

Denitratisoma oestradiolicum gen. nov., sp. nov., a 17 β -oestradiol-degrading, denitrifying betaproteobacterium

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A Gram-negative, motile, denitrifying bacterium (strain AcBE2-1^T) was isolated from activated sludge of a municipal wastewater treatment plant using 17 β -oestradiol (E2) as sole source of carbon and energy. Cells were curved rods, 0.4–0.8 \times 0.8–2.0 μ m in size, non-fermentative, non-spore-forming, oxidase-positive and catalase-negative. E2 was oxidized completely to carbon dioxide and water by reduction of nitrate to a mixture of dinitrogen monoxide and dinitrogen, with the intermediate accumulation of nitrite. Electron recoveries were between 90 and 100 %, taking assimilated E2 into account. With nitrate as the electron acceptor, the bacterium also grew on fatty acids (C₂ to C₆), isobutyrate, crotonate, DL-lactate, pyruvate, fumarate and succinate. Phylogenetic analysis of its 16S rRNA gene sequence revealed that strain AcBE2-1^T represents a separate line of descent within the family *Rhodocyclaceae* (*Betaproteobacteria*). The closest relatives are the cholesterol-degrading, denitrifying bacteria *Sterolibacterium denitrificans* DSM 13999^T and strain 72Chol (= DSM 12783), with < 93.9 % sequence similarity. The G + C content of the DNA was 61.4 mol%. Detection of a quinone system with ubiquinone Q-8 as the predominant compound and a fatty acid profile that included high concentrations of C_{16:1 ω 7c}/iso-C_{15:0} 2-OH and C_{16:0}, in addition to C_{18:1 ω 7c} and small amounts of C_{8:0} 3-OH, supported the results of the phylogenetic analysis. On the basis of 16S rRNA gene sequence data in combination with chemotaxonomic and physiological data, strain AcBE2-1^T (= DSM 16959^T = JCM 12830^T) is placed in a new genus *Denitratisoma* gen. nov. as the type strain of the type species *Denitratisoma oestradiolicum* gen. nov., sp. nov.

The natural steroid hormone 17 β -oestradiol (E2) belongs to the C-18 steroids and exhibits an aromatic ring A, which shows phenolic properties. In comparison with its biosynthetic precursor cholesterol, in E2 the angular methyl group between rings A and B (C-19) and the aliphatic side chain are absent (Breitmaier & Jung, 1995). This lipophilic signal

compound regulates metabolism, growth and reproduction in vertebrates. E2, excreted by humans and livestock, is frequently detected in the environment and is likely to cause endocrine-disrupting effects in aquatic wildlife (Sumpter & Johnson, 2005). The main sources of E2 are municipal effluent discharge (Ternes *et al.*, 1999), runoff from agricultural production and farmyard manure applied as an organic fertilizer (Hanselman *et al.*, 2003). The aerobic degradation of E2 has been studied in aerobic bacterial genera such as *Novosphingobium* (Fujii *et al.*, 2002, 2003), *Rhodococcus* (Yoshimoto *et al.*, 2004) and *Nocardia* (Coombe *et al.*, 1966). Recent studies have demonstrated that E2 is degraded under denitrifying conditions during conventional wastewater treatment processes and during

Abbreviation: E2, 17 β -oestradiol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AcBE2-1^T is AY879297.

An expanded phylogenetic tree showing the affiliation of the 16S rRNA gene sequence from strain AcBE2-1^T to selected reference sequences of members of the *Betaproteobacteria* is available as a supplementary figure in IJSEM Online.

laboratory-scale experiments (Andersen *et al.*, 2003; Joss *et al.*, 2004). Nothing is yet known about the bacteria responsible for mineralizing steroidal hormones under anoxic conditions. Here we report the description of a novel denitrifying bacterium, designated strain AcBE2-1^T, which degrades E2 completely with nitrate as the electron acceptor.

