**Empedobacter stercoris** sp. nov., isolated from an input sample of a biogas plant

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Two Gram-stain-negative, rod-shaped bacteria, strains 990B6_12ER2AT and 994B6_12ER2A, were isolated during microbiological analysis of a mixed manure sample which was used as input material for a German biogas plant. Phylogenetic identification based on nearly full-length 16S rRNA gene sequences placed the isolates into the family Flavobacteriaceae within the phylum Bacteroidetes. Strains 990B6_12ER2AT and 994B6_12ER2A shared identical 16S rRNA gene sequences and showed highest 16S rRNA gene sequence similarity to the type strains of *Empedobacter falsenii* (97.3 %) and *Empedobacter brevis* (96.8 %).

The major cellular fatty acids of strains 990B6_12ER2AT and 994B6_12ER2A were iso-C₁₅ : ₀, summed feature 3 (C₁₆ : ₁₀₇c and/or C₁₆ : ₁₁₆c) and iso-C₁₇ : ₀ 3-OH.

The polyamine pattern contained predominantly sym-homospermidine and the quinone system was menaquinone MK-6. Major polar lipids were phosphatidylethanolamine, one unidentified aminolipid and one unidentified polar lipid not containing an amino residue, a phosphate residue or a sugar moiety. In addition, moderate to minor amounts of several unidentified lipids were detected. The DNA G+C content was 31.7 and 29.0 mol%, for strains 990B6_12ER2AT and 994B6_12ER2A, respectively. On the basis of phylogenetic, chemotaxonomic and physiological analysis we propose a novel species of the genus *Empedobacter, Empedobacter stercoris* sp. nov. (type strain 990B6_12ER2AT=CIP 110833T=LMG 28501T).
strains was suspended twice in calf serum albumin and stored at −20 °C. After purification, the two strains showed the same pigmentation and morphology as the original colonies. After incubation of 5 days on R2A at 25 °C, strain 990B6_12ER2AT changed colony pigmentation from cream to yellowish. Strain 994B6_12ER2A formed yellow colonies which did not change pigmentation with time of incubation. Strains could also be cultured at 25 °C on R2A (Oxoid), nutrient agar (Oxoid), tryptic soy agar (TSA; Becton Dickinson), PYE (0.3 % yeast extract and 0.3 % casein peptone and 15 g agar 1−1, pH 7.2), CASO agar (Carl Roth), K7 (0.1 %, w/v, yeast extract, peptone and glucose, 15 g agar 1−1, pH 7.2), medium 65 (according to DSMZ), DEV agar (DEV; Merck), Luria–Bertani (LB; Sigma-Aldrich), malt agar (Merck) and marine agar 2216 (MA; Becton Dickinson), but not on MacConkey (Merck).

Genomic DNA for phylogenetic identification was obtained from a loop of biomass suspended in DNase- and RNase-free water (Carl Roth) and subjected to three cycles of freezing (−20 °C) and subsequent heating (100 °C, 1 min). Nearly full-length 16S rRNA gene sequences were obtained by PCR amplified with primers 8F (5′-AGAGTTTGATCCTG-GCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACG-3′); Lane, 1991). PCRs were performed in a total volume of 25 μl containing 1 × DreamTaq PCR buffer, 0.2 mM of each dNTPs 0.2 μM of each primer, 0.04 mg BSA 1−1, and 0.5 U DreamTaq DNA polymerase, and as template 1 μl of the cell lysate supernatant. All chemicals except primer were obtained from Fermentas (now Thermo Scientific). PCR conditions were as follows: 95 °C 3 min, 28 cycles of 95 °C 30 s, 57.3 °C 30 s and 72 °C 1.5 min, and finally 72 °C 15 min. PCR purification and Sanger sequencing with primers 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GATTAGATACCTGGTAG-3′) (Coloqhoun, 1997) were performed by LGC Genomics, Berlin, Germany. DNA sequences were processed manually in MEGA 5 (Tamura et al., 2011) based on the electropherograms by removing unclear 5′and 3′ ends. Final sequences were 1447 nt long spanning 16S rRNA gene positions 46 to 1505 (according to the Escherichia coli numbering; Brosius et al., 1981). A first phylogenetic placement of the strains was obtained by BLAST analysis in the EzTaxon-e type strain database (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012).

