Deefgea rivuli gen. nov., sp. nov., a member of the class Betaproteobacteria

Erko Stackebrandt,1 Elke Lang,1 Sylvie Cousin,1 Orsola Päuker,1 Evelyne Brambilla,1 Reiner Kroppenstedt1 and Heinrich Lünsdorf2

1DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, Inhoffenstr. 7b, 38124 Braunschweig, Germany
2Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany

Two strains, designated WB 3.4–79T and WB 3.3-25, were isolated from a hard-water sample collected from the Westerhöfer Bach, Lower Saxony, Germany. The strains shared 100% DNA–DNA relatedness, indicating membership of the same species. This close relationship was supported by identical 16S rRNA gene sequences and high similarities in fatty acid composition and biochemical characteristics. The G+C content of the genomic DNA of strain WB 3.4–79T was 48.5 mol% and the predominant ubiquinone was Q-8. Major polar lipids were phosphatidylethanolamine and phosphatidylglycerol. Major fatty acids (>10%) were C16:0 and C16:1ω7c. Polyhydroxybutyrate and polyphosphate granules as well as unidentified enterosomes and a polar organelle are visible by electron microscopy. Comparative 16S rRNA gene sequence analysis indicated that the isolates were placed within the class Betaproteobacteria, remotely related to Chitinibacter tainanensis DSM 15459T, Silivimonas terrae KCTC 12358T, Formivibrio citricus DSM 6150T and Iodobacter fluviatilis DSM 3764T. On the basis of phylogenetic and phenotypic distinctness, we propose a novel genus, Deefgea gen. nov., with Deefgea rivuli sp. nov., as the type species. The type strain of Deefgea rivuli is strain WB 3.4–79T (=DSM 18356T =CIP 109326T).

During the investigation of the prokaryotic diversity of a hard-water creek, with emphasis on the participation of bacteria in the formation of tufa, hundreds of organisms were isolated from rivulet water which, on R2A agar, showed either the capacity for swarming or colonies that had a slimy appearance. Most of the swarming bacteria (~40%) and many of the slime-producing bacteria were members of the genus Flavobacterium (Brambilla et al., 2007; Cousin et al., 2007), while other strains with the slimy phenotype were affiliated to various classes of the Proteobacteria on the basis of partial 16S rRNA gene sequences (unpublished results). Phylogenetic analysis demonstrated the close affiliation of most water isolates to described species (>98% 16S rRNA gene sequence similarity), and only a few were more distantly related. In this communication we present the characterization of two of these strains which are affiliated to the class Betaproteobacteria.

Strains WB 3.4–79T and WB 3.3-25 were isolated from sampling site 3 (Brambilla et al., 2007) of the Westerhöfer Bach on the western slopes of the Harz Mountains in Lower Saxony, Germany. Creek water was spread on R2A plates (Difco) which were incubated at 10 or 18°C for 4 days. Single slimy colonies on the plates were purified by transferring them onto new plates and incubating again under the same conditions. The isolates was cultured routinely on R2A agar at 28°C and maintained as a glycerol suspension (20%, w/v) at −70°C. Strains were preserved in the long term as freeze-dried cultures or in liquid nitrogen.

Ultrastructural analysis

Mid-exponential phase cells of strain WB 3.4–79T were prepared as ‘whole-mount’ samples either shadow-cast (Fig. 1a, b) or negatively stained (Fig. 1c), following a general protocol (Yakimov et al., 1998; Golyshina et al., 2000). Cells were also embedded in epoxy resin (Spurr, 1969) and ultrathin-sectioned. Energy-filtered transmission electron microscopy (EFTEM) was done with a CEM 902 instrument (Zeiss) at 80 kV in the elastic bright-field mode (Lünsdorf et al., 2001, 2006).

On ultrastructural analysis by EFTEM, whole-mount bacterial cells typically appeared as rods, 1.9–3.7 μm in length (mean 2.8 ± 0.7 μm, n = 12) and 0.7–0.9 μm in width (mean 0.8 ± 0.1 μm, n = 21). Cells generally showed one and
occasionally two flagella inserted at one cell pole [monopolar, mono(bi)trichous flagellation], which measured 3.3–5.3 μm in length (mean 4.4 ± 0.7 μm, n = 12) (Fig. 1a–c). Individual cells were surrounded by a slime layer, variable in thickness. In negatively stained cells, the slime layer can be visualized as an electron-dense halo (Fig. 1c) and in a platinum-shadowed preparation as a greyish amorphous rim at the cell periphery that occasionally showed radiant extrusions (Fig. 1a, open arrowheads).