Techniques for the preparation of media and cultivation of bacteria under anoxic conditions have been described elsewhere (Widdel & Bak, 1992). E2 was added under sterile conditions to empty culture vessels in defined amounts dissolved in acetone (stock solution of 20 g E2 l⁻¹). After complete evaporation of the solvent, the medium was dispensed anoxically. A mixed activated sludge sample, taken from two aeration basins of a municipal wastewater treatment plant, was pretreated with 50 mg cycloheximide l⁻¹ to prevent growth of eukaryotes. After removal of this cycloheximide, enrichment cultures were established by inoculating an oxygen-free, bicarbonate-buffered freshwater medium (Widdel & Bak, 1992) containing 1 mM E2 as the sole source of energy and 5 mM nitrate as the electron acceptor. This medium also contained trace element solution SL10 (Widdel *et al.*, 1983), selenite tungstate solution (Widdel *et al.*, 1983), seven-vitamin solution (Widdel & Pfennig, 1981) and 0.5 mM Na₂SO₄ as sulfur source under a N₂/CO₂ (80:20, v/v) atmosphere. The pH was 7.1–7.4. Isolation and routine cultivation of strain AcBE2-1^T were performed at 28 °C in the dark. Cultures were briefly shaken once per day to allow for homogeneous distribution of bacteria and substrate crystals. Soluble substrates and electron acceptors were added anoxically from sterile, neutralized stock solutions or were added prior to autoclaving.

Aerobic growth was tested in potassium phosphate-buffered medium (20 mM, pH 7.2) at 28 °C with shaking at 120 r.p.m. Dependence of growth on pH was tested in anoxic freshwater medium buffered with 20 mM potassium phosphate or 10 mM HEPES. Glycerol phosphate (1.5 mM) was added to HEPES-buffered medium to account for a lack of phosphate. The temperature range (tested at 4–50 °C) for growth was determined in bicarbonate-buffered medium. In all growth experiments described in this paragraph 5 mM sodium acetate and 5 mM nitrate were used and the increase in optical density at 578 nm was determined by use of a Perkin Elmer 550 SE spectrophotometer.

Purity was checked by microscopic examination after incubation in medium containing E2 as well as in medium with 5 mM fumarate, 5 mM pyruvate, 0.05% (w/v) yeast extract, 5 mM D(+)-glucose or 0.7 g peptone l⁻¹. For maintenance, stock cultures grown on E2 were stored at 4 °C and transferred every 6–8 weeks. Long-term storage of bacteria was achieved as stocks in glycerol (25%, v/v) at –80 °C under oxic conditions. Gram type was determined as described by Süßmuth *et al.* (1987) and using the KOH test (Gregersen, 1978). *Staphylococcus epidermidis* DSM 1798 and *Nitrosomonas europaea* ATCC 19718 were used as controls. Cytochrome oxidase activity was determined with

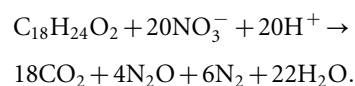
a Merck Bactident oxidase strip. Detection of catalase activity was carried out by using a standard method (Gerhardt *et al.*, 1994). Purity and cell dimensions were determined by phase-contrast microscopy using a Leitz microscope. Specimens for scanning electron microscopy were prepared according to a modification of the protocol of Bruce *et al.* (1999) and were viewed using a Philips XL 30 ESEM FEG scanning electron microscope at 20 kV.

Isoprenoid quinone analysis was performed by reversed-phase TLC according to the method of Collins (1985). The fatty acid profile of strain AcBE2-1^T (method according to Kämpfer & Kroppenstedt, 1996) was analysed from biomass obtained after growth on bicarbonate-buffered medium with 5 mM sodium acetate and 5 mM nitrate.

Analysis of the G + C content of the DNA was performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) using the HPLC method and conditions as described by Mesbah *et al.* (1989) and Tamaoka & Komagata (1984). Purification and enzymic digestion of the DNA were performed according to Cashion *et al.* (1977) and Mesbah *et al.* (1989).

For determination of the 16S rRNA gene sequence, standard protocols were used (Kuever *et al.*, 2001). Sequencing was performed by AGOWA (Berlin, Germany). The 16S rRNA gene sequence of strain AcBE2-1^T was compared against the NCBI database (<http://www.ncbi.nlm.nih.gov>) using the BLAST tool (Altschul *et al.*, 1997). Sequences that were not included in the ARB database of the Technical University Munich were loaded into the database and aligned using the ARB_ALIGN tool (Ludwig *et al.*, 2004). Alignment was visually inspected and corrected manually using the sequence editor ARB_EDIT. Tree topologies were evaluated by performing maximum-parsimony, neighbour-joining and maximum-likelihood analyses with different sets of filters. Only sequences of at least 1300 nt were used for the calculation of different trees.