Detailed phylogenetic analysis was performed in ARB release 5.2 (Ludwig et al., 2004) using the ‘All-Species Living Tree’ project (LTP) (Yarza et al., 2008) database LTPs115 (March 2014). 16S rRNA gene sequences of the novel strain were aligned with the SILVA incremental aligner (SINA; Pruesse et al., 2012) based on the SILVA seed alignment (http://www.arb-silva.de; Pruesse et al., 2007) and implemented into the LTP database. Reference type strain sequences not implemented in the LTP database were added in the same manner by using sequences published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The alignment used for phylogenetic analysis was checked manually. Sequence similarities were calculated with the ARB neighbour-joining tool, without an evolutionary model. A maximum-likelihood tree was calculated with RAxML version 7.04 (Stamatakis, 2006) using GTR-GAMMA and rapid bootstrap analysis, and a maximum-parsimony tree using DNAPARS v3.6 (Felsenstein, 2005). Both trees were based on 100 resamplings (bootstrap analysis; Felsenstein, 1985) and sequences between gene termini 55 and 1471 (Escherichia coli numbering; Brosius et al., 1981).

Strains 990B6_12ER2AT and 994B6_12ER2A shared identical 16S rRNA gene sequences. The next most closely related type strains were Empedobacter falsenii NF 993T and Empedobacter brevis LMG 4011T with 97.3 and 96.8 % similarity, respectively, to strains 990B6_12ER2AT and 994B6_12ER2A. Phylogenetic trees showed that strains 990B6_12ER2AT and 994B6_12ER2A formed a distinct cluster with Empedobacter falsenii NF 993T and Empedobacter brevis LMG 4011T, clustering closest to Empedobacter falsenii NF 993T (Fig. 1). DNA–DNA hybridization studies for species differentiation were not performed between the novel strains and the type strain of the most closely related species, Empedobacter falsenii, because the 16S rRNA gene sequence similarity was far below 16S rRNA gene sequence similarity values suggested recently as thresholds for species differentiation by Meier-Kolthoff et al. (2013) (98.2–99.0 %) and Kim et al. (2014) (98.65 %).

The 16S rRNA gene sequence identical strains 990B6_12ER2AT and 994B6_12ER2A were further differentiated by genomic fingerprinting using enterobacterial repetitive intergenic consensus (ERIC)-PCR and randomly amplified polymorphic DNA (RAPD)-PCR with primers A, B and C according to the protocol of Glaeser et al. (2013). RAPD and BOX-PCR fingerprint patterns showed slight but distinct differences between the two strains, indicating that the two isolates represented two genetically closely related but different strains (Fig. 2).

High molecular mass genomic DNA was extracted for more detailed genotypic analysis by the method of Pitcher et al. (1989). The DNA G+C content was determined by the DNA melting temperature method established by Gonzalez & Saiz-Jimenez (2002) with modifications as described previously (Glaeser et al., 2013). The genomic DNA G+C content of strains 990B6_12ER2AT and 994B6_12ER2A was 31.7 and 29.0 %, respectively.

