**Hydrobacter penzbergensis** gen. nov., sp. nov., isolated from purified water

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A Gram-negative, oxidase- and catalase-positive bacterium, designated strain EM 4T, which varied in shape from rod-shaped to curved or helical with frequently observed bulb-shaped protuberances, was isolated from purified water. 16S rRNA gene sequence analysis indicated that the novel strain belongs to the family Chitinophagaceae within the phylum Bacteroidetes; the closest relative among bacterial species with validly published names was determined to be *Sediminibacterium salmoneum* NBRC 103935T, with 93.4 % sequence identity. The main fatty acids of strain EM 4T were iso-C15:0, iso-C15:1 and iso-C17:0 3-OH. The polar lipid profile consisted of phosphatidylethanolamine, aminolipids, aminophospholipids and unknown lipids; the quinone system consisted of menaquinone MK-7. 16S rRNA gene sequence analysis and the polar lipid and fatty acid profiles suggest that the strain represents a novel genus and species, for which the name *Hydrobacter penzbergensis* gen. nov., sp. nov. is proposed. The type strain of *Hydrobacter penzbergensis* is strain EM 4T (=DSM 25353T=CCUG 62278T).


Strain EM 4T and the closely related strains EM 4.1, EM 4.3 and EM 4.4 were isolated from a purified water system at Roche Penzberg after plating on R2A agar (Oxoid) plates and incubation at 30–35 °C for 7 days. Subcultivation was carried out on R2A agar at 30–35 °C for 32–48 h (standard conditions). Strain EM 4T was able to grow at 15–37 °C, but not at 40 or 10 °C. Colonies of strain EM 4T on R2A agar were tiny, pale yellow, convex, entire to undulate and shiny and reached a diameter of 1–2 mm after 1–2 days of incubation. Strain EM 4T was not able to grow on tryptic soy agar, brain heart infusion agar, PYG agar or MacConkey agar.

To ascertain the morphology of strain EM 4T, cells in the exponential growth phase were analysed with scanning and transmission electron microscopic techniques. For chemical fixation, cells were incubated in 2.5 % glutaraldehyde-containing fixative buffer (75 mM sodium cacodylate, 2 mM MgCl2, pH 7.0) for 1 h at room temperature. Afterwards, samples were rinsed several times in fixative buffer and post-fixed at room temperature for 1 h with 1 % osmium tetroxide in fixative buffer. After two washing steps in water, the cells were stained en bloc for 30 min with 1 % (v/v) uranyl acetate in 20 % (v/v) acetone. Dehydration was performed with a graded acetone series. Samples were then infiltrated and embedded in Spurr’s low-viscosity resin. Ultrathin sections were cut with a diamond knife and mounted onto uncoated copper grids. The sections were post-stained with aqueous lead citrate (100 mM, pH 13.0).
Transmission electron micrographs were taken with an EM 912 electron microscope (Zeiss) equipped with an integrated Omega energy filter operated at 80 kV in the zero-loss mode. For scanning electron microscopy, drops of the sample were placed onto a glass slide, covered with a coverslip and frozen rapidly with liquid nitrogen. The coverslip was then removed with a razor blade and the glass slide was immediately fixed with 2.5 % (v/v) glutaraldehyde in 75 mM cacodylate buffer (pH 7.0), post-fixed with 1 % (v/v) osmium tetroxide in fixative buffer, dehydrated in a graded series of acetone solutions and critical-point dried after transfer to liquid CO2. Specimens were mounted on stubs, coated with 3 nm platinum using a magnetron sputter coater and examined with a Zeiss Auriga scanning electron microscope operated at 1–2 kV.

Cells of EM 4T varied widely in length, diameter and shape, from rod-shaped to curved or helical (Fig. 2a–c). Even at low magnifications, bulb-shaped protuberances were observed frequently in scanning electron microscope preparations (Fig. 2b).