Strain AcBE2-1^T was isolated by two subsequent serial dilution series with E2 as the sole electron donor and nitrate as electron acceptor. Cells of AcBE2-1^T showing the site of insertion of its flagellum are shown in Fig. 1. Physiological studies of the anaerobic metabolism of E2 revealed that it was oxidized completely to carbon dioxide and water by reduction of nitrate to a mixture of dinitrogen monoxide and dinitrogen, with the intermediate accumulation of nitrite (data not shown). The degradation equation is as follows:



The doubling time with E2 as electron donor was 23–25 h at 30 °C and pH 7.2. Electron recoveries were between 90 and 100%, taking assimilated E2 into account (data not shown). Further physiological characteristics are listed in Table 1 and in the species description below.

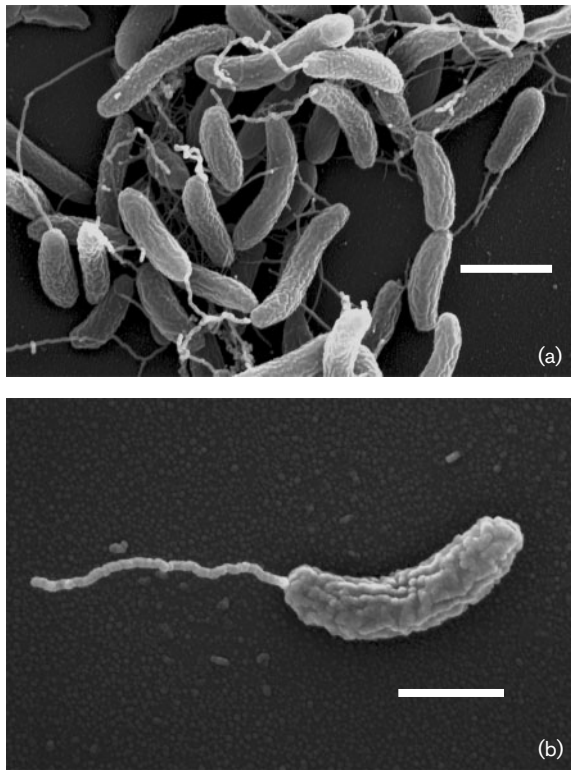


Fig. 1. Scanning electron micrographs of cells of strain AcBE2-1^T grown on 5 mM acetate and 5 mM nitrate. Bars, 1 μ m (a), 0.5 μ m (b).

Comparative 16S rRNA gene sequence analysis showed that strain AcBE2-1^T belongs to the order *Rhodocyclales* within the *Betaproteobacteria* (Fig. 2). Highest sequence similarity was to *Sterolibacterium denitrificans* DSM 13999^T (Tarlera & Denner, 2003) and the partially described strain 72Chol (Harder & Probian, 1997), with similarity values below 93.9%. Together with clone sequences obtained from several habitats, strain AcBE2-1^T forms a separate cluster distinct from known lineages, such as *Dechloromonas*–*Ferribacterium*–*Quadricoccus*–*Azonexus*, *Azovibrio*, *Propioniovibrio*–*Rhodocyclus*, *Azospira*, *Azoarcus*–*Thauera* and *Zoogloea* (see Supplementary Fig. S1, available in IJSEM Online). The only described organisms within this cluster are *Sterolibacterium denitrificans* and strain 72Chol. The most similar 16S rRNA gene sequences obtained were from borehole water (AY741708 and AY741714; 95.6% sequence similarity) and a series of sequences that resemble nitrogen-fixing isolates obtained from wild rice (AY235685, AY235684, AY235688 and AY235687; sequence similarity of 95.5–95.7%). Interestingly, the latter sequences were only revealed during a BLAST search at low ranking positions, because the main fragment showed relatively low sequence similarity (<94%). Taken together with the high level of sequence similarity of a short fragment (97%), the overall sequence similarity given above was reached. The only described species within this cluster seemed to be specialized in steroid degradation, indicating

