids, quinones, polar lipids and polyamines, this isolate was found to be substantially different from the genera mentioned above. On the basis of these results, a novel genus and species is proposed for this strain. The name *Elstera litoralis* gen. nov., sp. nov. is suggested, with strain Dia-1^T (=DSM 19532^T=LMG 24234^T) as the type strain of the type species.

Lake Constance is a mesotrophic, phosphate-limited hard-water pre-alpine lake (Schmieder *et al.*, 2005), and the second largest freshwater lake in Europe. The infra-littoral zone consists of stones that are covered with biofilms. The shallow upper-littoral zone of lentic periphyton communities is often dominated by diatoms and green algae (Stevenson *et al.*, 1996). Diatoms and bacteria are the pioneers in developing photic biofilms (Rao *et al.*, 1997), and the extracellular polysaccharides (EPS) produced by the diatoms are an important source of organic carbon for heterotrophic bacteria in such biofilms (Bruckner *et al.*, 2008). In turn, the diatom cells also profit from the presence of heterotrophic bacteria in the culture (Bruckner *et al.*, 2008). In an attempt to analyse metabolic interactions between diatoms and accompanying bacteria in more depth, we aimed to cultivate biofilm-associated bacteria living in close cooperation with diatoms. Strain Dia-1 was isolated from a stone biofilm as a numerically dominant bacterium after initial growth with isolated diatom EPS.

Diatoms were isolated from biofilms growing on stones at 20–40 cm water depth in the littoral zone (47° 41' N; 9° 11' E) of Lake Constance, Germany. Unialgal, non-axenic and axenic diatom cultures were established by repeated screening and/or antibiotic treatment, and were

maintained in Diatom Mineral Medium (DM; Watanabe, 2005) at 16 °C in a 16:8 h light–dark cycle using cool-white fluorescent tubes (50 µmol photons m⁻² s⁻¹), in stationary culture without shaking or aeration.

Unialgal axenic diatoms were isolated as previously described (Bahulikar & Kroth, 2008). Four of these axenic diatom strains, *Cymbella microcephala* (isolates B-04, I-04 and D-23) and *Cymbella minuta* (isolate I-51) were grown in 1 l flasks containing 600 ml DM and were allowed to grow to stationary phase. Possible bacterial contamination was checked microscopically following staining with SYBR Green (Invitrogen Inc.) according to the manufacturer's instructions. After confirming the diatom cultures were axenic, cells were removed by centrifugation at 10 000 r.p.m. for 10 min and the spent medium was used in experiments for enrichment of heterotrophic bacteria. The medium was checked microscopically for remnants of diatom cells or any contaminating bacteria. Soluble EPS were quantified by a carbohydrate assay (DuBois *et al.*, 1956) before and after bacterial growth.

For isolation of biofilm-associated bacteria, stones from the littoral zone (20–30 cm water depth) of Lake Constance, Germany were collected on 19 August 2006. Epilithic biofilms were scraped off and pooled in a Falcon tube. Approximately 2.5 ml biofilm material was diluted with DM to 25 ml and vortexed vigorously for 2–3 min until most of the particles were well-suspended. The suspension was further mixed by pipetting and was serially diluted in 1:10 steps up to 10⁻⁸, with vortexing for 1–2 min at each

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Abbreviation: DM, Diatom Mineral Medium.

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step to disperse the bacteria evenly. A 5 ml aliquot of each diluted suspension was used to inoculate 45 ml spent medium from diatom cultures containing soluble EPS in 100 ml flasks. Flasks were incubated at 25 °C in the dark for 15 days without shaking, with mixing of contents after 3–4 days. The final optical density of the cultures was recorded after 2 weeks, and samples were taken on days 8 and 15 for quantification of soluble EPS as described above. Non-inoculated flasks served as controls and were incubated in the same manner.

The two highest dilution cultures showing positive growth were used for isolation of the numerically dominant bacteria utilizing the diatom-produced EPS in the spent media. Bacteria from lower dilutions were also plated to check for the overall diversity. Approximately 20 µl samples of these dilutions were streaked on two different agar plates [1 : 2- or 1 : 10-diluted nutrient broth solidified with 1.5% (w/v) agar (Difco) and containing 10 mM glucose] and incubated at 23 °C until colonies were visible. Bacteria were examined with a phase-contrast microscope (Axiophot; Zeiss) and restreaked until homogeneous colonies and microscopically pure cultures were obtained. Cells were observed and photographed with a phase-contrast microscope (Axiophot; Zeiss) and a cooled charge-couple device camera (MagnaFire; INTAS).