Analysis of ultrathin sections showed the cell wall of strain WB 3.4-79<sup>T</sup> to be outlined by an outer and cytoplasmic membrane (Fig. 1e), characteristic of Gram-negative bacteria. In contact with the cytoplasmic membrane at the cytoplasmic face, a regularly structured layer was occasionally found, which is isomorphic to 'polar organelles'. These have been described in *Sphaerotilus natans* (and other bacteria), and have been assumed to play a role in energy supply in this polytrichous monopolarly flagellated bacterium, based on their ATPase and cytochrome oxidase activities (Tauschel, 1985). Additionally, three morphotypes of intracellular inclusions, polyhydroxybutyrate (phb), polyphosphate (pp) and enterosomes (es) (Fig. 1d, e), are present. Electron-transparent polyhydroxybutyrate inclusions filled most of the cellular volume and were often torn out during the sectioning process. Polyphosphate granules, intensely stained and small in size, were interspersed as small clusters within the cytoplasm or were associated with the surface of large, electron-dense inclusions, 170–200 nm in size. Electron energy loss spectroscopy (EELS) revealed distinctly the presence of phosphorus in both small and large dark cellular inclusions (see Supplementary Fig. S1 in IJSEM Online). EELS also revealed the electron density to be based mainly on uranium, added as a stain during the dehydration process. A closer view of these large inclusions revealed the interior to be filled with a fine particulate matrix that, after low-frequency filtering or fast Fourier transformation, revealed a faint structural order, indicative of residual crystallinity (see Supplementary Fig. S1f–h). Residual crystallinity can also be deduced from the

---

**Fig. 1.** Transmission electron microscopy of strain WB 3.4-79<sup>T</sup> showing 'whole mount' shadow-cast (a, b) and negatively stained (c) bacteria. Cells appear enveloped in a greyish slime matrix, which occasionally shows fibre-like protrusions (open arrowheads). Darkish spots within this matrix represent drying artefacts. An individual flagellum (fl) is inserted at a cell pole, which appears slightly flattened (c, double arrows) relative to the opposite, conical cell end (large arrow). (d, e) Uranyl acetate-post-stained ultrathin sections reveal cells to contain large amounts of polyhydroxybutyrate (phb), often torn out of the section, leaving holes behind (asterisks). Large electron-dense inclusions represent 'enterosomes' (es) associated with or adjacent to clusters of polyphosphate granules (pp). po, Polar organelle; cm, cytoplasmic membrane; om, outer membrane. Bars, 1.0 μm (a–d) and 150 nm (e).
polyhedral contours of these inclusions. EELS and high-resolution elemental mapping showed the presence of phosphorus and nitrogen as the main constituents of these inclusions, supplemented by small amounts of calcium (Supplementary Fig. S1b–e). Nitrogen, as an indicator element of proteins, and the weak structural order of the particulate matrix indicate that these dark inclusions can be considered ‘enterosomes’ in the general sense (Cannon et al., 2001). The presence of a distinct outer proteinaceous shell could not be observed and thus they differ at the structural level from typical ‘polyhedral bodies’. Further biochemical and physiological analysis has to be done to differentiate whether these inclusions represent ‘carboxyosomes’ in a specific sense (Cannon et al., 2001) or, because of the presence of calcium and phosphate, analogues to the ‘acidocalciosomes’ of Agrobacterium tumefaciens (Seufferheld et al., 2003) or general ‘metabolosomes’ (Brinsmade et al., 2005).

**Genomic and phylogenetic characterization**

Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen) following the manufacturer’s instructions. The 16S rRNA gene was amplified as described by Rainey et al. (1996) using the primers 10–30 forward and 1500 reverse. PCR products were purified with the QiAquick PCR purification kit (Qiagen) and sequenced directly by using the CEQ Dye Terminator cycle sequencing kit and on a CEQ 8000 Genetic Analysis system. 16S rRNA gene sequences were aligned with corresponding sequences from the DSMZ database using the ae2 editor (Maidak et al., 1997). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). A distance analysis dendrogram was reconstructed by the neighbour-joining and maximum-likelihood algorithms (Felsenstein, 1993) and by the algorithm of De Soete (1983). Bootstrap analysis was used to evaluate the tree topology by performing 500 resamplings (Felsenstein, 1985).

