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 analyze 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. Strain Dia-1 was 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

An alphaproteobacterium, strain Dia-1T, 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-1T 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-1T (=DSM 19532T=LMG 24234T) as the type strain of the type species.

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

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An alphaproteobacterium, strain Dia-1T, 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-1T 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-1T (=DSM 19532T=LMG 24234T) 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

†These authors contributed equally to this work.

Abbreviation: DM, Diatom Mineral Medium.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Dia-1 is EU678309.
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 bact-
eria 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 photo-
graphed with a phase-contrast microscope (Axiophot; Zeiss)
and a cooled charge-couple device camera (Magnafire;
INTAS).

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

For growth tests on API 20NE and Biolog PM1 micro-
plates, 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 fu¨r
Bakteriologie, Mykologie und Hygiene, Veterinarmedizi-
nische Universita¨t, Vienna, Austria (Busse & Auling, 1988;
Stolz et al., 2007).

Fixation of molecular nitrogen was investigated in mineral
medium devoid of ammonium or nitrate. Nitrogenase activ-
ity was assayed by the acetylene reduction assay (Stewart
et al., 1967). To check for the presence of nif genes, geno-
mic 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 Chl: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-
1T were downloaded from the NCBI database and aligned
using the sina aligner (http://www.arb-silva.de/aligner/). Phy-
logenetic 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 diazo-
 trophicus PAI 5T 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-1T.

Strain Dia-1T 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-1T 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-1T 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
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-1T showed similarity to representatives of the Roseomonas group *cervicalis* which is synonymous with *Azospirillum brasilense*. The major fatty acids were 18:1ω7c (55%), 18:12-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-1T was closest related to representatives of the genera *Azospirillum* (90–91% gene sequence similarity), *Skenmanella* (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-1T 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-1T was similar in all trees.

The DNA G+C content of strain Dia-1T 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-1T 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 *Skenmanella* 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-1T represents a novel species of a new genus for which
Table 1. Comparison of strain Dia-1T with related genera from Fig. 2

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>3</th>
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<td>Chemotrophic</td>
<td>Chemotrophic</td>
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<td>+</td>
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<td>Q-10, Q-8</td>
<td>Q-9</td>
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<td>+</td>
<td>-</td>
<td>ND</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td></td>
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<td>W</td>
<td>V</td>
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<tr>
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<td>-</td>
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<tr>
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<td>-</td>
<td>V</td>
<td>-</td>
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</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>61.0 ± 1.5</td>
<td>65.5</td>
<td>64.0–71.0</td>
<td>67.2</td>
<td>68.3–69.9</td>
</tr>
</tbody>
</table>

Taxa: 1, Dia-1T; 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.

Description of Elstera gen. nov.

Elstera (Elstera. 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-xylene, L-lyxose, D-ribose, D-mannitol, D-sorbitol, 2-methyl-D-galactoside, N-acetyl-D-glucosamine, methyl β-D-galactoside, d-glucuronic acid, D-galactonic acid, D-glucose 6-phosphate, myo-inositol, glycerol, ethanol, L-malate, L-glutamate, L-alanine, L-serine, L-proline, glycin L-proline; but weak or no growth observed in presence of trehalose, D-maltose, melibiose, 2-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-o-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, acetocacetate, 2-hydroxybutyrate, 2-hydroxyglutaric acid-γ-lactone, 2-ketobutyrate, tricarballylic acid, γ-glycerophosphate, mucic acid, L-threonine, D-threonine, L-aspartate, L-asparagine, L-glutamine, D-alanine, D-serine, D-serine, D-aspartate, tyramine, L-α-alanylglycine, glycin L-glutamate, glycin L-aspartate, thymidine, 2-deoxyadenosine, p-hydroxyphenylacetate, phenyl-ethanamine, 1,2-propanediol, 2-aminoethanol, Tween 20, 40 and 80. Major cellular fatty acids are 18:1ω7c, 18:1 ω9c and 16:0.

The type strain Dia-1T (=DSM 19532T =LMG 24234T), 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%.

Acknowledgements

The authors want to thank Hans-Jürgen Busse, Vienna, Austria, for analysis of polyamines. We also thank Antje Wiese for technical help in characterization of the strain. This study was supported by the Deutsche Forschungsgemeinschaft, Bonn, with Sonderforschungsbereich (SFB) 454, and research funds of Universität Konstanz.

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