that substrates of this type lead to the enrichment of members of this cluster. All other members of this cluster resemble clone sequences of hitherto uncultivated bacteria (see Supplementary Fig. S1 in IJSEM Online), and information regarding their physiological properties is lacking. Several clone sequences within this cluster belong to the *Proteobacteria* and probably contribute to wastewater treatment (Juretschko *et al.*, 2002; Loy *et al.*, 2005; Crocetti *et al.*, 2000). It would be of interest to determine the physiology of these bacteria and to investigate their ecological significance.

Analysis of quinones revealed a spot that corresponded to ubiquinone-8 (Q-8). This quinone system is a characteristic feature of the *Betaproteobacteria* (Collins & Jones, 1981; Yokota *et al.*, 1992). The fatty acid profile of strain AcBE2-1^T consisted mainly of C_{16:0} (29.8%) and summed feature 3 (C_{16:1 ω 7c} and/or iso-C_{15:0} 2-OH; 53.4%). The fatty acids C_{14:0} (0.6%), C_{16:1 ω 5c} (1.5%), C_{18:1 ω 5c} (0.3%), C_{18:1 ω 7c} (11.8%) and C_{18:0} (0.3%) were found in minor amounts. C_{8:0} 3-OH (2.3%) was the only hydroxylated fatty acid detected. This fatty acid profile is clearly different from that of *Sterolibacterium denitrificans*, for which C_{16:0}, summed feature 3 and C_{18:1 ω 7c} were also detected in major amounts. However, *Sterolibacterium denitrificans* also contained C_{10:0} 3-OH, C_{16:0} 3-OH and (in minor amounts) C_{8:0} 3-OH (Tarlera & Denner, 2003).

The reason for erecting a new genus to accommodate strain AcBE2-1^T was the high sequence divergence from related genera and phenotypic features that were characteristically different from those of related taxa. In contrast to its nearest relatives *Sterolibacterium denitrificans* DSM 13999^T and strain 72Chol, strain AcBE2-1^T was able to use E2, short-chain fatty acids and several citric acid cycle intermediates, but not cholesterol or long-chain fatty acids. On the basis of 16S rRNA gene sequence analysis in combination with chemotaxonomic and physiological data, strain AcBE2-1^T is considered to be the type strain of a novel species in a new genus, for which the name *Denitratisoma oestradiolicum* gen. nov., sp. nov. is proposed.

Description of *Denitratisoma* gen. nov.

Denitratisoma (De.ni.trati.so'ma. L. pref. *de-* away from; N.L. n. *nitratis* nitrate; N.L. neut. n. from Gr. neut. n. *soma* body; N.L. neut. n. *Denitratisoma* a body that reduces nitrate).

Cells are Gram-negative, non-spore-forming, motile, curved rods (0.4–0.8 \times 0.8–2.0 μ m in size) with rounded ends, and occur singly or in pairs. In cultures grown on sodium acetate and nitrate, more elongated cells with light-refracting inclusions predominate (length up to 2.0 μ m). Metabolism is strictly oxidative. Nitrate is reduced to a mixture of N₂O and N₂; nitrite accumulates intermediately. Oxidase-positive and catalase-negative. Anaerobic oxidation of E2 to CO₂ with nitrate as electron acceptor, but no growth with cholesterol or C-19 steroids such as testosterone or 4-androstene-3,17-dione. Electron acceptors nitrate, nitrite or (per)chlorate are used; sulfate or sulfite

Table 1. Physiological, cytological and morphological properties of strain AcBE2-1^T and related taxa

Strains: 1, AcBE2-1^T; 2, *Sterolibacterium denitrificans* Chol-1S^T (=DSM 13999^T); 3, 72Chol (=DSM 12783). Concentrations (in mM) of electron donors and acceptors in the medium of strain AcBE2-1^T are given in parentheses in the first column. +, Growth; (+), weak growth; -, no growth; mp-mt, monopolar-monotrichous; ND, not determined. Data were taken from Tarlera & Denner (2003), Harder & Probian (1997) and Kniemeyer (1998), unless specified otherwise. All three strains are negative for Gram stain and positive for cytochrome oxidase (the latter was confirmed in this study for strain 72Chol); they were also negative for utilization of 17 α -ethinyloestradiol (1), testosterone (1) and 19-nor-testosterone (1) (these latter characters were confirmed in this study for strains Chol-1S^T and 72Chol). Cell lysis occurred during growth of strain AcBE2-1^T on testosterone and 4-androstene-3,17-dione.