Phenotypic characteristics of the strains were investigated as followed. Gram staining was performed by the modified Hucker method according to the method of Gerhardt et al. (1994). Cell morphology was determined by light microscopy using an Axioskop2 microscope (Zeiss). AxioVision Rel. 4.7. (Carl Zeiss) software was used for cell size measurements. Gliding motility was tested by the hanging drop method as described by Bernardet et al. (2002). Catalase activity was tested by the observation of gas bubbles after the addition of a few drops of 3 % H2O2 to fresh biomass grown on an agar plate. Oxidase activity was determined with the Microbiology Bactident oxidase test strip (Merck). The production of flexirubin-type pigments was tested with the KOH method according to the protocol of Fautz & Reichenbach (1980).
Temperature-dependent growth was tested on TSA at 4, 10, 15, 20, 25, 28, 30, 36, 45, 50 and 55 °C; salinity- and pH-dependent growth was tested at 28 °C in tryptic soy bouillon (TSB) supplemented with 1 to 12 % (w/v) NaCl (in 1 % intervals) or adjusted to pH 4.5 to 12.5 (in 1 pH unit intervals) with 6 M HCl and 1 M NaOH after autoclaving. Further physiological properties were tested using the API20NE, APIZYM and API50CH test strips (bioMérieux) as described by the manufacturer. Bacterial biomass for API20NE and APIZYM was suspended in 0.9 % (w/v) NaCl. Acid production was tested with API 50CH using the CHB/E medium (bioMérieux). Test strips were analysed after 3, 4 and 7 days’ incubation time, respectively.

Cells of strains 990B6_12ER2A and 994B6_12ER2A were rod-shaped with a cell size of 1.08(±0.2) μm × 0.59(±0.1) μm and 1.25(±0.2) μm × 0.73(±0.1) μm, respectively. Optimal growth was obtained between 25 and 36 °C on R2A without or with 1 % (w/v) NaCl and at a pH between pH 6.5 and 10.5. Neither of the strains grew on MacConkey agar, which is in line with the type strains of Chishuiella changwenlii and Weeksella virosa, but not with those of Empedobacter falsenii.

**Fig. 1.** Maximum-likelihood tree based on nearly full-length 16S rRNA gene sequences showing the phylogenetic relationships of strains 990B6_12ER2A and 994B6_12ER2A among the type strains of the most closely related genera within the family Flavobacteriaceae. Numbers at nodes represent bootstrap values >70 % (obtained from 100 resamplings). Filled circles at nodes represent nodes which were also present in the maximum-parsimony tree with bootstrap values >70 %. Three type strains of the family Cryomorphaceae, Phaeocystidibacter luteus PG2S01T (HQ434766), Owenweeksia hongkongensis UST20020801T (AB125062) and Cryomorpha ignava 1-22T (AF170738), were used as an outgroup. The number shown in the outgroup cluster indicates the number of sequences included in the cluster. Bar, 0.1 substitutions per nucleotide position.
and *Empedobacter brevis*. Comparison of the novel strains in physiological tests showed variable results. Strains 990B6_12ER2AT and 994B6_12ER2A differed in enzyme activity for trypsin and x-glucosidase and in colony pigmentation. Phenotypic properties are given in the species description and differentiating phenotypic characteristics with the most closely related taxa are listed in Table 1.

Susceptibility testing to 12 veterinary relevant antibiotics was performed according to Schauss et al. (2015). The following antibiotics were tested at the concentration ranges given in parentheses: the β-lactam antibiotics amoxycillin (0.5–64 mg l\(^{-1}\)), oxacillin (0.25–32 mg l\(^{-1}\)), ceftiofur and ceftirine (0.25–32 mg l\(^{-1}\)) both ± clavulanic acid (4 mg l\(^{-1}\)), the sulfonamides fleroxacin (0.5–64 mg l\(^{-1}\)) and trimethoprim/sulfamethoxazole (0.0625–1.875–8/152 mg l\(^{-1}\)), and sulfamethoxazole (4–256 mg l\(^{-1}\)) and the macrolide tylosin (0.125–16 mg l\(^{-1}\)), tetracycline (0.25–32 mg l\(^{-1}\)) and the fluorochinolon enrofloxacin (0.0625–8 mg l\(^{-1}\)). Inhibition of growth was used as a criterion to differentiate between susceptible and resistant phenotypes. Both strains were susceptible to all concentrations tested of amoxycillin, oxacillin, ceftiofur/± clavulanic acid and enrofloxacin, and resistant to florfenicol (MIC, 1 mg l\(^{-1}\)) and sulfamethoxazole (MICs, 8 and <4 mg l\(^{-1}\) for strains 990B6_12ER2AT and 994B6_12ER2A, respectively). Strain 990B6_12ER2AT was also resistant to ceftirone (MIC, 1 mg l\(^{-1}\)), tetracycline (MIC, 4 mg l\(^{-1}\)), tylosin (MIC, 8 mg l\(^{-1}\)) and trimethoprim/sulfamethoxazole (MIC, 0.25/4.75 mg l\(^{-1}\)), while strain 994B6_12ER2A was susceptible to these respective antibiotics.

