Anaerobic degradation of steroid hormones by novel denitrifying bacteria

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Michael Fahrbach

aus Bad Mergentheim (Baden-Württemberg)

Berichter: Professor Dr. Juliane Hollender
Professor Dr. Andreas Schäffer

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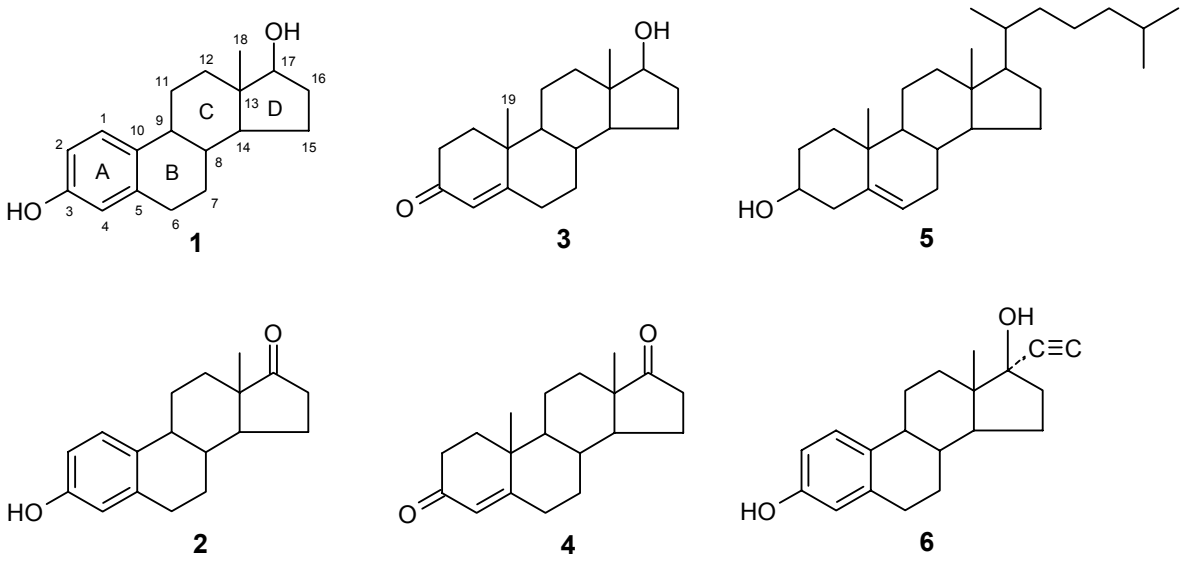
% (v/v)	% volume per volume
% (w/v)	% weight per volume
ARB	Software for sequence data analysis
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CASO agar	Casein-peptone soymeal-peptone agar. The medium is identical with trypticase soy agar (TSA)
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate
Da	Dalton (molecular weight)
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase I	Endonuclease that nonspecifically cleaves DNA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	Dithiotreithol
E1	Estrone
E2	Estradiol
EE2	Ethinyl estradiol
ESI	Electrospray ionization
FAD	Flavin-adenine dinucleotide
FISH	Fluorescence *in situ* hybridization
G+C content of the DNA	Guanine+Cytosine content of the DNA
GC	Gas chromatography
HEPES	*N*-[2-hydroxyethyl] piperazine-*N*'-[ethansulfonic acid]
HPLC	High performance liquid chromatography
IEF	Isoelectric focussing
IMG system	Integrated Microbial Genomes system
IPG strip	Immobilized pH gradient strip
JCM	Japan Collection of Microorganisms
Log K_{OW}	Log octanol-water partition coefficient
MPN	Most probable number
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NA	No data available
NAD(P)	Nicotinamide-adenine dinucleotide (phosphate)
ND	Not determined
PCR	Polymerase chain reaction
pI	Isoelectric point
R2A agar	A low-nutrient solid medium
RNA	Ribonucleic acid
RNase	Endonuclease that nonspecifically cleaves RNA
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SL	Trace element solution
T2	Testosterone
TCA	Trichloroacetic acid
TGS buffer	Tris-glycine-sodium dodecylsulfate buffer
TLC	Thin layer chromatography
Tris	Tris-[hydroxymethyl]-aminomethane

1 Introduction

1.1 General information on steroids

Steroids are isoprenoic compounds that are of great importance in biology, medicine, and chemistry and that are found in multiple forms in the environment (Fig. 1). One class comprises the sterols with cholesterol **5** as the main representative in animals required to build and maintain the cell membranes. Plant sterols (phytosterols) like *β*-sitosterol (not shown) also act as structural components in herbal cell membranes. Another class are the steroid hormones derived biosynthetically from cholesterol, including the estrogens (estradiol **1** and estrone **2**) and the androgens (testosterone **3** and 4-androstene-3,17-dione **4**). These signal compounds are regulating metabolism, growth and reproduction in vertebrates. Estradiol, formed by the ovary and placenta, is the most potent naturally occurring steroid hormone in female organisms, whereas testosterone is the most important one in male organisms, formed by the testis (Koolman et al., 1998).



1 **3** **5**

2 **4** **6**

Fig. 1. Molecular structures of estradiol **1**, estrone **2**, testosterone **3**, 4-androstene-3,17-dione **4**, cholesterol **5**, and ethinyl estradiol **6**. The nomenclature of the carbon atoms and the different rings is shown.

Like their biosynthetic precursor cholesterol, the four rings of the steroid skeleton of estrogens and androgens are in trans-trans-trans conformation (Breitmaier & Jung, 1995). One main difference between cholesterol and steroid hormones is the absence of the

aliphatic side chain (Fig. 1). The aromatic ring A in estradiol shows phenolic properties. Oxygen-dependent aromatization of testosterone to estradiol results in the removal of the methyl group 19 between ring A and B (Stryer, 1996). Steroid hormones of lower estrogenic activity are estrone and 4-androstene-3,17-dione. Ethinyl estradiol **6** is a synthetic derivative of estradiol and is the key component of oral contraceptives. The acetylene residue at position 17 circumvents an oxidation at this carbon atom and makes this compound more recalcitrant in the environment. Basically, steroids share similar chemical characteristics (Table 1), as they display low water solubility and comparatively high melting points. All these molecules possess one or two quaternary carbon atoms, C-10 and C-13.

Table 1. Selected physicochemical properties of steroidal hormones

Steroid homone	Molecular weight [g/mol]	Water solubility [mg l^{-1}]	Log K_{OW}	Melting point [°C]	References
17*β*-Estradiol	272.4	3.9-13.3	3.1-4.0	171	Hanselman et al., 2003
Estrone	270.4	0.8-12.4	3.1-3.4	259	Hanselman et al., 2003
17*α*-Ethinyl estradiol	296.4	32.0	3.6-4.1	183	Lee et al., 2003
Testosterone	288.4	18.0-25.0	3.2	155	Lee et al., 2003
4-Androstene-3,17-dione	286.4	37.0-41.0	NA*	173	Lee et al., 2003
Cholesterol	386.6	2.0	NA*	149	Windholz et al., 1983

* No data available.

1.2 Steroid hormones in the environment

1.2.1 Natural and anthropogenic sources and deposits

Estradiol and estrone that were detected in the aquatic environment mainly originate from municipal effluent discharge (Ternes et al., 1999b), runoff from agricultural production, and farmyard manure applied as organic fertilizer (Hanselman et al., 2003). The importance of estrogens from animal sources has to date been of less concern than their discharge from wastewater treatment plants, but runoff from manure largely contributes to the entry in the environment (Hanselman et al., 2003; Raman et al., 2004). Cattle and poultry manure have also been reported as a source of the environmental loadings of testosterone (Lee et al., 2003). It is clear that intensive animal breeding could generate large quantities of both the steroidal estrogens and androgens from urinary and fecal deposition and indeed high concentrations (up to 2 µg l^{-1}) of estradiol and testosterone have been found in runoff from poultry manure (Finlay-Moore et al., 2000). Further significant sources of androgens appear

to include pulp and paper mill effluents and sewage treatment effluents (Jacobsen et al., 2005). Another origin could be the microbial conversion of phytosteroidal compounds (e.g., *β*-sitosterol) to steroid hormones including 4-androstene-3,17-dione in anoxic aquatic sediments (Jenkins et al., 2004).

In addition, steroids can be found as fossil fuel markers in coals, petroleum and sedimentary rocks. These markers represent modified molecules of biochemical precursors like cholesterol and other steroids, formed by microbial degradation, pressure, temperature and mineral catalysis, while being buried for millions of years in deep sediments. In most cases these steroids are thermodynamically more stable than the steroids found in the organisms but possess the basic structure of their predecessors. They can be used to determine the constitution of a community of organisms in a specific time of earth history, or to trace contamination of soils, plants and groundwater by petrol and petroleum derived products (Mackenzie et al., 1982; Payet et al., 1999).

1.2.2 Potential impact on the environment

Steroid hormones are frequently detected in the environment and are likely to cause endocrine disrupting effects in aquatic wildlife (Sumpter & Johnson, 2005; Hanselman et al., 2003) at concentrations in the nanogram per liter range. Endocrine disrupting chemicals (EDCs) have been defined as “exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the substance in the body of an organism responsible for the maintenance of homeostasis and the regulation of developmental processes” (Kavlock, 1991). The potential endocrine effects of estrogens, such as vitellogenin production and feminization of male fish, have been well documented (Panter et al., 1998; Jobling et al., 1998). Although the study of estrogens has received considerable attention, much less effort has been directed at studying the potentional endocrine-disrupting effects of androgens such as testosterone. Information of androgens in the environment is limited, but aquatic organisms downstream of pulp and paper mills have demonstrated biological responses consistent with exposure to these substances, including masculinization of female fish (Howell & Denton, 1989; Thomas et al., 2002).

Besides their potential impact on internal physiological signal pathways, steroid hormones also do seem to have a disrupting effect on chemical signalling pathways between different organisms. These external signalling pathways include such fundamental processes like the nodulation in leguminous roots mediated by phytoestrogens (Fox, 2004). Phytoestrogens like the flavonoid luteolin act as recruiting signals to attract soil bacteria of the genus *Sinorhizobium,* responsible for the symbiotic nitrogen fixation. Recent investigations have shown that many of the same synthetic and natural chemicals that disrupt endocrine

signalling in vertebrates also disrupt the binding of phytoestrogens to the bacterial nodulation D protein (NodD) receptor. As a result the potential binding of estrogens to the bacterial NodD receptor could have a destabilizing influence on the establishment of this vital symbiotic relationship.

1.2.3 Fate of steroid hormones

Concern over the potential negative ecological effects of steroid hormones from human- and animal-derived wastes has resulted in an increased interest regarding the mobility and perstistence of these compounds in the environment. Removal of steroid hormones from water, sediments, and soils is expected to be largely the result of a combination of sorption and biodegradation. Being predominantly hydrophobic organic compounds of low volatility, sorption to the solid phase is likely to be a significant process. Studies by Holthaus et al. (2002) about the sorption of estradiol to river sediments revealed that less than 1 % of the present steroid are predicted to be removed from the aqueous phase by suspended sediments. Andersen et al. (2003) reported, that sorption of estradiol and estrone to activated sludge and anoxic sewage sludge occurred to a minor degree and the sorbed steroids appear to decrease slightly along the treatment train. The strong decrease of dissolved estrogens along the treatment train despite the almost equal amounts of sorbed estradiol and estrone in the activated sludge of the same sample indicates slow sorption kinetics and no equilibrium between the sorbed and dissolved estrogens. Soils were also found to bind estrogens (Hanselman et al., 2003; Lee et al., 2003). In addition, androgens have been shown to be sorbed to soils or to be accumulated in sediments (Lee et al., 2003; Jenkins et al., 2003).