Ultrathin sections revealed a typical Gram-negative architecture for the 25 nm-thick bacterial envelope, which consisted of a 6–7 nm-thick outer membrane, an electron-dense peptidoglycan layer measuring 3–4 nm and a cytoplasmic membrane with a thickness of 6–7 nm (Fig. 2d). The outer membrane frequently undulated, with distances of 10–35 nm to the peptidoglycan layer, which resulted in local changes in the periplasmic space (Fig. 2d), which was in continuity with the bulb-shaped protrusions (Figs 2c, e, f). Osmiophilic globules with diameters of up to 170 nm were frequently observed in the cytoplasm, typically close to or attached to the cytoplasmic membrane (Fig. 2c; circle). Additional membranous structures were observed frequently at the inner face and parallel to the cytoplasmic membrane (Fig. 2d–f).

Strain EM 4T exhibited Gram-negative behaviour and showed catalase and oxidase activity. The Gram-reaction was tested by the non-staining KOH method as described by Fluharty (1967) and by testing for aminopeptidase using ready-made sticks (Merck). Oxidase activity was tested according to Tindall et al. (2007) and catalase with 10 % H2O2. Motility of cells was tested by observing swarming in soft agar (containing 1.0 g yeast extract, 0.1 g K2HPO4 and 2.0 g agar per litre), incubated at 22 °C for up to 5 days, and by using the hanging-drop technique (Skerman, 1967).

Tolerance of NaCl was tested in R2A liquid medium supplemented with 0, 1, 3 and 5 g NaCl l−1 for 14 days. Strain EM 4T grew without NaCl; growth with 1–5 g NaCl
1−1 was negative. The pH range for growth was pH 5.8–7.9; no growth was observed at pH 5.7 or 8.0. Flexirubin-type pigments were detected by flooding a small mass of bacterial cells with 10 % KOH (Bernardet et al., 2002). Conventional biochemical tests were performed according to standard methods (Tindall et al., 2007).

Since strain EM 4T was isolated from pure water, adaptations to very low NaCl and substrate concentrations seemed likely. Following this idea, where possible, the solute concentrations during testing were reduced. Strain EM 4T (and strains EM 4.1, EM 4.3 and EM 4.4) was inactive in API 20 NE tests (Table S1, available in the online Supplementary Material). Even in 1 : 3-diluted AUX medium, no substrate degradation was observed in API 20 NE. Positive reactions occurred only for hydrolysis of aesculin and β-galactosidase activity (p-nitrophenyl β-D-galactopyranosidase). Positive reactions of strain EM 4T could be achieved in mineral medium with several substrates at 0.2 % substrate concentration (Stanier et al., 1966) (see species description). The API strips were inoculated according to the instructions of bioMérieux using a suspension with a turbidity of McFarland 1.5. Strain EM 4T was strictly aerobic; no growth was observed on R2A agar under anaerobic conditions and no acid production in oxidation/fermentation of glucose in Hugh–Leifson’s medium (OF glucose) (Cowan & Steel, 1974) was observed. Anaerobic growth was tested by using anaerobic agar (Difco cat. no. 253610) and also by using Merck Microbiology Anaerocult A Mini. The strain can be differentiated from its phylogenetic neighbours by several reactions (Table 1).

The 16S rRNA gene sequence of strain EM 4T showed very high identity to those of strains EM 4.1, EM 4.3 and EM 4.4 (99.9–100.0 %). Strain EM 4T was selected as a representative strain for further studies. The determined nearly full-length 16S rRNA gene sequence of strain EM 4T comprising 1348 nt was inserted into a SILVA database (Pruesse et al., 2007) based upon the SSU Ref release 111 and its respective alignment of about 630 000 bacterial reference sequence entries according to the SILVA taxonomy, using the ARB software package version 5.5 (Ludwig et al., 2004). The sequence of strain EM 4T was aligned according to the SILVA reference alignment and inspected manually, taking into account secondary structure information and positional variability of the rRNA. A phylogenetic maximum-likelihood tree with 100 bootstrap replicates was reconstructed based on 1705 common alignment columns (no additional filtering of highly variable positions) using RAxML version 8.1 (Stamatakis, 2014) with the GTR nucleotide substitution model and the Gamma model of rate heterogeneity applied. To maximize the stability of the tree topology, the