Biomass for fatty acid analysis was grown on TSA for 48 h at 28 °C. All strains were approximately in the same growth phase (late exponential growth phase) when the biomass was harvested. Fatty acid extraction and analysis was performed according to the method of Kämper & Kroppenstedt (1996) by fatty acid separation with a 5898A gas chromatograph (Hewlett Packard). Peaks were integrated automatically and fatty acid names and percentages were determined with Sherlock version 2.1 (TSBA version 4.1; MIDI). Major cellular fatty acids of strains 990B6_12ER2AT and 994B6_12ER2A were iso-C\(_{15:0}\) summed feature 3 (C\(_{16:1}\)ω7c and/or C\(_{16:0}\)ω6c) and iso-C\(_{17:0}\) 3-OH, which are characteristic for the genus *Empedobacter* (Vandamme et al., 1994). The most pronounced difference in the fatty acid pattern between strains 990B6_12ER2AT and 994B6_12ER2A was the presence of C\(_{15:0}\) (6 %) in the fatty acid pattern of strain 990B6_12ER2AT and the absence in the fatty acid pattern of strain 994B6_12ER2A. Fatty acid profiles of the two novel strains and the most closely related type strains are given in Table 2.

Analyses of quinones, polar lipids and polyamines were carried out with biomass that had been grown on 3.3 × PYE [1.0 % (w/v) peptone from casein, 1.0 % (w/v) yeast extract, pH 7.2]. Biomass subjected to polyamine analysis was harvested at the late exponential growth phase as recommended (Busse & Auling, 1988) whereas biomass used to extract quinones and polar lipids was harvested at the stationary growth phase. Polyamines were extracted as described by Busse & Auling (1988) and analysed by HPLC as described by Busse et al. (1997). Quinones and polar lipids were extracted and analysed as described by Tindall (1990a, b) and Altenburger et al. (1996). The HPLC apparatus was used as described by Stolz et al. (2007). The polar lipid profile consisted of the major compounds phosphatidylethanolamine and the two unidentified lipids aminolipid AL1 and lipid L3. In addition, moderate to minor amounts of four lipids (L1, L2, L4, L5), four unidentified aminolipids (AL2–AL5) and one unidentified glycolipid (GL) were detected in strain 990B6_12ER2AT (Fig. 3). The quinone system contained exclusively menaquinone MK-6. The polyamine pattern was composed of sym-homospermidine (24.2 µmol (g dry weight)\(^{-1}\)), spermidine, (0.9 µmol (g dry weight)\(^{-1}\)), spermine (0.5 µmol (g dry weight)\(^{-1}\)), putrescine and cadaverine [each 0.2 µmol (g dry weight)\(^{-1}\)]. The presence of menaquinone MK-6 and a polyamine pattern with the predominant compound sym-homospermidine have also been reported for the two established species of the genus *Empedobacter*, *Empedobacter brevis* and *Empedobacter falsenii* (Dees et al. 1986; Zhang et al., 2014b). Overall, the polar lipid profile of 990B6_12ER2AT also showed a high degree of similarity to those of *Empedobacter brevis* and *Empedobacter falsenii* (Zhang et al., 2014a). However, presence of some minor lipids and especially of the unidentified glycolipid (Fig. 3) clearly distinguished strain 990B6_12ER2AT from its closest relatives.