Steroid hormones are mainly excreted from humans and livestock as soluble conjugates (e.g. sulfate- and glucuronic acid esters), which are cleaved during wastewater treatment (Ternes et al., 1999a). Recent studies demonstrated that unconjugated, active estrogens are degraded under oxic conditions during normal activated sludge process and lab scale experiments (Layton et al., 2000; Ternes et al., 1999a). However, other investigations indicated only a partially elimination of estrogens, depending on facility and location of the respective wastewater treatment plant and residual amounts could reach surface and groundwater (Ternes et al., 1999b; Desbrow et al., 1998; Belfroid et al., 1999). Layton et al. (2000) have performed a series of biodegradation studies with radio-labeled estradiol and testosterone in laboratory assays inoculated with activated sludge obtained from different wastewater treatment plants. Differences in mineralization of estradiol by sludge from a municipal compared to that from an industrial plant was observed. In contrast to estradiol, testosterone was mineralized to carbon dioxide in all investigated plants.

In recent years, co-metabolic transformations of estrogens in activated sludge and by the nitrifying bacterium *Nitrosomonas europaea* were described (Vader et al., 2000; Shi et al., 2004). It was proposed that the enzyme ammonium monooxygenase unspecifically hydroxylates steroid hormones like estradiol or ethinyl estradiol to derivatives with lower estrogenic activity. However, co-metabolism is mostly used to describe a microbial process for which no exact explanation exists (Wackett, 1996). Furthermore, the relevance of *Nitrosomonas europaea* for wastewater treatment is disputable, since it is known that other nitrifying bacteria (e.g., *Nitrosomonas mobilis* and *Nitrosomonas marina*) are mostly abundant and functionally important during sewage sludge treatment (Wagner et al., 2002).

1.3 Microbial degradation of steroid hormones and sterols

Steroid hormones of human and animal origin are being delivered into the environment over thousands of years, especially to an increasing extent due to growing population and more intensive farming. Several bacteria have acquired pathways to make use of these compounds as growth substrates. Certain bacteria are able to grow on steroid hormones or sterols as the sole source of carbon and energy by the expression of a set of steroid-catabolizing enzymes.

1.3.1 Aerobic degradation

Under air, steroids can be degraded by certain bacterial species of different genera (Fujii et al., 2002; Fujii et al., 2003; Yoshimoto et al., 2004; Kieslich, 1985). Although bacteria are generally capable of growing on estradiol or estrone as sole source of carbon and energy, only a few degradation mechanisms have been proposed (Coombe, 1966). On the other hand, the degradation pathway of testosterone, 4-androstene-3,17-dione in *Comamonas testosteroni* (Talalay et al., 1952; Tamaoka et al., 1987) and of cholesterol in various genera (Kieslich, 1985), known as 9,10-seco pathway, was studied in great detail since the 1960s (Shi & Whitlock, 1968; Horinouchi et al., 2004).

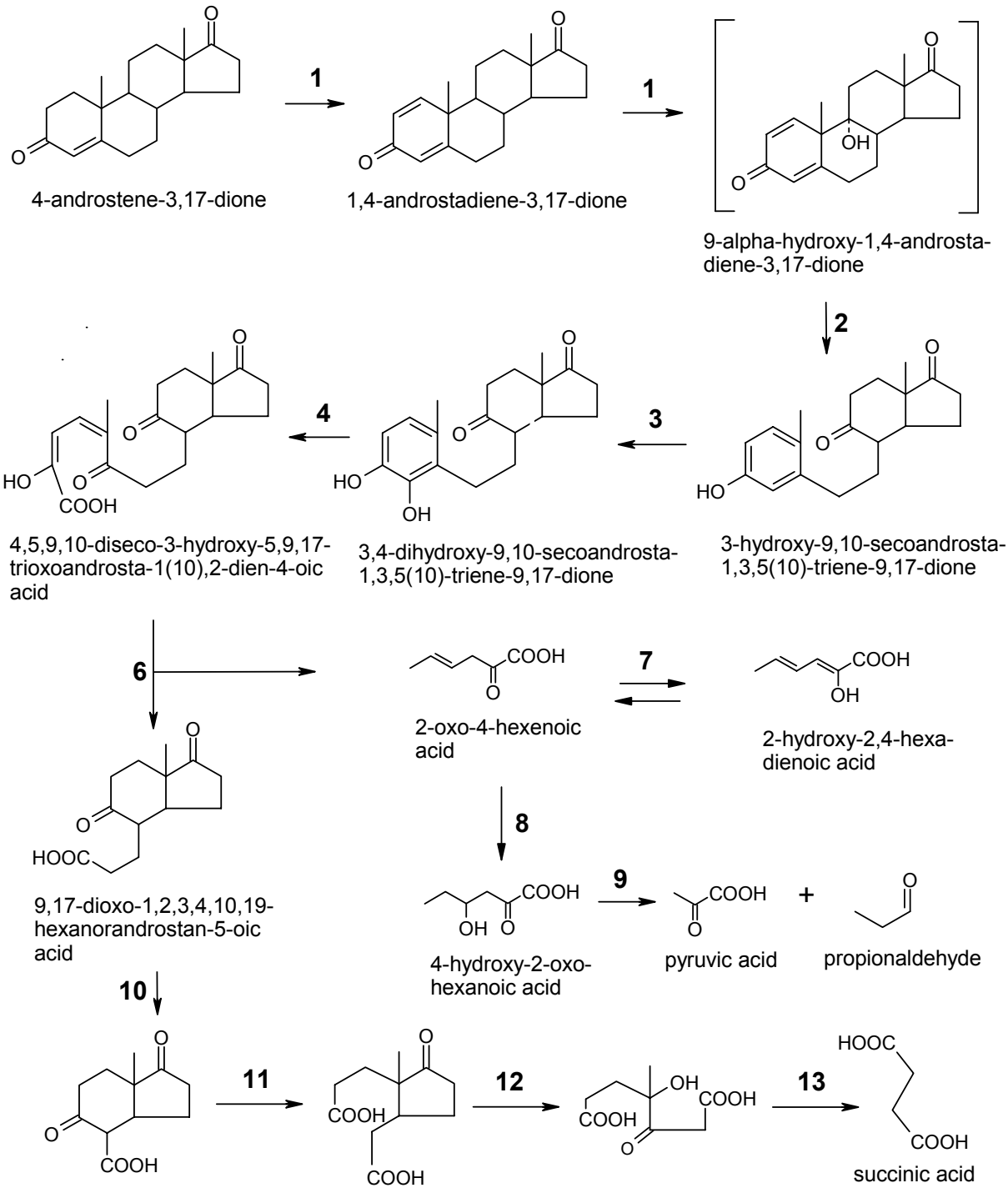


Fig. 2. Proposed aerobic cholesterol and testosterone degradation pathway (9,10-seco-pathway). The side-chain cleavage of cholesterol and the dehydrogenation of the 17*β*-hydroxyl group on testosterone is not shown. Steps 1 to 6 have been taken from Horinouchi et al. (2004), while the final steps from 7 to 13 have been taken from Kieslich (1985). The degradation steps 10 to 13 have yet to be clarified. Numbers displayed refer to the description of the reaction steps in the text.

This oxygen-dependent pathway with its mode of cleavage of the steroid nucleus appears to be a general degradation pathway of C-19 steroids and cholesterol in aerobic bacteria (Fig. 2). Cholesterol and testosterone both enter this pathway, the former via side chain cleavage, the latter by oxidation. After Δ^1-dehydrogenation to 1,4-androstadiene-3,17-dione (step **1**), 9*α*-hydroxylation of the C-9 atom is followed by the nonenzymatic transformation of 9α-hydroxy-1,4-androstadiene-3,17-dione into 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (step **2**). Furthermore, the phenolic ring A is opened by *meta*-cleavage (steps **3** and **4**) yielding 4,5,9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-dien-4-oic acid. This compound is hydrolyzed (step **6**) to 2-oxo-4-hexenoic acid and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid. Only the ketoform (2-oxo-4-hexenoic acid) of the two tautomeres **7** is further metabolized to 4-hydroxy-2-oxo-hexanoic acid, which is finally cleaved to propionaldehyde and pyruvic acid (steps **8** and **9**). 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid is thought to be further degraded (steps **10**, **11**, **12**, **13**) to succinic acid (Schubert et al., 1969). Still these last steps have to be further investigated (Horinouchi et al., 2004). Especially the quaternary carbon atom in the product of step **11** poses a problem for further degradation, as well as the following tertiary alcohol (Hylemon & Harder, 1999).

Interestingly, bacteria that are using the 9,10-seco pathway are unable to metabolize estradiol or estrone, since existence of the C-19 carbon atom is a prerequisite for the cleavage of ring B. For example, when 19-nor-androst-4-ene-3,17-dione is incubated with *Comamonas testosteroni*, estrone accumulates in the medium (Levy & Talalay, 1959). Another important feature is that the genes for steroid degradation in *Comamonas testosteroni* are not constitutively expressed, but are induced by their respective steroid substrates (Möbus et al., 1997; Marcus & Talalay, 1956; Skowasch et al., 2002). In addition, studies of the mechanisms regulating the steroid-inducible gene expression revealed, that regulator proteins, binding proteins or intermediate compounds produced in the course of testosterone degradation are likely involved in microbial steroid metabolism (Xiong & Maser, 2001; Xiong et al., 2003; Pruneda-Paz et al., 2004; Thomas et al., 1989; Horinouchi et al., 2001).

1.3.2 Anaerobic degradation

Anoxic environments develop where organic matter is degraded through microbial activity, and oxygen has only limited access, for example, during the denitrification step in wastewater treatment or in sediments of lakes and rivers. Steroids that occur in such environments have in common with aliphatic hydrocarbons and monoaromatic compounds a chemical inertness that makes them recalcitrant substrates for bacteria. As described above,

the aerobic biodegradation of steroids requires molecular oxygen to form hydroxylated products from oxygenase-catalyzed reactions. The subsequent cleavage of carbon-carbon bonds also requires oxygen. Less is known about the biochemical reaction principles in steroid metabolism employed in the absence of molecular oxygen. In addition, quaternary carbon atoms are hardly degradable without the co-substrate oxygen and detailed knowledge about mechanisms involved is still lacking (Hylemon & Harder, 1999; Kniemeyer et al., 1999; Probian et al., 2003).

The most known transformation of steroids in anoxic habitats occurs during the enterohepatic circulation in mammals by intestinal anaerobic bacteria. Cleavage of alkylaryl ether linkages, dehydroxylations, and oxidation or reduction at C-17 were found, but a breakdown of the steroid nucleus by these bacteria to gain energy was not described (Groh, 1993). For the first time Taylor et al. (1981) showed that bacteria are capable of degrading cholesterol under denitrifying conditions. Two denitrifying *Betaproteobacteria*, strain 72Chol and *Sterolibacterium denitrificans* capable of oxidizing cholesterol completely (Harder & Probian, 1997; Tarlera & Denner, 2003) were recently described. Although both bacteria were extensively characterized, information about the metabolic pathway and enzymes involved is still not available.