Strain Dia-1^T was isolated from the final plated dilution of the biofilm sample where EPS was used as the carbon source. Growth of strain Dia-1^T in VM-ethanol medium (Reinhold-Hurek & Hurek, 2000) was investigated.

For growth tests on API 20NE and Biolog PM1 microplates, fresh colonies grown in 1 : 10-diluted nutrient broth supplemented with 10 mM glucose were used. Results were recorded after incubation for 4–6 days at 20–22 °C.

Analysis of total fatty acids, respiratory quinones and polar lipids were carried out by the Identification Service of the DSMZ and Dr B. J. Tindall, DSMZ, Braunschweig, Germany. Cells were grown in 1 : 10 diluted nutrient broth with 10 mM glucose at 22 °C into late exponential phase, pelleted and freeze-dried before lipid analysis. Fatty acid methyl esters were extracted from the cells following saponification and methylation, and were subjected to gas chromatography. The gas-chromatographic elution profile of the fatty acid methyl esters was compared with the fatty acid patterns in the database of the Microbial Identification System (MIS, MIDI), and qualitative and quantitative composition of the profile were given. Polyamines were extracted and analysed by Dr H.-J. Busse, Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, Vienna, Austria (Busse & Auling, 1988; Stolz *et al.*, 2007).

Fixation of molecular nitrogen was investigated in mineral medium devoid of ammonium or nitrate. Nitrogenase activity was assayed by the acetylene reduction assay (Stewart *et al.*, 1967). To check for the presence of *nif* genes, genomic DNA of strain Dia-1 was extracted using a modified

CTAB method (Murray & Thompson, 1980). Cells were grown, pelleted, suspended in CTAB extraction buffer and heated at 65 °C for 30 min followed by ChI:IAA extraction and ethanol precipitation. Partial amplification of *nifH* gene was carried out using a nested approach described by Zani *et al.* (2000).

DNA was extracted, used for amplification of the 16S rRNA gene and sequenced as previously described (Rahalkar *et al.*, 2007). The DNA G + C content was analysed by the Identification Service, DSMZ, Braunschweig, Germany, following the method of Mesbah *et al.* (1989).

16S rRNA gene sequences of type strains related to strain Dia-1^T were downloaded from the NCBI database and aligned using the SINA aligner (<http://www.arb-silva.de/aligner/>). Phylogenetic analysis of the 16S rRNA gene sequences of strain Dia-1 and related strains was performed with MEGA software version 5 (Tamura *et al.*, 2011). *Gluconacetobacter diazotrophicus* PAI 5^T was used as an outgroup.

Dilution cultures of homogenized biofilm material were incubated in medium with EPS of diatoms as sole source of carbon and energy for up to 15 days. Growth was observed up to the final dilution in the form of biofilms at the bottom of the flasks. After streaking on 1 : 10-diluted nutrient broth agar plates containing 10 mM glucose, various colonies were observed, and one of the most common colony types was used for isolating strain Dia-1^T.

Strain Dia-1^T was isolated from agar plates originally inoculated with the 10^{−8} dilution by repeated streaking on the same agar medium. It also grew well in 1 : 2-diluted nutrient broth supplemented with 10 mM glucose, and in VM medium with ethanol as sole carbon source at room temperature (20–23 °C), both in liquid medium and on solid medium plates. In liquid VM media without shaking, the strain initially formed small aggregates or white flocks. On agar plates, milky white to cream-coloured colonies were formed within 3–4 days, which turned light yellow at the periphery after extended incubations. Cells were slightly curved, non-motile rods and tended to form aggregates (Fig. 1). Gas vesicles were formed occasionally inside the cells in ageing cultures.

Strain Dia-1^T grew well in VM-ethanol medium and did not grow in nutrient broth only, i.e. a sugar or ethanol was required for growth. Growth tests with the API 20NE system and the Biolog microplate system (PM1 microplate) showed that the strain grew in the presence of a wide variety of sugars, and some organic acids and alcohols (see species description). Strain Dia-1^T grew optimally at 20–25 °C, with a doubling time of approximately 40 h. Temperatures up to 30 °C were tolerated, but no growth was observed at 37 °C or at 15 °C. The optimum pH for growth was pH 6.5–7.0; with the limits of growth at pH 5.5 and 8.0.