On the basis of genotypic, phenotypic and chemotaxonomic data, the two strains investigated (990B6_12ER2AT and 994B6_12ER2A) are two representatives of a novel species of the genus *Empedobacter* with the proposed name *Empedobacter stercoris* (type strain 990B6_12ER2AT=CIP 110833=LMG 28501\(^{T}\)). The two strains were isolated from the same
sample. They shared identical 16S rRNA gene sequences but differed in RAPD- and ERIC-PCR patterns, colony pigmentation, fatty acid composition and antibiotic susceptibility.

Empedobacter stercoris sp. nov.

Empedobacter stercoris (ster’co.ris. L. gen. n. stercoris of faeces).

Cells are Gram-stain-negative, non-motile and rod-shaped with a size of 1.1–1.3 µm × 0.6–0.7 µm. Colonies are small in size (<1 mm) and cream (type strain) or yellow after 48 h of growth on R2A agar at 28 °C. Cells are oxidase-positive. Production of flexirubin-type pigments is strain specific. Culturable on a broad range of media at 28 °C: R2A, nutrient agar, TSA, PYE, CASO, K7, medium 65, DEV, LB, MA, slightly on malt agar and glycine/arginine agar but not on MacConkey agar. Grows on TSA medium between 15 and 36 °C, slightly at 10 °C but not at 4, 45, 50 or 55 °C, well at a pH range of pH 6.5 to 11.5, slightly at pH 5.5 and 12.5 but not at pH 4.5, and at a salt concentration of 0–3 % (w/v) NaCl, but not at 4 % and higher. Optimal growth occurred at 28 °C in the presence of 0–1 % NaCl and at neutral pH (around pH 7) on R2A agar.

### Table 1. Differentiating phenotypic characteristics of strains 990B6_12ER2A<sup>T</sup> and 994B6_12ER2A and the type strains of the most closely related species

<table>
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<td>33.8–34.4§</td>
<td>38.2∥</td>
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*Data were obtained from Zhang et al. (2014a).
†Variable data were reported by Holmes et al. (1978, 1986), Hollis et al. (1995) and Kämpfer et al. (2006).
§Vandamme et al. (1994).
∥Kämpfer et al. (2006).
||Holmes et al. (1986).
In API 20NE, positive for gelatinase activity and indole production. Positive enzyme activities (API ZYM) for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, a-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase, negative for lipase (C14), esterase (C4), cystine arylamidase, a-galactosidase, N-acetyl-b-glucosaminidase, a-mannosidase and a-fucosidase, and variable for trypsin, a-glucosidase and b-glucuronidase activity. In API 50CHB acids are not produced from tested substrate.

The fatty acids profile is characterized by the presence of several branched and hydroxyl fatty acids with highest amounts of iso-C15:0, summed feature 3 (C16:1ω7c and/or C16:1ω6c) and iso-C17:0 3-OH.

The polar lipid profile consists of the major compounds phosphatidylethanolamine and two unidentified aminolipids AL1 and lipid L3. In addition, moderate to minor amounts of four lipids, four unidentified aminolipids and one unidentified glycolipid are present. The quinone system contains exclusively menaquinone MK-6. The polyamine pattern is composed of the major polyamine sym-homospermidine and minor amounts of spermidine, spermine, putrescine and cadaverine.

Susceptible to amoxicillin, oxacillin, ceftiofur + clavulanic acid, cefquinome + clavulanic acid, and enrofloxacin, variable to cefquinome, tetracycline, tylosin and trimethoprim/sulfamethoxazole, and resistant to florfenicol and sulfamethoxazole. The DNA G+C content is 29–32 mol%.

The type strain, 990B6_12ER2AT (=CIP 110833T =LMG 28501T), was isolated from a mixed sample of manure and slurry from dairy cattle and breeding sows mixed in a ratio of 2 : 1, which was used as input material in a German biogas plant.

### Acknowledgements

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References