The mineralization of estradiol has been reported in the denitrifying step of wastewater treatment plants and in anoxic river sediments, but the responsible bacteria and further oxidation products of estrone are still unknown (Andersen et al., 2003; Joss et al., 2004). In fact, the loss of estradiol was only accompanied by a corresponding accumulation of estrone (Jürgens et al., 2002). Czajka & Londry (2006) also reported an oxidation of estradiol to estrone but no further degradation under different redox conditions (even with nitrate as the electron acceptor), and suggested an accumulation of estrogens in anoxic environments. An inhibitory or toxic effect of the reductant sodium sulfide on denitrifying bacteria in the mineral medium used by Czajka & Londry (2006) could not be ruled out.

Accumulation of androgens such as 4-androstene-3,17-dione most likely caused by microbial conversion of phytosteroids, was observed in anoxic river sediments that receive effluent from paper mills (Jenkins et al., 2003; Jenkins et al., 2004). Unfortunately, no information about the mineralization of estrogens and androgens under anoxic conditions is available in the literature.

1.4 Aim of dissertation

As mentioned above, denitrification is an important step for the overall elimination of steroid hormones in the sewage sludge process. Up to now, nothing is known about the bacteria responsible for mineralizing estradiol, ethinyl estradiol or testosterone under anoxic conditions and the mechanisms by which these compounds are degraded. The aims of this thesis are (I) to evaluate the biodegradability of steroidal homones in the absence of molecular oxygen with nitrate or sulfate as the electron acceptor, (II) the physiological characterization of isolated bacteria from sewage sludge that have the ability to use these compounds as the sole energy source, and (III) the elucidation of the bacterial enzymes that might be involved in anaerobic steroid hormone degradation.

2 Materials and Methods

2.1 Microbiological methods

2.1.1 Sources of bacteria

Denitratisoma oestradiolicum strain AcBE2-1^T (= DSM 16959^T = JCM 12830^T) was enriched and isolated in this work from activated sludge of the municipal wastewater treatment plant in Aachen-Soers (Germany). A mixed sludge sample, taken from two aeration basins was pretreated for 12 h by stirring at 25 °C in the presence of 50 mg cycloheximide l^{-1} to prevent growth of eukaryotes. Removal of cycloheximide was achieved by centrifugation at 30,000 x g for 5 min at 25 °C. The pellet was washed once and resuspended in sterile tap water. Enrichment cultures were established by inoculating 60 ml of oxygen-free and bicarbonate-buffered freshwater mineral medium containing 1 mM estradiol as the sole source of energy and 5 mM nitrate as the electron acceptor with 5 ml pretreated sludge. *Steroidobacter denitrificans* strain FS^T (the bacterium has not been deposited at DSMZ and JCM yet) was enriched and isolated in this work under the same cultivation conditions from untreated anoxic digested sludge of the municipal wastewater treatment plant in Aachen-Soers (Germany).
An overview of all enrichment cultures established in this work with either estradiol or ethinyl estradiol are listed in Table 9A and 10A (Appendix). The cholesterol-degrading denitrifying bacteria *Sterolibacterium denitrificans* Chol-1S^T (DSM 13999^T) and strain 72Chol (DSM 12783) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

2.1.2 Media and growth conditions

General composition of anoxic freshwater mineral medium. For enrichment, isolation and routine cultivation of strains AcBE2-1^T and FS^T an oxygen-free and bicarbonate-buffered freshwater medium was used. The medium contained in 1 l of distilled water: 1.0 g NaCl, 0.4 g $MgCl_2$ x 6 H_2O, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 0.5 g KCl, and 0.15 g $CaCl_2$ x 2 H_2O. The medium also contained 5 mM $NaNO_3$ as electron acceptor, 0.5 mM Na_2SO_4 as sulfur source, 30 ml $NaHCO_3$ (1 M, autoclaved under CO_2), 1 ml trace element solution SL10 (Widdel et al., 1983), 1 ml selenite tungstate solution (Widdel et al., 1983), and 0.5 ml seven-vitamin solution (Widdel & Pfennig, 1981) under a N_2/CO_2 (80:20, v/v) atmosphere (Biogon C20, Linde, Höllriegelskreuth, Germany). The final pH of the medium was adjusted to 7.2-7.4.

For routine cultivation and maintenance, steroid hormones were added under sterile conditions to empty culture vessels in defined amounts dissolved in acetone (stock solutions of 20 g estradiol l^{-1}, 20 g testosterone l^{-1}, 20 g 4-androstene-3,17-dione l^{-1}, and 10 g estrone l^{-1}, respectively). After complete evaporation of the solvent, the medium was dispensed anoxically. Crystals of steroid hormones were minced and detached from the glass wall by ultrasonic treatment. For quantitative growth experiments, acetone was omitted and the steroid hormones were added as solid pure substances, although both bacteria do not use acetone as carbon and energy source. All other modifications of this freshwater mineral medium are given in the respective chapters. Soluble substrates and electron acceptors were anoxically added with plastic syringes from sterile, neutralized stock solutions or were added prior to autoclaving. Bacterial cultures were incubated at 28 °C or 30 °C in the dark.

Media used for quantitative experiments with strain FS^T contained freshly and anoxically prepared sodium ascorbate (4 mM) as a reductant, which did not serve as growth substrate. The medium was preincubated for at least 48 h and remains colorless during growth, indicating anoxic conditions. In contrast to the latter bacterium, strain AcBE2-1^T accumulates nitrite during growth. In the presence of nitrite, the medium with ascorbate turns yellow, even in sterile control medium with anoxically added sodium nitrite. Ascorbate is oxidized to dihydroascorbate by reacting with nitrite or molecular oxygen (Windholz et al., 1983). In the presence of 1 mM titanium(III) citrate, prepared according to Zehnder & Wuhrmann (1976), both strains grew only poorly. Titanium(III) citrate is known to act inhibitory to facultative anaerobic bacteria (Zehnder & Wuhrmann, 1976). The titanium(III) citrate complex forms a blue-to-violet solution with sterile freshwater mineral medium without sodium nitrate or nitrite and it remains colored over several years. But the solution decolorized slowly after addition of nitrate or nitrite, indicating an oxidation process. Other reductants like 1 mM L-cysteine, 4 mM thioglycolate, and 0.5-1 mM ferrous sulfide did not support growth on steroid hormones. Intermediate formed nitrite might react chemically with ferrous sulfide. Oxygen penetrating through the butyl rubber stoppers was not responsible for ferrous sulfide oxidation in inoculated bottles since no oxidation could be detected in sterile control bottles.

Composition of further media. Aerobic growth of strains AcBE2-1^T and FS^T on steroid hormones was tested in potassium phosphate-buffered (20 mM, pH 7.2) or HEPES-buffered medium (10 mM, pH 7.2) at 28 °C and 120 r.p.m. containing the same additions as described above for the bicarbonate buffered mineral medium with the exception of sodium nitrate. In addition, potassium phosphate-buffered (20 mM, pH 7.2) steroid agar medium was used that contained in 1 l of distilled water: 1.74 g K_2HPO_4, 1.36 g KH_2PO_4, 1.0 g NaCl, 0.4 g $MgCl_2$ x 6 H_2O, 0.25 g NH_4Cl, 0.5 g KCl, 0.15 g $CaCl_2$ x 2 H_2O, 15 g agar-agar, and

0.07 g Na_2SO_4 as sulfur source. The steroid agar medium also contained 1 mM of either estradiol or testosterone dissolved in DMSO (50 mM stock solution), 1 ml trace element solution SL10 (Widdel et al., 1983), 1 ml selenite tungstate solution (Widdel et al., 1983), and 0.5 ml seven-vitamin solution (Widdel & Pfennig, 1981). Liquid cultures were also streaked on either R2A (Merck 100416) or CASO (Merck 105458) agar plates. Furthermore, aerobic growth of strain FS^T was tested in liquid CASO-Bouillon (Merck 105459) or Nutrient Broth (Difco).

The dependence of growth on pH was conducted in anoxic freshwater medium buffered with 30 mM bicarbonate, 20 mM potassium phosphate or 10 mM HEPES in a range from pH 5.8-8.5. Glycerol phosphate (1.5 mM) was added to HEPES-buffered medium to substitute lacking phosphate. The temperature range (from 4 to 50 °C) of growth was determined in bicarbonate-buffered medium. In all experiments decribed in this paragraph acetate (strain AcBE2-1^T) or heptanoate (strain FS^T) were used as substrates and nitrate as the electron acceptor. The increase in optical density at 578 nm was determined by use of a Perkin Elmer 550 SE spectrophotometer.

Sterolibacterium denitrificans Chol-$1S^T$ (DSM 13999) and strain 72Chol (DSM 12783) were routinely cultivated in an oxygen-free freshwater mineral medium containing 1 mM cholesterol and 5 mM sodium nitrate. Unlike the medium described above, it contains 50 ml $NaHCO_3$ (1 M, autoclaved under CO_2) and an eleven-vitamin solution (see DSMZ medium 461).

2.1.3 Anoxic cultivation techniques

Details about general techniques for preparation of media and cultivation of bacteria under anoxic conditions have been described elsewere (Widdel & Bak, 1992).

Preparation of anoxic mineral medium. Oxygen-free mineral medium was prepared in specifically constructed vessels developed by Widdel (1980). After autoclaving and cooling under a N_2/CO_2 atmosphere (80:20, v/v), bicarbonate solution, trace element solution, selenite-tungstate and vitamin solution were added from sterile stock solutions while the medium was kept under anoxic gas. To ensure anoxic growth conditions, 4 mM sodium ascorbate was used as a reducing agent in the case of strain FS^T. After having supplied all additives, the pH was adjusted to 7.2-7.4 by adding either a 0.5 M solution of Na_2CO_3 or a 1 M solution of HCl.

Culture vessels. The enrichment cultures and pure cultures were routinely cultivated either in butyl rubber-sealed serum bottles of 120 ml volume or in 22 ml screw-capped culture

tubes containing 60 ml and 10 ml medium, respectively. For preparation of cell suspensions, bacteria were grown in infusion bottles of 1200 ml volume, filled with 1000 ml medium and sealed with butyl rubber septa. Infusion bottles (150 ml and 320 ml) sealed with butyl rubber septa and additional sealing disks were used for quantitative growth experiments.
Tubes and bottles were incubated at 28 °C or 30 °C in the dark. Infusion bottles filled with 100-1000 ml medium and culture tubes were incubated in a horizontal position to facilitate contact between bacteria and settled steroid crystals. Cultures were briefly shaken twice per day to allow for homogeneous distribution of bacteria and substrate crystals.

2.1.4 Isolation, purity control and maintenance

To obtain pure cultures, decimal dilution series with steroid hormones and nitrate were prepared into bicarbonate-buffered mineral medium up to 1 and 10^{-9}, from dilution 10^{-7} starting in three parallels. Alternatively, agar dilution series with bicarbonate-buffered medium containing 5 mM nitrate were prepared according to a modification of the protocol of Widdel & Bak (1992). The almost insoluble steroid hormones were added to the medium in form of sterile and anoxic DMSO stock solutions (50 mM) obtaining a final concentration of 1 mM. Purity was checked by microscopic examination after incubation in medium containing estradiol or testosterone 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}. In addition, R2A (Merck 100416) or CASO (Merck 105458) agar plates were used for purity tests.
For maintenance, stock cultures grown on estradiol or testosterone were stored at 4 °C and transferred every 6-8 weeks. Long-term storage of bacteria was achieved as stocks in glycerol (10-25 %, v/v) at - 80 °C under oxic conditions. After reactivation of the frozen cultures, lag phases can take up to 6 weeks. Strain AcBE2-1^T can also be stored at - 80 °C without any cryo protectant.