Nearly complete 16S rRNA gene sequences (1508 nt) were obtained from strains WB 3.4-79T and WB 3.3-25. As the two sequences were identical, only that of strain WB 3.4-79T was analysed further. Preliminary comparison against the 16S rRNA gene sequences, showing the nearest neighbours (Supplementary Fig. S2), indicated that strain WB 3.4-79T belonged to the Chitinibacter–Iodobacter branch of the family Neisseriaceae, class Betaproteobacteria. The sequence of strain WB 3.4-79T was then included in the DSMZ database of 16S rRNA gene sequences and similarity values were determined. On the basis of these values, the closest described type strains were Chitinibacter tainanensis BCRC 17254T (93.8 %), Formivibrio citricus DSM 6150T (92.6 %), Silvimonas terrae KCTC 12358T (91.9 %) and Iodobacter fluviatilis ATCC 33051T (92.3 %). The 16S rRNA gene sequence similarity between strain WB 3.4-79T and other members of the order Neisseriales was less than 90 %. Strain WB 3.4-79T and the four type strains indicated above formed a monophyletic clade with a high bootstrap value (99 %), which was in accordance with the different treeing algorithms employed. While strain WB 3.4-79T branched adjacent to Chitinibacter tainanensis BCRC 17254T according to the neighbour-joining and De Soete analyses (Fig. 2), maximum-likelihood analysis showed strain WB 3.4-79T and Formivibrio citricus DSM 6150T to be phylogenetic neighbours (Supplementary Fig. S2).

Data from the literature (compiled by Stackebrandt & Ebers, 2006) indicate that strains may belong to different genospecies even if they share 100 % 16S rRNA gene sequence similarity. Therefore, the genomic relatedness between the two novel strains was determined by the spectrophotometric DNA–DNA reassocation method. DNA was isolated as described by Marmur (1961) and was fragmented by ultrasonication (Bandelin Sonoplus) for 2 min at 170 μm. DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications of Huß et al. (1983) and Escara & Hutton (1980), using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multiclcell changer and a temperature controller with in-situ temperature probe (Varian). The G + C content of the DNA of the two strains was determined according to Mesbah et al. (1989). The DNA relatedness of strains WB 3.4-79T and WB 3.3-25 was 100 %. The G + C content of strain WB 3.4-79T was 48.5 mol % (two determinations).

**Analyses of chemotaxonomic properties**

Except for fatty acid analysis, biomass for chemotaxonomic studies was prepared by growing the strain in shake flasks in

---

**Fig. 2.** Additive phylogenetic tree (neighbour-joining), based on 16S rRNA gene sequences, showing the nearest neighbours of strain WB 3.4-79T within the family Neisseriaceae, class Betaproteobacteria. Numbers at branch points indicate percentage bootstrap values from 1000 datasets. Only bootstrap values greater than 60 % are shown. Bar, 5 % difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting any two organisms.
Phenotypic characterization

Cultural properties such as colony size, shape and colour were determined after 3 days incubation at 28 °C on R2A (Difco 218263). Conventional biochemical tests were performed according to standard methods (Smibert & Krieg, 1994). The data obtained are indicated in the species description and in Table 2. Accumulation of poly-β-hydroxybutyrate was determined as described by Ostle & Holt (1982). Physiological and biochemical tests were performed on the two isolates and Chitinibacter tainanensis DSM 15459T at 28 °C using API 20NE, API 20E and API 50CH strips (bioMérieux) and Biolog GN microplates (Oxoid). All tests were inoculated with cells grown on R2A. API strips were used according to the manufacturer’s instructions; utilization reactions were observed after 5 days. Acid production from carbohydrates was tested on API 2NE and API 20E supplemented with 0.5, 1, 2, 4, 6, 8 and 10 % (w/v) NaCl at 28 °C. Good growth was observed between 4 and 32 °C over 72 h. The pH range for growth was tested in buffered R2A at pH 5.8–8.5, with an optimum around pH 7.3–7.6.

Cultural characteristics

The novel strains grew well on R2A agar, Bacto tryptic soy broth agar without glucose (BTS; BD 286226), H3P agar (DSMZ medium 428) and Columbia agar with 5 % sheep blood (BD 254005) at 28 °C. The temperature optimum was tested for strain WB 3.4-79T in BTS medium for 72 h, using a temperature gradient incubator model TN-3 (Toyo Kagaku Sangyo) in 1 °C steps, ranging from −1 to 40 °C. Growth was observed between 4 and 32 °C over 72 h. The optimum temperature was 23–28 °C. Cells began to lyse about 29 °C after 22 h; growth was delayed by 24 h at temperatures between 4 and 12 °C. Motility was observed over the whole temperature range.