![Fig. 2. Scanning and transmission electron micrographs of cells of strain EM 4T. The cells varied widely in shape, from rod-shaped to curved or helical (a–c). Bulb-shaped protuberances were frequently observed (b). The bacterial envelope revealed a typical Gram-negative architecture, with a 6–7 nm-thick outer membrane (om), an electron-dense peptidoglycan (pg) layer measuring 3–4 nm and a cytoplasmic membrane (im) with a thickness of 6–7 nm (d). The outer membrane was in continuity with the bulb-shaped protrusions (c, e, f). Osmiophilic globules with diameters of up to 170 nm were frequently observed in the cytoplasm typically close to or attached to the cytoplasmic membrane (c; circle). Additional membranous structures were frequently observed at the inner face and parallel to the cytoplasmic membrane (d–f). Bars, 1 μm (a), 100 nm (b, c, e, f) and 50 nm (d).](image-url)
**Table 1.** Differential characteristics for strain EM 4<sup>T</sup> and closely related strains

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<sup>*</sup>LY, Light yellow; O, orange; PY, pale yellow; SP, salmon pink.

<sup>†</sup>Data taken from: a, Qu & Yuan (2008); b, Shiratori et al. (2009); c, Kämpfer et al. (2011).

For determination of the G+C content, DNA of strain EM 4<sup>T</sup> was degraded to nucleosides by using P1 nuclease and bovine intestinal mucosa alkaline phosphatase, as described by Mesbah et al. (1989). The nucleosides were separated by reversed-phase HPLC (Shimadzu apparatus) according to the method described by Tamaoka & Komagata (1984), and the G+C content was calculated from the ratio of deoxyguanosine to thymidine. The DNA G+C content of strain EM 4<sup>T</sup> was found to be 44.9 mol%.

For analysis of fatty acids, cells were grown on R2A agar for 1 day at 28 °C. Whole-cell fatty acid methyl esters were obtained using methods described previously (Kämpfer & Kroppenstedt, 1996) and separated using a gas chromatograph (model 5898A; Hewlett Packard). Peaks were integrated automatically and fatty acid names and percentages were determined using the Microbial Identification standard software package (MIDI; Sasser, 1990). The major fatty acids (ratio of about 10 % or more) of strain EM 4<sup>T</sup> were iso-C<sub>15</sub>:0, iso-C<sub>15</sub>:1<sup>ω</sup>-OH and iso-C<sub>17</sub>:0<sup>3</sup>-OH, confirming the taxonomic relationship of the strain within the class Flavobacteria (Table 2). Strain EM 4<sup>T</sup> and the related strains differed from related type strains in the presence of larger amounts of iso-C<sub>16</sub>:0, C<sub>15</sub>:0 and iso-C<sub>16</sub>:0<sup>3</sup>-OH (for details, see Table 2).

For extraction of quinones and polar lipids, biomass was grown in R2A medium. Quinones and polar lipids were extracted and analysed as described by Tindall (1990a, b). Respiratory lipoquinones were separated into their different classes (menaquinones, ubiquinones, etc.) by TLC on silica gel (Macherey-Nagel, art. no. 805 023), using hexane/ tert-butyl methyl ether (9 : 1, v/v) as solvent. UV-absorbing bands corresponding to the different quinone classes were removed from the plate and analysed further by HPLC. This step was carried out on an LDC Analytical HPLC (Thermo Separation Products) fitted with a reversed-phase column (Macherey-Nagel; 2 mm × 125 mm, 3 μm, RP18) using methanol/heptane (9 : 1, v/v) as the eluant. Respiratory lipoquinones were detected at 269 nm. The quinone system of EM 4<sup>T</sup> consisted of menaquinone MK-7. Polar lipids were separated by two-dimensional silica gel TLC (Macherey-Nagel, art. no. 818135). The first direction was developed in chloroform/methanol/water (65 : 25 : 4, by vol.) and the second in chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). Total lipid material was detected using molybdophosphoric acid, and specific functional groups were detected using spray reagents specific for defined functional groups. Full details are given in Tindall et al. (2007). The polar lipid profile consisted of phosphatidylethanolamine, aminolipids, aminophospholipids and unknown lipids (Fig. 3), similar to profiles of close relatives in the family Chitinophagaceae (Fig. S2).