2.1.5 Characterization of isolated bacteria

Purity and cell dimensions were determined by phase-contrast microscopy using a DMRB Leitz light microscope. Specimens for scanning electron microscopy were prepared by cultivating bacteria in the presence of glas coverslips inside the culture vessels (Bruce et al., 1999). Attached cells were fixed overnight at room temperature in 3 % (v/v) glutaraldehyde in 20 mM phosphate buffer (pH 7.2). The slides were rinsed three times in phosphate buffer for 10 min. Cells were postfixed in 2 % (w/v) OsO_4 overnight at 4 °C and washed three times for 10 min with destilled water before they were dehydrated in a graded ethanol series (25-100 %, v/v) for 10 min each. After a third transfer in 100 % ethanol, dehydrated cells

were critical point dried, coated with gold, and then viewed with a Philips XL 30 ESEM FEG scanning electron microscope at 20 kV.

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).

2.1.6 Phylogenetic analysis

PCR amplification and the phylogenetic analysis of the 16S rRNA gene was performed by Dr. Jan Küver and colleagues (Bremen Institute for Materials Testing, Foundation Institute for Materials Science, Bremen, Germany). 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 strains AcBE2-1^T and FS^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, neighbor-joining and maximum-likelihood analysis with different sets of filters. No filters were used for the tree calculation of strain FS^T. Only sequences of at least 1300 nt were used for the calculation of different trees.

2.1.7 MPN dilution series

The number of viable estradiol-oxidizing, nitrate-reducing bacteria in activated sludge of the wastewater treatment plant Aachen-Soers was estimated by using the three-tube most probable number (MPN) technique performed in culture tubes containing oxygen-free bicarbonate-buffered mineral medium with 1 mM estradiol and 5 mM nitrate. One dilution series was inoculated with cycloheximide-treated sludge according to the enrichment culture of strain AcBE2-1^T as described above. In a second series cycloheximide was omitted and untreated sludge was used. Three replicate 10-fold dilutions of sludge samples in mineral medium were prepared. Both MPN preparations were incubated at 28 °C in the dark for six month. Tubes were primarily scored positive on the basis of turbidity. Furthermore, consumption of estradiol and nitrate was tested in subcultures obtained from positive tubes.

The numbers of bacteria per gram (dry weight) of sludge were then calculated by standard procedures (Bast, 2001).

2.1.8 Quantification of steroid hormone oxidation and denitrification

Quantitative growth experiments to measure the consumption of steroid hormones and nitrate, the formation of nitrite, dinitrogen monoxide, dinitrogen, and cell mass were carried out in 150 ml infusion bottles, gassed with He/CO_2 (80:20, v/v) and tightly sealed with butyl rubber stoppers. The stoppers were covered with sealing disks and aluminium screw caps to minimize leakage. Each bottle contained 100 ml medium with 500 µmol nitrate, but varying amounts of steroid (0-100 µmol). In experiments with strain FS^T, 4 mM sodium ascorbate was added to the medium as reducing agent. Media were inoculated with 1 ml of a preculture using syringes flushed with helium. Cultures were incubated at 30 °C in the dark and were briefly shaken twice per day to allow for homogeneous distribution of bacteria and substrate crystals. After inoculation and briefly before reaching the stationary phase, gas samples were taken for N_2O and N_2 analysis. Immediately after the gas was sampled and steroid crystals settled to the bottom of the bottles, samples of the aqueous phase were taken anoxically for determination of nitrate, nitrite, and protein. For quantification of the residual steroid substrate, NaOH was added to cause alkaline lysis of bacterial cells. After acidification with HCl (pH 1.0), steroids were extracted three times with ethyl acetate. The organic phases were combined and filled up to 100 ml. Dilutions in acetonitrile were directly analyzed by HPLC. An extraction efficiency of 93.7 ± 4.0 % (mean ± standard diviation, n=3) was determined using ethinyl estradiol as an internal standard which is not degraded by both bacteria. Growth of the bacteria was monitored continously in a control culture with 100 µmol steroid hormone and 500 µmol nitrate. All assays were analyzed and evaluated after the electron acceptor nitrate was completely consumed in this control culture.

2.1.9 Degradation time course

Enrichment cultures. After several transfers, a degradation time course with dissolved estradiol was performed in order to ensure that steroid degradation was coupled to nitrate reduction. Dissolving of estradiol was achieved by autoclaving the medium, containing approximately 20 µM estradiol and 1 mM nitrate. Duplicate assays were incubated at 28 °C in the dark. 1 ml samples were withdrawn anoxically with a nitrogen flushed syringe and filtered through a 0.2 µm cellulose membrane filter (Schleicher & Schuell, Dassel, Germany) for HPLC analysis and ion chromatography. Continuous monitoring of the estradiol turnover

was performed by reversed-phase HPLC with fluorescence detection. Nitrate consumption and nitrite formation was determined by ion chromatography.

Pure cultures. Anaerobic growth on steroid hormones, electron acceptor conversion and gas formation was investigated either in 320 ml or 150 ml infusion bottles containing 100 ml or 200 ml mineral medium under a He/CO_2 (80:20, v/v) atmosphere. 1 mM estradiol or 1 mM testosterone were added as the electron donors and 5 mM nitrate as the electron acceptor. In experiments with strain FS^T, 4 mM sodium ascorbate were added to the medium as reducing agent. The medium was inoculated (3 %, v/v) with a preculture grown on either estradiol or testosterone. In order to quantify gas formation, no samples were withdrawn because of the requirement of a constant volume over time. Therefore, parallel assays for the determination of nitrate depletion, nitrite formation, and cell mass increase were investigated simultaneously. Gas samples were withdrawn anoxically with a helium flushed syringe. The data of both assays were diagrammed in one figure. For both assays, triplicate determinations were established in experiments with strain AcBE2-1^T. Experiments with strain FS^T were conducted only in duplicate assays (only for gas measurements). Note, that continuous steroid consumption could not be determined in these experiments due to high substrate amounts and the weak water solubility of steroid hormones.

2.2 Biochemical methods

2.2.1 Preparation of cell-free extracts for proteomics

Sodium acetate grown cultures of strain AcBE2-1^T were transferred over ten times before they were harvested in the late exponential growth phase under anoxic conditions in an anaerobic chamber (Coy, Laboratory Products Inc., Grass Lake, USA) at an optical density around 0.14. Centrifugation was performed at 11,000 x g for 20 min and 4 °C. If estradiol was used as growth substrate, bacteria were harvested after consumption of 5 mM nitrate (optical density was not measured).

Cells were washed once with anoxic potassium phosphate buffer (20 mM, pH 7.5) and were resuspended in small amounts of Tris-HCl buffer (circa 3 ml for a pellet resulting from 1000 ml of culture). The Tris-HCl buffer (10 mM, pH 7.5) contained 5 mM $MgCl_2$ x 6 H_2O, 100 µg RNase ml^{-1}, 150 µg DNase I ml^{-1}, and 100 µg lysozyme ml^{-1}. Cell suspensions were incubated under anoxic conditions on ice for 30 min until the cells were broken in a cooled French pressure cell at 137 MPa (5 passages). The crude extract was separated from cell debris by centrifugation for 30 min at 20,000 x g and 4 °C. Protein determination was carried

out by the method of Bradford (1976) with a Bio-Rad assay and bovine serum albumin as the standard.

2.2.2 Two-dimensional gel electrophoresis

Preparation of protein samples for two-dimensional gel electrophoresis was performed as follows. Protein samples from cell-free extracts were precipitated in 8 µl TCA on ice for 1 h (600 µg protein for a pH range from 3-10; 400 µg protein for a pH range from 5-8). The pellets were suspended and incubated for 1 h in 500 µl ice-cold acetone at - 20 °C after centrifugation for 45 min at 20,000 x g and 4 °C. Actetone was removed by centrifugation for 45 min at 20,000 x g and 4 °C. Vacuum dried pellets were resolved in 125 µl rehydration buffer (9 M urea, 4 % (v/v) CHAPS, 65 mM DTT, 0.4 % (v/v) carrier ampholytes, and 1 µl bromophenol blue ml^{-1}), loaded on 7 cm ReadyStrip IPG strips (Bio-Rad, Munich, Germany) with pH ranges of either 3-10 or 5-8, and covered with mineral oil. The IPG strip is based on the use of immobilized pH gradients, in which polycarboxylic acid ampholytes are immobilized on supports to reproducibly create stable pH gradients. Active rehydration of the IPG strips was performed overnight in a IEF cell (PROTEAN IEF cell, Bio-Rad, Munich) at 50 V and 20 °C. Samples were separated in the first dimension by isoelectric focusing in four steps: 500 V (250 Vh), 1000 V (500 Vh), 8000 V (6500 Vh), and 500 V (hold). Prior to the second dimension, the IPG strips were equilibrated two times for 15 min with gentle shaking in 10 ml of a solution containing Tris-HCl buffer (50 mM, pH 8.8), 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, and a trace of bromophenol blue. DTT (1 %, w/v) was added to the first, and iodoacetamide (2.5 %, w/v) to the second equilibration step. After equilibration, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as the second dimension separation was performed on a vertical system (mini-PROTEAN 3 cell, Bio-Rad, Munich, Germany) by placing the strips on a agarose (0.5 %, w/v; 55 °C) overlayed polyacrylamide gel (12 %). Electrophoresis was performed with voltages of 120 V for 20 min and 180 V for 40 min in TGS buffer (Tris-Glycine-SDS electrophoresis running buffer), respectively. Molecular mass standards were separated to serve as size markers (Bio-Rad, Munich, Germany). Upon completion of SDS-PAGE, the gels were fixed and stained overnight with a blue silver micellar solution (Coomassie blue G-250) as described by Candiano et al. (2004). Destaining was performed in distilled water.

It should be mentioned that limited solubility of membrane proteins may prevent their appearance in these gels, so proteins potentially involved in membrane-associated reactions or transport may not be observed using these cell-free extracts. Fractionation of the cell-free extracts could be obtained by ultracentrifugation at 100,000 x g.