The pH range for growth was tested in buffered R2A medium at 28 °C between pH 5.1 and 9.5, with steps of about 0.3 pH. Growth of strain WB 3.4-79T occurred at pH 5.8–8.5, with an optimum around pH 7.3–7.6.

Salt tolerance of strain WB 3.4-79T was tested on R2A supplemented with 0.5, 1, 2, 4, 6, 8 and 10 % (w/v) NaCl at 28 °C. Good growth was observed after 3 days incubation at 0.5 %, while growth was weak on agar containing 1 % NaCl. No growth occurred at 2 % NaCl or above.

Tests for chemolithoautotrophic growth were done in mineral medium 81 (DSMZ, 2001) at 28 °C for 7 days under reduced (H2/N2/CO2/air; 70 : 10 : 10 : 10 by vol.) and aerobic conditions. Hydrogen and oxygen served as electron donor and acceptor, respectively. Growth was positive under both conditions and cells grew to similar densities.
Table 2. Characters that distinguish strains WB 3.4-79T and WB 3.3-2.5 from their closest phylogenetic relatives

Strains: 1, WB 3.4-79T and WB 3.3-2.5; 2, Chitinibacter tainanensis DSM 15459T; 3, Silvimonas terrae KCTC 12358T; 4, Formivibrio citricus DSM 6150T. Phenotypic tests were based on API 20NE and API 50CH strips (columns 1 and 2), API 20NE and API 32GN strips (Yang et al., 2005) (column 3) or API 20A strips (Tanaka et al., 1991) (column 4). W, Weak reaction; ND, not determined or no data available.

<table>
<thead>
<tr>
<th>Property</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Rods, straight to slightly curved</td>
<td>Rods, straight to slightly curved</td>
<td>Rods, singly or in pairs</td>
<td>Curved rods, singly or in pairs</td>
</tr>
<tr>
<td>Number of polar flagella</td>
<td>One, rarely two</td>
<td>One or two</td>
<td>One</td>
<td>One</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Relation to O₂</td>
<td>Facultatively anaerobic</td>
<td>Strictly aerobic</td>
<td>Facultatively anaerobic</td>
<td>Strictly anaerobic</td>
</tr>
<tr>
<td>Anaerobic growth on R2A or in R2A broth</td>
<td>+</td>
<td>w</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose in OF test</td>
<td>-</td>
<td>W</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose in API 20NE strips</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Succrose in API 20E strips</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Growth at 40°C</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
<td>+ (after 5 days)</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Chitin hydrolysis</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Utilization of (API 50CH):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Succrose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>N-Acetylgalcosamine</td>
<td>w</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Assimilation of β-hydroxybutyric acid (Biolog)</td>
<td>w</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>48.5</td>
<td>56</td>
<td>58</td>
<td>59</td>
</tr>
</tbody>
</table>

Anti-biotic-sensitivity tests were performed by using filter-paper discs containing 15 different antibiotics and concentrations. Discs were placed on R2A plates spread with WB 3.4-79T culture and were then incubated at 28°C for 3 days. Susceptibility was scored as positive at zone diameters above 13 mm, intermediate susceptibility at 10–12 mm and resistance at less than 10 mm. The reactions are indicated in the species description.

Strains WB 3.4-79T and WB 3.3-25 are virtually identical in all taxonomic criteria used. They were isolated from the same site and may be considered clones, enriched on different growth media. The novel taxon can be distinguished from all neighbours by its significantly lower G+C content and in addition from Chitinibacter tainanensis (Chern et al., 2004) by the lack of chitin and gelatin hydrolysis, lack of growth at 40°C, lack of C16:0 3-OH and C19:0 methyl fatty acids and by some phenotypic reactions. It differs from Silvimonas terrae (Yang et al., 2005) by the lack of hydrolysis of chitin, starch and cellulose, lack of growth at 40°C, lack of C17:0 cyclo and C14:0 3-OH fatty acids and in the quantities of C16:0 fatty acid and those summed in features 4 and 7. While strains WB 3.4-79T and WB 3.3-25 are facultatively anaerobic, Formivibrio citricus (Tanaka et al., 1991) is strictly anaerobic. Due to this feature, only the API 20A (anaerobe) tests could be performed on the latter organism. Based on phylogenetic, cultural and physiological distinctness, we propose the genus Deefgea gen. nov. and Deefgea rivuli sp. nov. as new taxa, with strain WB 3.4-79T as the type strain of Deefgea rivuli.