Nil or very weak growth was found in mineral medium free of bound nitrogen compounds. The acetylene reduction test was negative even after prolonged incubation for

Fig. 1. Phase-contrast micrograph of aggregated cells of strain Dia-1^T grown in 1:10-diluted nutrient broth medium containing 10 mM glucose. Bar, 5 µm.

2 days. The *nifH* gene could not be amplified using a direct or a nested PCR approach (Zani *et al.*, 2000).

According to the FAME profile, strain Dia-1^T showed similarity to representatives of the *Roseomonas* group *cervicalis* which is synonymous with *Azospirillum brasilense*. The major fatty acids were 18:1 ω 7c (55%), 18:1 2-OH (12%) and 16:0 (10%).

Ubiquinone Q-10 was found to be the only respiratory quinone. Analysis of polar lipids identified phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and two aminolipids as dominant

lipid constituents. The following polyamines were found (µmol g⁻¹): putrescine, 60.4; cadaverine, 0.5; spermidine, 1.8; *sym*-homospermidine, 13.5; and spermine, 1.0.

According to the 16S rRNA gene sequence analysis, strain Dia-1^T was closest related to representatives of the genera *Azospirillum* (90–91% gene sequence similarity), *Skermanella* (88–89%), *Rhodocista* (87–88%) and *Dongia* (88–89%) (Fig. 2). The robustness of the tree was confirmed by minimum evolution, neighbour-joining and maximum-likelihood methods (1000 replications each) in MEGA5 software, and the 16S rRNA gene sequence of strain Dia-1^T was added to the 'All-Species Living Tree' LTPs104 (March 2011) (Yarza *et al.*, 2008). All methods yielded trees of similar topology and the position of strain Dia-1^T was similar in all trees.

The DNA G+C content of strain Dia-1^T was 61.0 ± 1.5 mol%.

Based on the 16S rRNA gene sequence analysis (Fig. 2) and the various phenotypic properties analysed (Table 1), strain Dia-1^T is proposed to represent a novel species in a new genus. It differs from members of the genus *Dongia* by the presence of catalase and oxidase, and the lack of nitrate reduction; from members of the genus *Azospirillum* by the lack of the characteristic ability to fix nitrogen; from *Azospirillum* and *Skermanella* by the lack of nitrate reduction; and from *Rhodocista* by a different metabolism. It differs from all these genera by a substantially lower DNA G+C content, and by a different pattern of substrate utilization (Table 1). We therefore conclude that strain Dia-1^T represents a novel species of a new genus for which

Fig. 2. Phylogenetic tree of strain Dia-1^T and related members of the family *Rhodospirillaceae* based on 16S rRNA gene sequences. The tree was constructed using the Minimum Evolution method. Bootstrap values of 1000 replications are shown next to the branches. Evolutionary distances were computed using the Tamura-Nei method with a total of 1207 positions used for analysis. Species present in the collapsed branch: *A. lipoferum* NCIMB 11861^T (Genbank accession no. Z29619), *A. melinis* TMCY 0552^T (DQ022958), *A. oryzae* COC8^T (AB185396), *A. zeae* N7^T (DQ682470), *A. doebereineriae* GSF71^T (NR025354), *A. thiophilum* BV-S^T (EU678791), *A. picis* IMMIB TAR-3^T (AM922283), *A. canadense* DS2^T (DQ393891), *A. rugosum* IMMIB AFH-6^T (AM419042), *A. brasilense* DSM 1690^T (GU256438). Bar, 1% sequence divergence.

Table 1. Comparison of strain Dia-1^T with related genera from Fig. 2

Taxa: 1, Dia-1^T; 2, *Dongia*; 3, *Azospirillum*; 4, *Skermanella*; 5, *Rhodocista*. Data for reference genera from Liu *et al.* (2010), Mehnaz *et al.* (2007), Sly & Stackebrandt (1999) and Kawasaki *et al.* (1992). +, Positive; w, weakly positive; –, negative; v, variable; ND, no data available.