2.2.3 *In-gel* digestion

The ideal protein digestion approach would cleave proteins at certain specific amino acid residues to yield fragments that are most compatible with mass spectrometric analysis. Specifically, peptide fragments of between about 6-20 amino acids are ideal for mass spectrometry and database comparisons. For *in-gel* digestion, stained protein spots were excised from the gel and digested with sequencing grade modified trypsin (Promega, Mannheim, Germany). Trypsin cleaves proteins at lysine (K) and arginine residues (R), unless either of these is followed by a proline (P) residue in the C-terminal direction. After washing the gel particles with 100 µl water, acetonitrile was added for 10 min in defined amounts (3-4 times equal the volume of gel particles). The particles were spinned down to remove the supernatant and dried in a vacuum centrifuge for 5 min. Gel particles were incubated at 56 °C for 30 min in 40 µl of a mixture of 10 mM dithiotreithol and 0.1 M NH_4HCO_3 (reduction step), spinned down, and treated with acetonitrile for 10 min as decribed above. Acetonitrile was replaced by 20 µl of a mixture of 55 mM iodoacetamide and 0.1 M NH_4HCO_3 and incubated for 20 min at 25 °C in the dark (alkylation step). The gel particles were washed with 150 µl of 0.1 NH_4HCO_3 for 15 min, treated with acetonitrile and were vacuum dried for 5 min. Rehydration was conducted at 4 °C with digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$ x 2 H_2O, 12.5 ng trypsin l^{-1}) for 45 min. After removal of the supernatant, the gel particles were covered with the same buffer but without trypsin and incubated overnight at 37 °C. Extraction of the resulting peptides was conducted by the addition of 10 µl 25 mM NH_4HCO_3 and incubation for 15 min at 37 °C with shaking. NH_4HCO_3 was replaced by defined amounts of acetonitrile (3 to 4 times equal the volume of gel particles) followed by incubation for 15 min at 37 °C with shaking. The acetonitrile supernatant was collected. A further extraction was performed by the addition of 40 µl formic acid (5 %, v/v) and incubation for 15 min at 37 °C with shaking. The gel particle were treated with acetonitrile a second time as described above. Both acetonitrile supernatants were combined and vacuum dried (3-4 h). Peptides were resuspended in 15 µl of a mixture of acetonitrile (5 %, v/v) and formic acid (0.1 %, v/v) for nano-HPLC and tandem mass spectrometry.

2.3 Analytical methods

2.3.1 Analysis of nitrite and nitrate by ion chromatography

Nitrate and nitrite were analyzed by a DX-100 ion chromatograph (Dionex, USA) with conductivity detection, using an AS14 anion exchange column (250 mm x 4 mm). The eluent was 1 mM $NaHCO_3$ and 3.5 mM Na_2CO_3 with a flow rate of 1.0 ml min^{-1}. Samples were

filtered through 0.2 µm cellulose membrane filter (Schleicher & Schuell, Dassel, Germany) before analysis. For nitrate and nitrite, the calibration ranged from 1.0 mg l^{-1} to 10.0 mg l^{-1}, with 10 calibration points. For nitrite, an additional ten-point calibration was used ranging from 0.1 mg l^{-1} to 1.0 mg l^{-1}. Limits of quantification (LOQ), allowing for for the quantification of analytes in samples were set as the lowest calibration point. Limits of quantification (LOQ) were at least 1.0 mg l^{-1} (16.1 µM) for nitrate and 0.1 mg l^{-1} (2.1 µM) for nitrite.
For a better separation of nitrite ions from chloride ions by chromatography, medium salt concentrations of 0.33 g NaCl l^{-1}, 0.17 g KCl l^{-1}, 0.13 g $MgCl_2$ x 6 H_2O l^{-1}, and 0.05 g $CaCl_2$ x 2 H_2O l^{-1} were adjusted (Chapter 2.1.2). In addition, 0.25 g NH_4Cl l^{-1} was substituted by 0.31 g $(NH_4)_2SO_4$ l^{-1}. This medium composition was exclusively used for the quantitative experiments with enrichment cultures and with strain AcBE2-1^T.

2.3.2 Analysis of dinitrogen monoxide and dinitrogen by gas chromatography

N_2O and N_2 in the headspace of cultures were quantified with a Perkin Elmer Autosystem gas chromatograph with thermal conductivity detector equipped with a Alltech CTR 1-column to separate CH_4, CO_2, CO, O_2, N_2O, and N_2. The packed column system consists of an outer and an inner column. The outer column consists of a 13X molecular sieve for the separation of CO, O_2, N_2, and CH_4. The inner column contains Porapak material for the separation of CH_4, CO_2, and N_2O. The carrier gas was He at a flow rate of 62.5 ml min^{-1}. The column temperature was set at 40 °C isothermal. Injector and detector temperatures were 65 °C and 140 °C, respectively. Gas samples of 100 µl volume were taken from the assays with a gas-tight syringe. A three-point calibration of the system was performed by using certified standard gas mixtures (Linde, Höllriegelskreuth, Germany). These included mixtures of 1 % (v/v) N_2O and 2 % (v/v) N_2, as well as a mixture of 10 % (v/v) N_2O and 40 % (v/v) N_2. Another standard gas mixture contained 5 % (v/v) N_2. 1000 ml infusion bottles were filled with standard gas underwater and sealed with butyl rubber stoppers covered with sealing discs. The total pressure in the bottle was determined with a pressure gauge to calculate the partial pressures of the corresponding standard gases. Additionally, gas standards with 5 % (v/v) N_2O were prepared in 320 ml infusion bottles from pure N_2O gas and helium gas using a gas-tight syringe. Each standard gas was used for calibration by means of triplicate determinations with an injection volume of 100 µl. All culture samples and standard gases were taken at 25 °C. Amounts of gas measured in the headspace were corrected by adding calculated amounts of dissolved gas, using the adapted Bunsen coefficient (D´Ans & Lax, 1983).

2.3.3 Analysis of steroid hormones by HPLC

Steroid hormones were quantified with the HPLC system HP1100 (Agilent, Massy, France) with UV-VIS diode array (210 nm for estrogens; 244 nm for androgens) and fluorescence (excitation 220 nm and emission 315 nm only for estrogens) detectors in series. Separation was achieved at 35 °C on a Thermo HyPurity C-18 column (150 mm x 2.1 mm; particle size 3 µm) with a flow rate of 0.2 ml min^{-1}. An acetonitrile-water gradient, from 30 % to 95 % acetonitrile in 16 min, was applied. Retention times were as follows: estradiol, 9.7 min; estrone, 11.3 min; testosterone, 12.0 min; and 4-androstene-3,17-dione, 13.5 min. Identification of intermediary formed estrone and 4-androstene-3,17-dione was proved by comparison of retention time and UV spectra with an external standard.

External standards for calibration were made of steroid stock solutions with acetonitrile as the organic solvent. For estradiol, testosterone and 4-androstene-3,17-dione, the calibration ranged from 0.1 mg l^{-1} to 10.0 mg l^{-1}, with 10 calibration points (coefficient of correlation r^2 0.9987, 1.0, and 1.0 respectively). For estrone, a ten-point calibration was used ranging from 0.2 mg l^{-1} to 10.0 mg l^{-1} (coefficient of correlation r^2 was 0.9998). Limits of detection (LOD), allowing for the detection of steroid hormones, were at least 0.03 mg l^{-1} (0.11 µM) for estradiol, 0.1 mg l^{-1} (0.35 µM) for testosterone, 0.1 mg l^{-1} (0.35 µM) for 4-androstene-3,17-dione and down to 0.05 mg l^{-1} (0.18 µM) for estrone, with a signal to noise ratio higher than 3.

2.3.4 Proteomic analysis

Nano-HPLC and tandem mass spectrometry (ESI-Q-TOF-MS) of peptides. ESI quadrupole-time-of-flight mass spectrometers interfaced to nano-LC are widely used in proteomics research. Their main advantage is the excellent mass resolution and accuracy in MS and MS/MS, and the broad distribution of fragments in MS/MS.

HPLC analysis was performed with a nano-HPLC system (DIONEX UltiMate™ LC Packings, Sunnyvale, USA) consisting of two pump systems (DIONEX UltiMate™, Switschos loading pump and Ultimate gradient pump), an autoinjector (DIONEX UltiMate™, Famos), a microtrap column (DIONEX, 300 µm i.d. x 5 mm, C18 PepMap100, 5 µm, 100 Å) connected to a capillary column (DIONEX, 300 µm i.d. x 15 cm, C18 PepMap100, 5 µm, 100 Å). As eluents, a mixture of 5 % (v/v) acetonitrile and 0.1 % (v/v) formic acid (eluent A) and a mixture of 80 % (v/v) acetonitrile and 0.1 % (v/v) formic acid (eluent B) were used at a flow rate of 250 nl min^{-1}. A gradient method was applied for analysis of a sample volume of 10 µl, starting with 5 % eluent B for 4.5 min (loading of the trapping column), rising to 80 % eluent B within 40.5 min (loading of the capillary column followed by peptide separation), and returning to 5 % B, followed by 5 min equilibration.

All MS/MS measurements were performed on a Q-TOF^{TM} 2 mass spectrometer (Micromass, Mancester, UK) equipped with a nano-ESI source. The applied voltage to the capillary to produce an electrospray was 1.25 kV, and cone voltage was 40 V or 50 V. The instrument was automatically switched from MS (full-scan) to MS/MS mode to acquire peptide MS/MS spectra. In this approach, the instrument was set by default in full-scan mode to detect peptide ions as they have emerged from the source. When peptide ions were detected, the instrument selected the most intense ion and subjected it to CID (collision induced dissociation) to obtain an MS/MS spectrum. The instrument then switched back to full-scan mode and selects the next most intense peptide ion. For the MS/MS analysis, each ionized peptide of a trypsin digested protein is selected after a first MS analysis was performed. Argon was introduced as a collision gas at a pressure of 10 psi. The collision energy was step increased to 30 eV from 10 eV. The instrument was calibrated externally, and no post-acquisition recalibration of MS/MS spectra was performed.

From all acquired MS/MS spectra, the sequence information of the respective peptides are derived. These spectra were analyzed and processed by the Proteinlynx software (Masslynx 4.0 software, Micromass) followed by a database search at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) with the Mascot program (Matrix Science, www.matrixscience.com, UK). Mass tolerance was set at 0.8 Da for the masses of peptide precursors and at 0.2 Da for the masses of fragment ions. In this approach, algorithms were applied to directly correlate MS/MS spectral data with peptide sequences in databases without actually interpreting each MS/MS spectrum individually (*de novo* sequence interpretation). How such software tools work is well described by Liebler (2002). Proteins containing at least one significant peptide were selected from database search results. In addition, peptide sequences longer than six amino acid residues, which were not assigned to protein hits by Mascot, were also compared with the database at the NCBI (search for short, nearly exact matches; substitution matrix PAM30) using the BLAST program (Altschul et al., 1997). All interesting protein hits were compared with the Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) (Markowitz et al., 2006) or the Enzyme Database BRENDA (http://www.brenda.uni-koeln.de) to adjust for physiological functions, molecular masses, and isoelectric points, respectively.

2.3.5 Protein determination

Since the measurement of the optical density was not possible in cultures growing on crystaline steroid hormones, increase in cell mass was monitored by protein determination. For protein determination, 1.5 ml culture sample was centrifuged at 20,000 x g for 10 min. The pellet was resuspended in 100 µl 0.5 M NaOH and boiled for 10 min. After

centrifugation, a volume of 20 µl was analysed according to Bradford (1976) using the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Bovine serum albumin standards were prepared in 0.5 M NaOH due to interference of the latter with the protein assay reagent. Obtained concentrations were corrected to account for a twofold higher response of the assay reagent to albumine in comparison with other proteins according to the manufacturer's instructions. For protein determination in crude extracts for two-dimensional gel electrophoresis NaOH was omitted in samples and albumin standards.

2.3.6 Chemotaxonomy

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

Isoprenoid quinone composition and the fatty acid profile was determined by Prof. Dr. Dr. Peter Kämpfer (Universität Gießen). The isoprenoid quinone analysis was performed by reverse phase TLC according to Collins (1985). The fatty acid profile of strain AcBE2-1^T (method according to Kämpfer & Kroppenstedt, 1996) was analyzed from biomass obtained after growth on bicarbonate-buffered medium with 5 mM sodium acetate and 5 mM nitrate. Cells of strain FS^T were obtained from testosterone grown cells.