Description of Deefgea gen. nov.

Deefgea [De.e.f.ge’a. N.L. fem. n. Deefgea arbitrary name derived from the acronym DFG for Deutsche Forschungsgemeinschaft (German Science Foundation)].

Cells are Gram-negative, rod-shaped (0.7–0.8 × 3–4 μm) and facultatively anaerobic. They occur mostly singly (Fig. 1a) and are motile by means of a single flagellum (Fig. 1b) or rarely two polar flagella. Colonies on R2A are circular, convex and pearl white. Catalase and oxidase are positive. Contain polyhydroxybutyrate and polyphosphate granules. The predominant quinone is Q-8. Polar lipids are phosphatidylethanolamine and phosphatidylglycerol. Major cellular fatty acids (> 10%) are C16:0 and C16:1ω7c. The G+C content of the DNA is 49 mol%. Based on 16S rRNA gene sequence analysis, Deefgea belongs to the class Betaproteobacteria, family Neisseriaceae, showing a distant relatedness to Chitinibacter tainanensis, Silvimonas terrae and Formivibrio citricus. The type species is Deefgea rivuli.

Description of Deefgea rivuli sp. nov.

Deefgea rivuli (ri’vu.li. L. gen. masc. n. rivuli of/from a rivulet, a small brook).

In addition to the characters that define the genus, it has the following characteristics. Cells have one round end and one pointed end; in older cultures, larger cells (4–7 μm) are occasionally present. Cells are highly motile at temperatures below 10°C. Colonies on R2A agar are 1–2.5 mm in
diameter, opaque with a smooth, shiny surface and butyrous with entire margins. Similar characteristics define colonies formed on Columbia agar (grey–white, unmbonate, 3.2–4 mm in diameter), H3P (cream–white, 0.5–0.7 mm) and BTS (cream–white, 1.0–2.2 mm). Unspecified enterosomes and a polar organelle are present in the cytoplasm. No acid production in OF test under oxic or anoxic conditions. Grows at 4 °C but not above 32 °C. No growth above 2% NaCl (w/v). pH optimum is between pH 7.3 and 7.6. Grows chemolithoautotrophically under aerobic and reduced conditions. Positive in API 20NE strips for reduction of nitrate to nitrite and utilization of glucose, N-acetylglucosamine and gluconate (all substrates utilized weakly). Tests positive in API 20E strips for acidification of glucose (only transiently after 24 h; recalkalization after 48 h) and acid production from sucrose. Substrates utilized in API 50CH with AUX medium are ribose, glucose, fructose, N-acetylglucosamine, sucrose and glutonate. Substrates positive in Biolog plates are dextrin, N-acetylglucosamin, D-fructose and β-D-mannose; weakly positive for D-mannose, methylpyruvate and D-mannobiose and β-hydroxybutyric acid. No degradation of chitin, cellulose, casein, starch or xylan. Resistant (per disc) to penicillin G (10 IU), oxacillin (5 μg), ampicillin (10 μg), cephalothin (30 μg), cefotaxime (30 μg), pipemdic acid (20 μg), erythromycin (15 μg), vancomycin (30 μg) and lincomycin (15 μg); intermediate susceptible to norfloxacin (10 μg), ofloxacin (5 μg) and colistin (10 μg); susceptible to tetracycline (30 μg), chloramphenicol (30 μg), imipenem (10 μg) and doxycycline (30 μg).

The type strain, WB 34-79^T (=DSM 18356^T = CIP 109326^T), was isolated from water of the Westerholfer Bach, near Westerholfer Bach, Lower Saxony, Germany (51°45′49″N 10°05′31.7″E).

Acknowledgements

This project is part of the Research Unit 571 ‘Geobiology of Organosols and Biofilms’ funded by the German Research Foundation (Sta 184/19-2; DFG-FOR 571; publication #1). We thank Hans Truper for his advice on the nomenclature of the novel species, Peter Schumann for the determination of the G+C content and Anja Frühling, Petra Aumann, Gabriele Potter, Jolanta Swiderski, Sabine Gronow and Stefan Spring for advice and support. The skilful work of TEM sample preparation by Ingeborg Kirsten (HZI, Department of Environmental Microbiology, Braunschweig) is gratefully acknowledged.

References