Major characteristics in the fatty acid profiles, the polar lipids and 16S rRNA gene sequence analysis suggest placement of EM 4<sup>T</sup> in the family Chitinophagaceae. In contrast to close relatives within this family, a characteristic feature of strain EM 4<sup>T</sup> was the frequently observed bulb-shaped protuberances. In conclusion, strain EM 4<sup>T</sup> should be regarded as the
type strain of the type species of the novel genus Hydrobacter gen. nov., for which we propose the name Hydrobacter penzbergensis sp. nov.

Description of Hydrobacter gen. nov.

Hydrobacter (Hy.dro.bac’ter. Gr. n. hydor water; N.L. masc. n. bacter a rod; N.L. masc. n. Hydrobacter a rod of water).

Cells are Gram-reaction-negative, aerobic and gliding, exhibit bulb-shaped protuberances and are non-spore-forming. The cells are rod-shaped to curved or helical and vary widely in length and diameter. Oxidase-positive. Growth occurs after 24 h of incubation on R2A agar at 28–30 °C. Sensitive to salt concentrations. Main fatty acids are iso-C15 : 0, iso-C15 : 1 and iso-C17 : 0 3-OH. Other fatty acids of the type strain of the type species are listed in Table 2. The polar lipid profile consists of phosphatidylethanolamine, aminolipids, aminophospholipids and unknown lipids. The quinone system consists of menaquinone MK-7. The DNA G+C content of the type strain of the type species is 44.9 mol%. The type species is Hydrobacter penzbergensis.

Description of Hydrobacter penzbergensis sp. nov.

Hydrobacter penzbergensis [penz.ber.gen’sis. N.L. masc. adj. penzbergensis referring to the town of Penzberg (Germany), where the type strain was isolated].

The description is the same as for the genus with the following additional characteristics. Cells reveal a typical Gram-negative architecture of a 25 nm-thick bacterial envelope, which consists of a 6–7 nm-thick outer membrane, an electron-dense peptidoglycan layer measuring 3–4 nm and a cytoplasmic membrane with a thickness of 6–7 nm. The outer membrane frequently undulates, with distances of 10–35 nm to the peptidoglycan layer, which results in local changes in the periplasmic space. Osmiophilic globules with diameters of up to 170 nm are frequently observed in the cytoplasm, typically close to or attached to the cytoplasmic membrane. Additional membranous structures are frequently observed at the inner face.
and parallel to the cytoplasmic membrane. On R2A agar, tiny, pale yellow, convex, entire to undulate and shiny colonies with a diameter of 1–2 mm are formed after 1–2 days of incubation. No growth on tryptic soy agar, brain heart infusion agar, PYG agar or MacConkey agar. Cells are catalase-positive and non-fluorescent. Does not reduce nitrate to nitrite. No growth at pH 5.7 or 8. No aerobic acid production in oxidation/fermentation of glucose in Hugh–Leifson’s medium. The only positive reactions in the API 20 NE strip are hydrolysis of ascinulin and β-galactosidase activity (p-nitrophenyl β-D-galactopyranosidase). In API 50 CHE tests (Table S2), positive for fermentation of D- and L-arabinose, fructose, rhamnose, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylgalcosamine, amygdalin, aesculin, salicin, cellubiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melizitose, raffinose, starch, gentiobiose, D-fucose and 5-ketogluconate. In API ZYM tests (Table S3), positive for alkaline phosphatase, esterase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-fucosidase. Degrades D- and L-arabinose, D-mannose, N-acetylglucosamine, cellubiose, maltose, melibiose, lactose, sucrose, trehalose, xylose, rhamnose, raffinose, D-fructose, D-galactose and L-glutamate. No production of hydrogen sulfide or indole. Hydrolyses starch and gelatin but not chitin, cellulose, tyrosine, DNA, casein or Tween 80. Temperature range for growth is 15–37 °C; no growth at 10 or 40 °C. The pH range for growth is pH 5.8–7.9; no growth at pH 5.7 or 8.0. Sensitive to higher substrate concentrations.

The type strain is strain EM 4T (=DSM 25353T=CCUG 62278T), isolated from a pure water system in Germany.

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References