2.4 Chemicals

All chemicals and gases were of analytical grade and highest purity available. 17*β*-estradiol, 17*α*-ethinyl estradiol and estrone were purchased from Sigma (Taufkirchen, Germany). Testosterone, 4-androstene-3,17-dione and cholesterol were purchased from Fluka (Taufkirchen, Germany). All gases were purchased from Linde (Höllriegelskreuth, Germany).

3 Results

3.1 Characterization of strain AcBE2-1^T

3.1.1 Enrichment and isolation

Enrichment cultures with 1 mM estradiol and 5 mM nitrate were inoculated with cycloheximide-treated activated sludge from a municipal wastewater treatment plant. After three weeks, turbidity formation and accumulation of nitrite was observed, but not in parallel enrichments without substrate. Subcultures with estradiol showed neither nitrate nor nitrite after 8-10 days of growth. In a degradation time course with 16.6 ± 0.31 µM (mean ± standard deviation, n=2) dissolved estradiol and 1154 ± 47 µM (n=2) nitrate, estrone was detected as an intermediate (up to 1.3 ± 0.04 µM, n=2) and was further degraded within 24 h (Fig. 3). Nitrite also accumulated at an average of 122 ± 0.01 µM (n=5), but was not further reduced after estradiol and estrone were completely consumed. Total nitrate consumption was 335 µM.

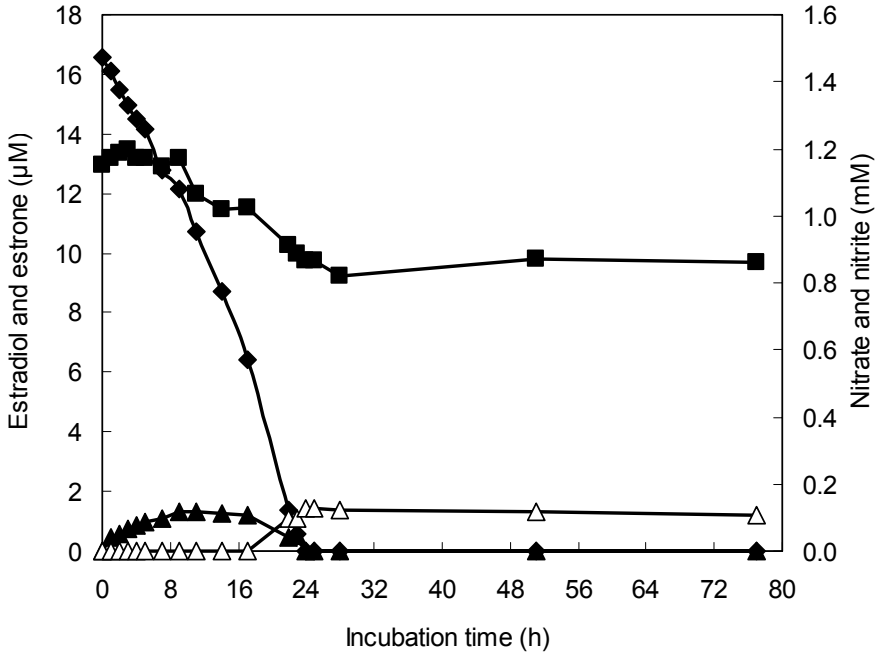


Fig. 3. Estradiol and estrone turnover with corresponding nitrate reduction during growth of the enrichment culture of strain AcBE2-1^T. Symbols are the average of duplicate determinations (Table 7A, Appendix): ♦, estradiol; ▲, estrone; ■, nitrate; △, nitrite.

After 8-10 transfers into fresh medium (inoculum size 2-5 %, v/v), motile curved rods dominated in the culture. A pure culture of an estradiol-oxidizing, denitrifying bacterium, strain AcBE2-1^T, was finally obtained by two subsequent serial dilutions in medium with 1 mM estradiol and 5 mM nitrate within 6 weeks of incubation.

3.1.2 Physiological, cytological, and morphological properties

Cells of AcBE2-1^T and the location of insertion of its flagellum are shown in Fig. 4. A comparison of morphological, cytological, and physiological properties of strain AcBE2-1^T with those of related bacteria is listed in Table 2. In contrast to its nearest relative *Sterolibacterium denitrificans* DSM 13999^T, strain AcBE2-1^T was able to use short-chain fatty acids and several citric acid cycle intermediates, but not cholesterol and long-chain fatty acids. Together with the utilization of the above mentioned substrates, strain AcBE2-1^T and *Dechlorosoma suillum* DSM 13638^T (Achenbach et al., 2001) share the capability to use (per)chlorate as electron acceptor for the oxidation of acetate. Further characteristics are listed in Table 2 and in the species description (Chapter 4.1).

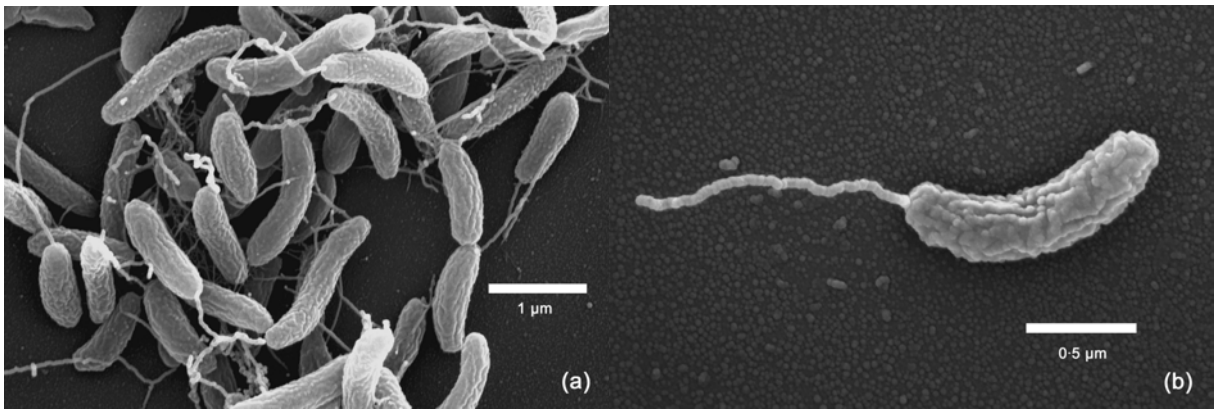


Fig. 4. Scanning electron micrographs of cells of strain AcBE2-1^T grown on 5 mM acetate and 5 mM nitrate. The magnification is 20000x (a) and 45000x (b), respectively.

Table 2. Physiological, cytological and morphological properties of strain AcBE2-1^T and related taxa

Strains: 1, AcBE2-1^T; 2, *Sterolibacterium denitrificans* Chol-S1^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; (+), faint or slow growth; -, no growth; mp-mt, monopolar-monotrichous; ND, not determined. Data were taken from Tarlera & Denner (2003), Harder & Probian (1997), 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 ethinyl estradiol (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 x length) (µm)	0.4-0.8 x 0.8-2.0	0.5-0.6 x 1.0-1.3	0.5-0.75 x 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 (°C)	4.0-38.0	15.0-35.0	10.0-32.0
Electron donors:‡			
Estradiol (1)	+	-†	-†
Estrone (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)	+	-	-†

continued on next page

Table 2 continued

Character	1	2	3
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 when nitrate was the electron acceptor (all three strains).

§ Testet with cholesterol as electron donor.

‖ Growth with acetate as electron donor but not with estradiol.

3.1.3 Environmental significance

MPN counting was used to estimate the numbers of estradiol-oxidizing, nitrate-reducing bacteria in activated sludge of the wastewater treatment plant Aachen-Soers. Two different assays with pretreated and untreated sludge were established. Growth was only observed in tubes inoculated with cycloheximide-treated sludge in accordance with the enrichment culture of strain AcBE2-1^T. Attempts to establish an enrichment culture with untreated activated sludge were in vain.

Turbidity as well as estradiol dependend nitrate-reduction in subcultures established from positive tubes showed that growth was not supported by organic compounds that may be present in activated sludge. It was estimated that approximately 6 x 10^3 estradiol-oxidizing, nitrate-reducing bacteria per gram (dry weight) of activated sludge were present.

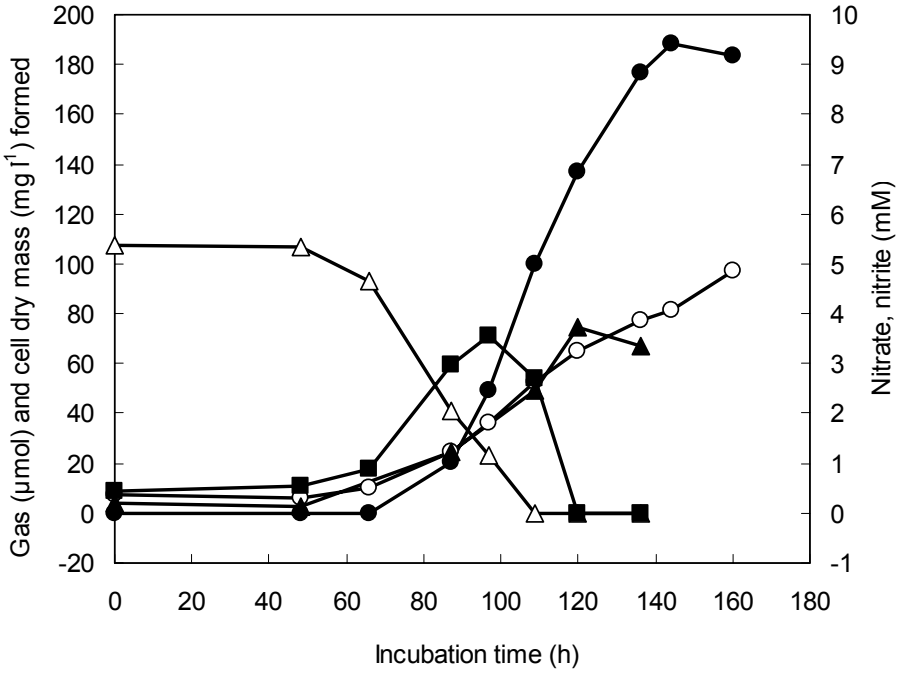


Fig. 5. Growth of strain AcBE2-1^T on 1 mM estradiol with 5 mM nitrate (100 ml mineral medium). Growth could be assayed as increase in protein content assuming that 50 % of the dry cell weight is protein. Symbols are the average of triplicate determinations (Table 4A, Appendix): ▲, cell dry mass; △, nitrate; ■, nitrite; ●, dinitrogen monoxide; ○, dinitrogen.

3.1.4 Quantification of steroid hormone degradation and denitrification

Strain AcBE2-1^T grew on 1 mM estradiol and 5 mM nitrate with a doubling time of 23 hours. Some nitrate was first reduced to nitrite (up to 3.5 mM), which was then further reduced to a mixture of dinitrogen monoxide and dinitrogen, respectively (Fig. 5). Estrone was detected only transiently in trace amounts. Growth yields and electron recoveries of estradiol degradation with nitrate as the electron acceptor are documented in Table 3. Because estradiol was almost water insoluble a continous monitoring of substrate consumption was not possible. Therefore, quantitative measurements of estradiol degradation were conducted in 5 assays containing different amounts of steroid (0-100 µmol) but constant amounts of nitrate (500 µmol). The increase in cell dry matter coincided with the amounts of estradiol dissimilated, shown by a constant growth yield of cultures grown on different amounts of substrate. Electron recoveries were between 90 and 100 %, taking assimilated estradiol into account. A complete dataset for analysis of the dinitrogen monoxide and dinitrogen is shown in Table 1A (Appendix).