Character	1	2	3
Cell morphology	Curved rod	Curved rod	Rod
Cell dimensions (width \times length) (μ m)	0.4–0.8 \times 0.8–2.0	0.5–0.6 \times 1.0–1.3	0.5–0.75 \times 1.0–2.2
Motility	Yes (mp-mt)	Yes (mp-mt)	Yes*
DNA G + C content (mol%)	61.4	65.3	ND
Catalase	Negative	Positive	Positive†
pH range for growth	6.4–8.5	5.8–8.0	6.0–8.2
Temperature range for growth ($^{\circ}$ C)	4.0–38.0	15.0–35.0	10.0–32.0
Electron donors:‡			
17 β -Oestradiol (E2) (1)	+	-†	-†
Oestrone (E1) (1)	+	-†	-†
4-Androstene-3,17-dione (1)	-	+†	-†
Cholesterol (1)	-	+	+
Acetate (5)	+	(+)+†	-†
Propionate (5)	+	(+)+†	(+)+†
Butyrate (5)	+	+†	-†
Isobutyrate (5)	+	-	-
Crotonate (1)	+	-	-†
Valerate (5)	+	(+)+†	(+)+†
Caproate (1)	+	-	(+)+†
Heptanoate (1)	-	(+)+†	(+)+†
Palmitate (0.5/1)	-	+	-
Stearate (0.5/1)	-	+	+
DL-Lactate (5)	(+)	-	-
Pyruvate (5)	+	-†	-
Fumarate (5)	+	-†	-
Succinate (5)	+	-	-†
Electron acceptors:			
Nitrate (5)	+	+†§	+§
Chlorate (5)	+	-†§	-†§
Perchlorate (5)	+	-†§	-†§
Oxygen	(+)	+§	+§
Sulfate (5)	-	-§	-†§

*Cells were motile after growth on cholesterol.

†Tested for the first time or repeated in this work.

‡Substrates tested with nitrate as the electron acceptor (all three strains).

§Tested with cholesterol as electron donor.

||Growth with acetate as electron donor but not with E2.

are not reduced. Optimal growth occurs at 28–30 $^{\circ}$ C and pH 7.0–7.2. Salinity range for growth is 0–1.0 % NaCl (w/v). Ubiquinone Q-8 is the only quinone. Major fatty acids are C_{16:1 ω 7c}/iso-C_{15:0} 2-OH and C_{16:0}. Minor components are C_{18:1 ω 7c} and C_{8:0} 3-OH (as the only hydroxylated fatty acid). The DNA G + C content of the type species is 61.4 mol%. The type species is *Denitratisoma oestradiolicum*.

Description of *Denitratisoma oestradiolicum* sp. nov.

Denitratisoma oestradiolicum (oes.tra.di.ol'i.cum. N.L. neut. n. *oestradiol* oestradiol; L. neut. suff. *-icum* belonging to; N.L. neut. adj. *oestradiolicum* belonging to oestradiol, referring to oestradiol utilization).