Characteristic	1	2	3	4	5
Metabolic type	Chemotrophic	Chemotrophic	Chemotrophic	Chemotrophic	Phototrophic
Oxidase	+	–	+	+	ND
Catalase	+	–	+	+	ND
Major quinones	Q-10	Q-10	Q-10	Q-10, Q-8	Q-9
N ₂ fixation	–	ND	+	–	ND
Nitrate reduction to nitrite	–	+	+	+	–
Utilization of:					
L-Arabinose	+	w	v	+	ND
D-Glucose	+	w	v	+	–
L-Rhamnose	+	–	v	+	ND
Sucrose	–	w	v	ND	–
Citrate	–	–	v	+	–
<i>myo</i> -Inositol	+	–	v	–	ND
DNA G + C content (mol%)	61.0 ± 1.5	65.5	64.0–71.0	67.2	68.3–69.9

the name *Elstera litoralis* gen. nov., sp. nov. is proposed. The type strain of the type species is strain Dia-1^T.

Description of *Elstera* gen. nov.

Elstera (Els'te.ra. N.L. fem. n. *Elstera* named after Hans-Joachim Elster, a German limnologist working on Lake Constance who was one of the first to establish the importance of the littoral zone for the lake ecosystem).

Cells are Gram-negative rods, catalase- and oxidase-positive. Chemoheterotrophic. Sugars, some organic acids and alcohols are preferred substrates. Ubiquinone Q-10 is the dominant quinone and putrescine is the dominant polyamine. The type species is *Elstera litoralis*.

Description of *Elstera litoralis* sp. nov.

Elstera litoralis (li.to'ra.lis. L. fem. adj. *litoralis* belonging to the shore or the littoral).

Exhibits the following properties in addition to those listed in the genus description. Cells are slightly curved rods, 1.0 × 2.0–5.0 µm in size, non-motile, with a minimum doubling time of approximately 40 h. Milky white to cream colonies form on diluted nutrient broth agar supplemented with glucose after 3–4 days. Growth occurs at 10–25 °C (optimum, 20–25 °C) and at pH 5.5–8.0 (optimum, pH 6.5–7.0). Negative result in tests for reduction of nitrate, sulfate, and iron(III), fixation of molecular nitrogen, and indole production from tryptophan. Grows in the presence of D-glucose, D-mannose, L-rhamnose, D-fructose, L-fructose, D-galactose, L-fucose, L-arabinose, D-xylose, L-lyxose, D-ribose, D-mannitol, D-sorbitol, α-methyl-D-galactoside, N-acetyl-D-glucosamine, methyl β-D-glucoside, D-glucuronic acid, D-galactonic acid, D-glucose 6-phosphate, *myo*-inositol, glycerol, ethanol, L-malate, L-glutamate, L-alanine, L-serine, L-proline, glycyl L-proline; but weak or no growth

observed in presence of trehalose, D-maltose, melibiose, α-D-lactose, lactulose, sucrose, D-psicose, cellobiose, maltotriose, dulcitol, adonitol, D-saccharic acid, D-galactonic acid-γ-lactone, D-fructose 6-phosphate, inosine, N-acetyl-β-D-mannosamine, glucuronamide, D-glucosaminic acid, L-galactonic acid-γ-lactone, formate, acetate, propionate, pyruvate, methylpyruvate, L-lactate, D-malate, fumarate, succinate, monomethylsuccinate, bromosuccinate, glycolate, glyoxylate, citric acid, *m*-tartrate, 2-oxoglutarate, acetoacetate, 2-hydroxybutyrate, 2-hydroxyglutaric acid-γ-lactone, 2-ketobutyrate, tricarballic acid, α-glycerophosphate, mucic acid, L-threonine, D-threonine, L-aspartate, L-asparagine, L-glutamine, D-alanine, D-serine, D-aspartate, tyramine, L-alanylglycine, glycyl L-glutamate, glycyl L-aspartate, thymidine, 2-deoxyadenosine, *p*-hydroxyphenylacetate, phenylethylamine, 1,2-propanediol, 2-aminoethanol, Tweens 20, 40 and 80. Major cellular fatty acids are 18:1ω7c, 18:1 2-OH and 16:0.

The type strain Dia-1^T (=DSM 19532^T=LMG 24234^T), was isolated from biofilms on stones in the littoral zone of Lake Constance, Germany. The DNA G + C content of strain Dia-1 is 61.0 ± 1.5 mol%.

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