Table 3. Quantification of estradiol oxidation and nitrate reduction by strain AcBE2-1^T with different amounts of substrate provided. The total amount of nitrate in each infusion bottle was 500 µmol (100 ml mineral medium)

Estradiol provided [µmol]	Estradiol consumed totally [µmol]	Nitrate consumed* [µmol]	Nitrite formed* [µmol]	Cell dry mass formed† [mg]	Estradiol dissimilated‡ [µmol]	Estradiol assimilated‡ [µmol]	Electron recovery [%]§	Growth yield Y_E [g/mol]¦
11.6	11.6	207.8	130.7	2.5	6.9	4.8	98	365
23.4	21.6	377.0	213.7	4.3	13.5	8.2	96	320
36.2	32.3	487.6	235.0	6.7	19.5	12.7	94	343
60.9	43.2	506.7	0	9.4	25.4	17.9	99	371
95.9	45.9	501.3	0	9.0	28.8	17.1	91	313

* Difference between compound measured at the beginning and at the end of incubation.

† Calculated via protein content after growth assuming that 50 % of the dry cell weight (X) was protein.

‡ Difference between estradiol consumed totally (ΔS) and estradiol assimilated (ΔS_B). The assimilated amount of estradiol was calculated by the equation (Chapter 8.2, Appendix):

$9\ C_{18}H_{24}O_2 + 46\ NH_4^+ + 22\ CO_2 + 30\ H_2O \rightarrow 46\ <C_4H_9O_2N> + 46\ H^+$

Thus, 1 mg of cell dry mass requires 0.0019 mmol estradiol. Dissimilated estradiol (ΔS_E) was calculated according to $\Delta S = \Delta S_E + \Delta S_B$.

§ The values were calculated from electrons recovered as cell dry mass or consumed by nitrate reduction (reduction of NO_3^- to NO_2^-, requirement of two mol electrons per mol nitrite formed; reduction of NO_3^- to a mixture of N_2O and N_2, requirement of approximately 4.5 mol electrons per mol gas formed) in relation to the total amount of electrons (corresponding to 100 %) released by a complete oxidation of totally consumed estradiol.

¦ The molar growth yield Y_E was calculated by taking into account estradiol consumption for cell synthesis ($Y_E = \Delta X / \Delta S_E$).

3.1.5 Phylogenetic analysis and chemotaxonomy

The comparative analysis of the 16S rRNA gene sequence showed that strain AcBE2-1^T belongs to the order *Rhodocyclales* within the *Betaproteobacteria* (Fig. 6). Highest sequence similarity was to *Sterolibacterium denitrificans* DSM 13999^T (Tarlera & Denner, 2003) and the partially described strain 72Chol DSM 12783 (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* (Appendix, Fig. 1A). The only described bacteria within this cluster are the cholesterol-degrading, denitrifying bacteria *Sterolibacterium denitrificans* and strain 72Chol. The most similar 16S rRNA gene sequences obtained were clone sequences 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, 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.

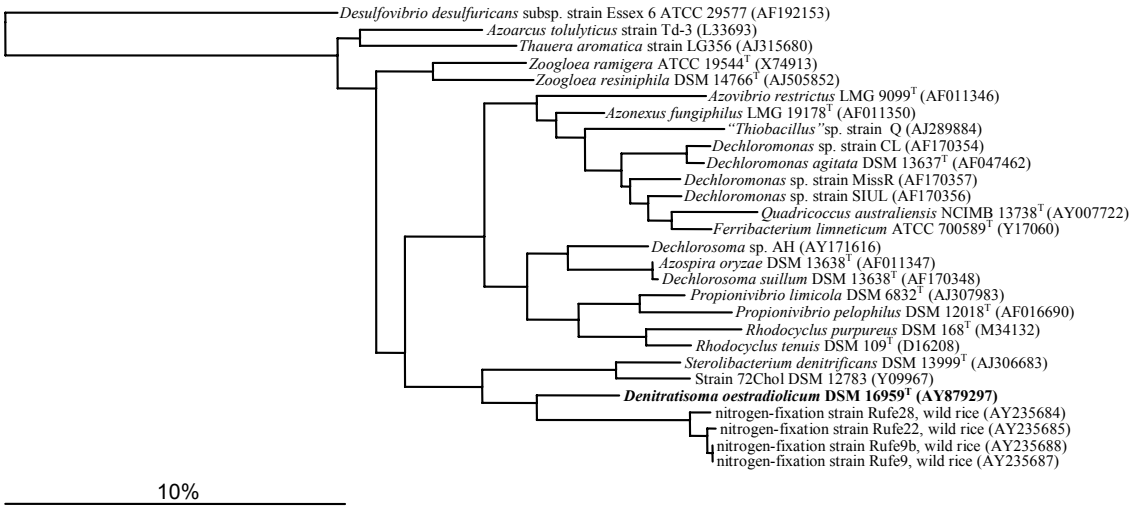


Fig. 6. Phylogenetic tree showing the affiliation of the 16S rRNA gene sequence from *Denitratisoma oestradiolicum* AcBE2-1^T to selected reference sequences of members of the *Betaproteobacteria*. The tree was calculated by maximum-likelihood analysis and corrected with a 50 % filter and a termini filter. The tree topology was nearly identical by using the neighbour-joining and maximum-parsimony analysis (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 shown in Fig. 1A (Appendix).

Analysis of quinones revealed a spot that corresponded to ubiquinone-8 (Q-8). The fatty acid profile of strain AcBE2-1^T consisted of mainly of $C_{16:0}$ (29.8 %), and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 53.4 %). The fatty acids $C_{14:0}$ (0.6 %), $C_{16:1}\omega5c$ (1.5 %), $C_{18:1}\omega5c$ (0.3 %), $C_{18:1}\omega7c$ (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.

3.2 Proteomic analysis of strain AcBE2-1^T

Cell suspension experiments showed that cells of strain AcBE2-1^T cultured on sodium acetate required an adaption phase of approximately 30 h to oxidize estradiol (data not shown). Partial amino acid sequence information from soluble estradiol-induced proteins was received from two-dimensional gel electrophoresis, *in-gel* digestion, and amino acid sequencing of peptides by tandem mass spectrometry.

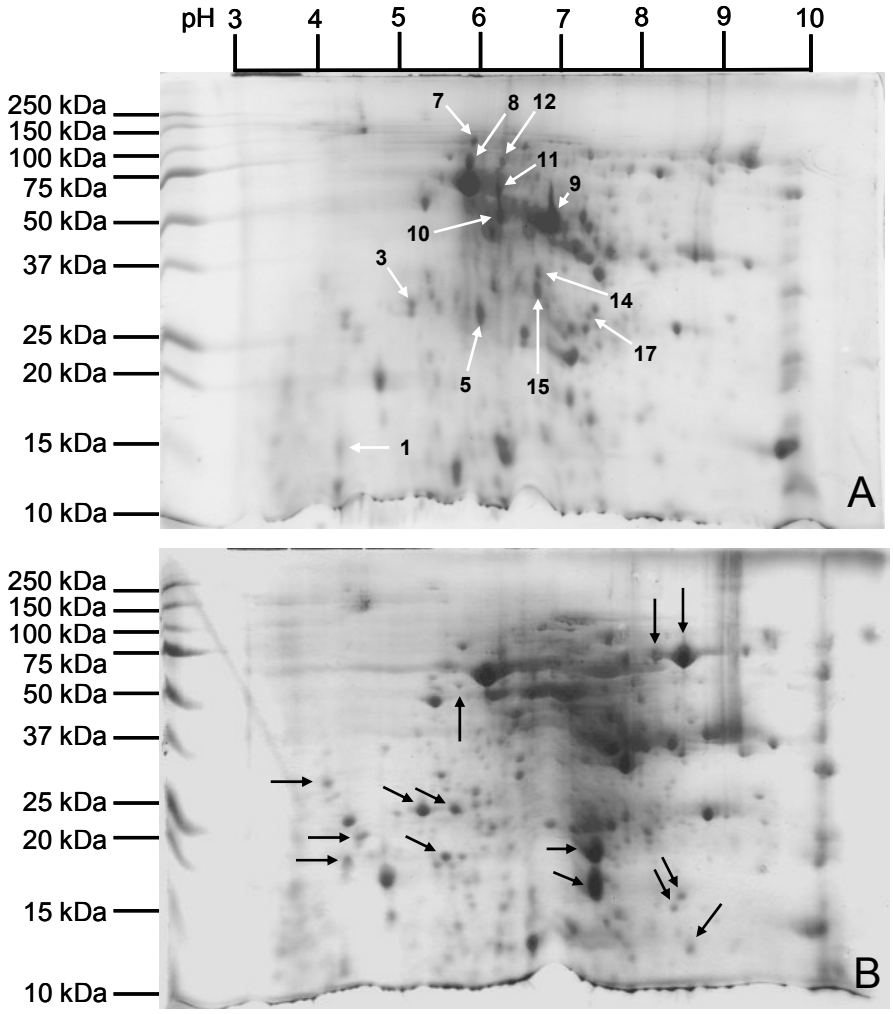


Fig. 7. Two-dimensional gel electrophoresis of soluble proteins (600 µg protein from cell-free extract each) of cells grown with nitrate plus estradiol (A) and sodium acetate (B). The proteins were separated on linear pH 3-10 IPG strips, followed by 12 % SDS-polyacrylamide gels. The proteins were stained with Coomassie blue (G-250) and were excised for trypsin digestion and amino acid sequencing. Gels were inspected visually and 18 estradiol-induced proteins were identified. Only proteins having similarity to known proteins are marked by white arrows and the numbers correspond to the spot numbers in Table 4, 11A, and 12A (Appendix). Repressed or downregulated proteins upon estradiol presence are marked by black arrows.

The electrophoresis pattern of acetate-grown cells served as the basis for comparison. Two different linear pH gradients were applied. At the beginning a pH interval of 3-10 for an overview of the total protein distribution was used (Fig. 7). An increased resolution between pH 5 and 8 was performed, since most proteins concentrated on this pH range (Fig. 8).