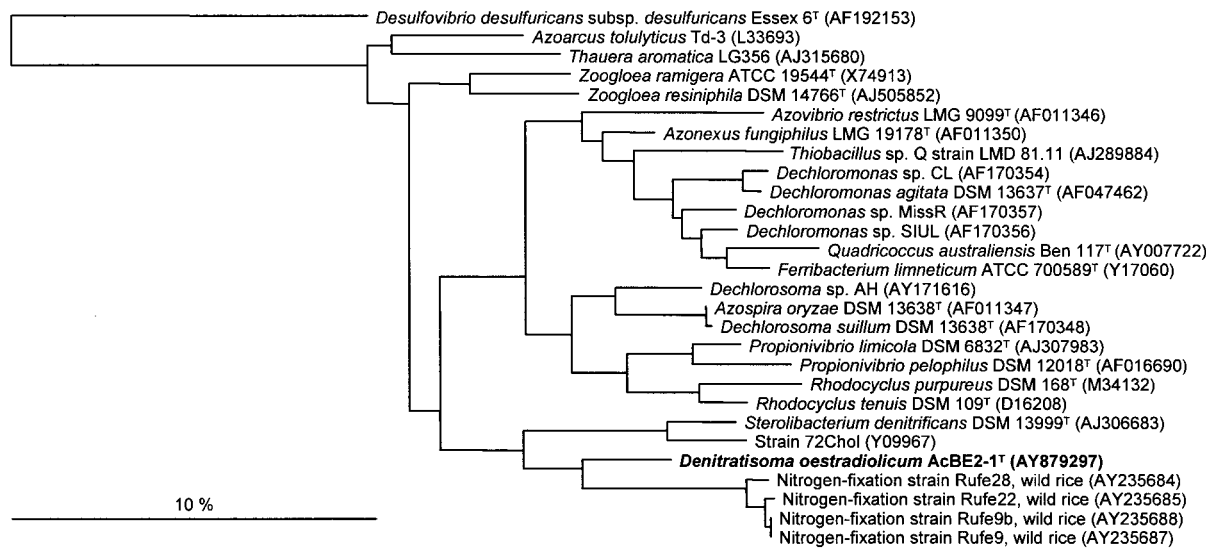


Fig. 2. Phylogenetic tree showing affiliation of the 16S rRNA gene sequence from *Denitratisoma oestradiolicum* AcBE2-1^T to selected reference sequences of members of the *Betaproteobacteria*. The tree was calculated by maximum-likelihood analysis and corrected with a 50% filter and a termini filter. Tree topology was nearly identical by using the neighbour-joining and maximum-parsimony analyses (not shown). The sequence of *Desulfovibrio desulfuricans* subsp. *desulfuricans* Essex 6^T was used as an outgroup. The accession numbers of sequences used are given in parentheses. Bar, 10% estimated sequence divergence. A tree containing a wider selection of reference sequences is available as Supplementary Fig. S1 in IJSEM Online.

Exhibits the following properties in addition to those given in the genus description. Fatty acid profile comprises C_{14:0} (0.6%), C_{16:0} (29.8%), C_{8:0} 3-OH (2.3%), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 53.4%), C_{16:1}ω5c (1.5%), C_{18:1}ω5c (0.3%), C_{18:1}ω7c (11.8%) and C_{18:0} (0.3%). Electron donors used with nitrate as the electron acceptor are listed in Table 1. No growth with the following substrates (not listed in Table 1): formate, laureate, isovalerate, oleate, adipate, pimelate, primary aliphatic alcohols (C₁ to C₄), 2-propanol, 1,2-propanediol, cyclohexanol, cyclopentanone, acetone, 2-butanone, citrate, L(+)-tartrate, ascorbate, L-cysteine, glutamate, D(+)-glucose, D(-)-fructose, D(+)-galactose, sucrose, D(+)-xylose, benzoate, 4-hydroxybenzoate, 3-hydroxybenzoate, benzene, toluene, phenol, *m*-cresol, *p*-cresol, catechol, resorcinol, 2,4-dihydroxybenzoate, 2,5-dihydroxybenzoate, 4-hydroxyphthalate, hydroquinol, hydroxyhydroquinol, peptone, yeast extract or thiosulfate, even after 12 months incubation. No aerobic growth with E2 or oestrone (E1). Substrates used with oxygen as the electron acceptor are acetate, pyruvate and fumarate. On exposure of cells to air, an expanded lag phase occurs, even after several transfers. Marginal and slow growth on R2A agar plates. The DNA G+C content of the type strain is 61.4 mol%.

The type strain, AcBE2-1^T (= DSM 16959^T = JCM 12830^T), was isolated from an enrichment culture inoculated with activated sludge from a municipal wastewater treatment plant in Aachen (Germany).

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