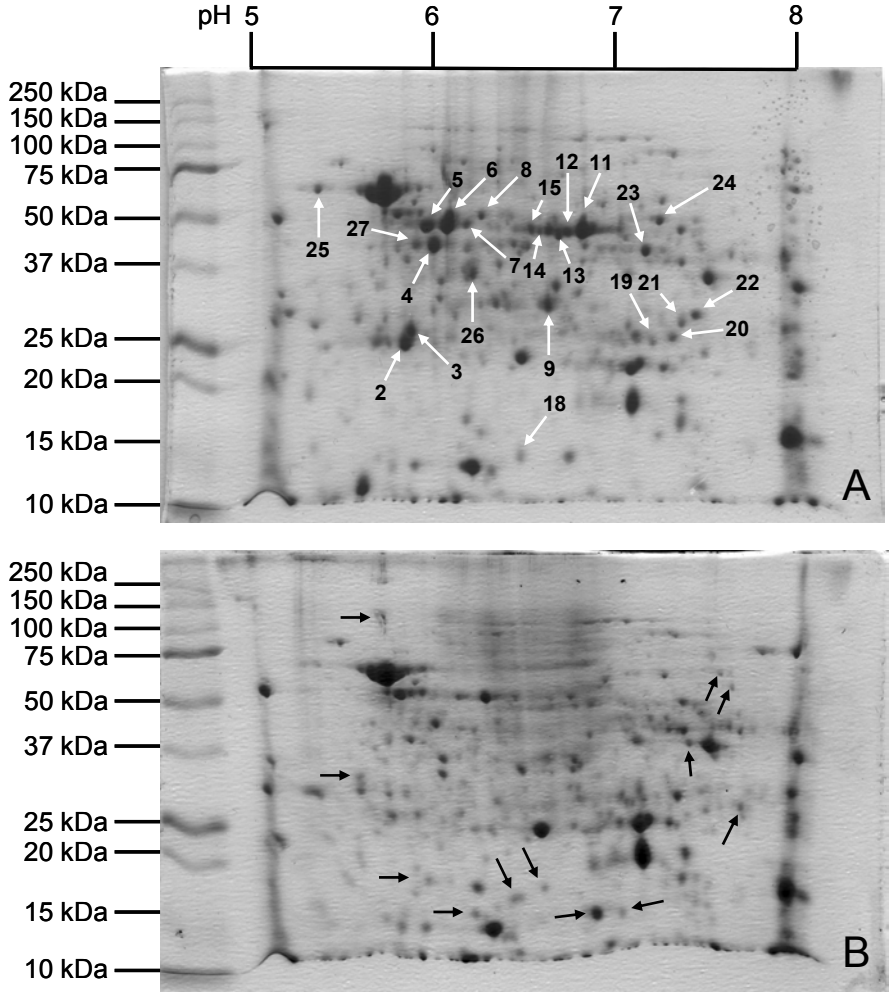


Fig. 8. Two-dimensional gel electrophoresis of soluble proteins (400 µg from cell-free extract each) of cells grown with nitrate plus estradiol (A) and sodium acetate (B). The proteins were separated on pH 5-8 IPG strips, followed by 12 % SDS-polyacrylamide gels. The proteins were stained with Coomassie blue (G-250) and were excised for trypsin digestion and amino acid sequencing. Gels were inspected visually and 27 estradiol-induced proteins were identified. Only proteins having similarity to known proteins are marked by arrows and the numbers correspond to the spot numbers in Table 5, 13A, and 14A (Appendix). Repressed or downregulated proteins upon estradiol presence are marked by black arrows.

In comparison with the gels prepared from cell-free extracts of acetate-grown cells, the estradiol gels showed several different new spots (Fig. 7 and 8, white arrows). On the other hand, several proteins were obviously repressed or downregulated upon estradiol presence (Fig. 7 and 8, black arrows). Note, that almost exclusively soluble proteins appear on the gels, since membrane proteins were precipitated during cell-free extract preparation.

Partial amino acid sequence information was determined by tandem mass spectrometry after trypsin digestion of excised estradiol-induced proteins. Database search for similarties of the obtained amino acid sequences with other proteins of *Bacteria* and *Archaea* revealed that several known proteins might be involved in anaerobic estradiol metabolism. The most important results are listed in Table 4 and 5 and will be discussed in detail because of their putative role in steroid degradation (Chapter 4.2). Further results are listed in the Appendix (Table 11A, 12A, 13A, and 14A) and will be partly discussed in Chapter 4.2.

Table 4. Estradiol-induced soluble proteins in *Denitratisoma oestradiolicum* AcBE2-1^T with highly significant identity to known proteins with regard to molecular mass, isoelectric point, degree of sequence identity, or function (gel with linear pH 3-10 IPG strip)*

AcBE2-1^T protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 2 MNMKSALEVR 11 MNMK+ LE R Sbjct 71 MNMKTPLEAR 80	7/10 (70%)	97	Enoyl-CoA hydratase/isomerase (IMG 401084200) (28.2 kDa; pI 5.0)	*Desulfitobacterium hafniense* DCB-2 Spot 5 (28.0 kDa; pI 6.0)
Query 1 IIVDTYGGAAPHGGGAFSGKDPSK 24 IIVDTYGGAAPHGGGAFSGKDPSK Sbjct 249 IIVDTYGGAAPHGGGAFSGKDPSK 272	24/24 (100%)	3e-13	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica* " RCB Spot 9 (50.0 kDa; pI 6.8)
Query 1 VADQISDAILDAILAQDKHSR 21 VADQISDAILDAILAQDKHSR Sbjct 19 VADQISDAILDAILAQDKHSR 39	21/21 (100%)	4e-11	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 9 (50.0 kDa; pI 6.8)
Query 1 FVVGGPQGDCGLTGR 15 FVVGGPQGDCGLTGR Sbjct 233 FVVGGPQGDCGLTGR 247	15/15 (100%)	2e-05	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 9 (50.0 kDa; pI 6.8)
Query 1 TAAYGHFGR 9 TAAYGHFGR Sbjct 357 TAAYGHFGR 365	9/9 (100%)	3.5	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica* „ RCB Spot 9 (50.0 kDa; pI 6.8)
Query 1 YLVNPTGR 8 YLVNPTGR Sbjct 225 YLVNPTGR 232	8/8 (100%)	18	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 9 (50.0 kDa; pI 6.8)
Query 1 HFDLRPK 7 HFDLRPK Sbjct 335 HFDLRPK 341	7/7 (100%)	68	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 9 (50.0 kDa; pI 6.8)
Query 1 AADAIPEIKK 10 AA+AIPEIKK Sbjct 66 AAEAIPEIKK 75	9/10 (90%)	5.2	1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (40.5 kDa; pI 7.9)	*Geobacillus kaustophilus* HTA426 Spot 11 (70.0 kDa; pI 6.2)
Query 1 HFGAMSGLAGILR 13 H+GA++G+A ILR Sbjct 170 HYGALTGMAAILR 182	8/13 (61%)	54	2-hydroxy-6-oxo-2,4-heptadienoate hydrolase (24.8 kDa; pI 6.5)	*Brucella melitensis* 16M Spot 14 (35.0 kDa; pI 6.6)

continued on next page

Table 4 continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 VPGVAERMR 9 VPGVAERMR Sbjct 128 VPGVAERMR 136	9/9 (100%)	1.9	Esterase/lipase-like (30.4 kDa; pI 7.1)	*Burkholderia* sp. 383 Spot 14 (35.0 kDa; pI 6.6)
Query 1 ILEMLA--SE 8 ILEMLA SE Sbjct 248 ILEMLARFSE 257	8/10 (80%)	456	Cyclohexadienyl dehydrogenase (33.8 kDa; pI 5.6)	*Mesorhizobium loti* MAFF303099 Spot 15 (32.0 kDa; pI 6.6)

* Peptide sequences which were not assigned to protein hits by the Mascot program were manually compared with the database at the NCBI (search for short, nearly exact matches; substitution matrix PAM30) using the BLAST program (Altschul et al., 1997). The percentage of identity was defined as the percentage of amino acids that are identical in two proteins. The line between the sequences shows identical and similar (+) amino acids whereas gaps are assign by a null (-). The lower the E value (expectation value), the more significant the score. The higher the score the better the alignment. The score for an alignment is calculated by summing the scores for each aligned position and the negative scores for gaps. Additional IMG numbers refer to defined genes or if paralogs of a gene are present in one genome.

Table 5. Estradiol-induced soluble proteins in *Denitratisoma oestradiolicum* AcBE2-1^T with highly significant identity to known proteins with regard to molecular mass, isoelectric point, degree of sequence identity, or function (gel with linear pH 5-8 IPG strip)*

AcBE2-1^T protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 DKQCDCVISLGGGSPHDCAK 20 DKQCDCVISLGGGSPHDCAK Sbjct 86 DKQCDCVISLGGGSPHDCAK 105	20/20 (100%)	6e-11	Alcohol dehydrogenase II (39.0 kDa; pI 4.9)	*Azoarcus* sp. EbN1 Spot 2 (25.0 kDa; pI 5.8)
Query 1 RSDAVKQELRA 11 RSDAVKQELRA Sbjct 142 RSDAVKQELRA 152	11/11 (100%)	0.10	2-hydroxychromene-2-carboxylate isomerase (23.0 kDa; pI 6.5)	*Rubrivivax gelatinosus* PM1 Spot 3 (26.0 kDa; pI 5.8)
Query 2 RLTSSLCANK 11 RL SSLCA K Sbjct 112 RL-SSLCAQK 120	8/10 (80%)	3043	3-demethylubiquinone-9 3-methyltransferase (IMG 635931690) (36.6 kDa; pI 5.7)	*Rhodoferax ferrireducens* DSM 15236 Spot 4 (40.0 kDa; pI 6.0)
Query 1 AFDQGEWRTMSPASR 15 AFDQGEWRTMSPASR Sbjct 69 AFDQGEWRTMSPASR 83	15/15 (100%)	1e-06	Aldehyde dehydrogenase (53.9 kDa; pI 4.9)	*Bacillus subtilis* strain 168 Spot 6 (48.0 kDa; pI 6.1)
Query 1 KDISIAVMGCVVNGPGEGK 19 KDISIAVMGCVVNGPGEGK Sbjct 319 KDISIAVMGCVVNGPGEGK 337	19/19 (100%)	2e-09	1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (40.7 kDa‡; pI NA†)	*Treponema denticola* ATCC 35405 Spot 6 (48.0 kDa; pI 6.1)
Query 1 DGSVTAGNASALNDGAAAVMLMSASKAR 28 DGSVTAGNASALNDGAAAVMLMSASKAR Sbjct 272 DGSVTAGNASALNDGAAAVMLMSASKAR 299	28/28 (100%)	2e-16	Acetyl-CoA acetyltransferase (IMG 625460160) (44.1 kDa; pI 6.7)	*Pseudomonas fluorescens* Pf-5 Spot 7 (48.0 kDa; pI 6.2)
Query 1 DLGREAMVASMRHK 14 DLGREAMVASMRHK Sbjct 496 DLGREAMVASMRHK 509	14/14 (100%)	2e-05	NAD-dependent aldehyde dehydrogenase (IMG 635560445) (55.0 kDa; pI 6.9)	*Burkholderia xenovorans* LB400 Spot 8 (50.0 kDa; pI 6.3)
Query 1 VP--KLGGKLINK 11 VP KL GKLI+K Sbjct 281 VPLMKLAGKLIDK 293	9/13 (69%)	215	NAD-dependent epimerase/dehydratase (IMG 636234830) (36.6 kDa; pI 9.9)	*Geobacter metallireducens* GS-15 Spot 9 (31.0 kDa; pI 6.5)
Query 1 SPNAACGRPR 10 SPNAACGRPR Sbjct 2 SPNAACGRPR 11	10/10 (100%)	0.45	20-beta-hydroxysteroid dehydrogenase, putative (28.2 kDa; pI 4.6)	*Sulfitobacter* sp. NAS-14.1 Spot 9 (31.0 kDa; pI 6.5)

continued on next page

Table 5 continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 ARIVASTVENVWPLLADGTIR 21 ARIVASTVENVWPLLADGTIR Sbjct 263 ARIVASTVENVWPLLADGTIR 283	21/21 (100%)	1e-11	Putative quinone oxidoreductase (33.4 kDa; pI 5.8)	*Corynebacterium diphtheriae* NCTC 13129 Spot 9 (31.0 kDa; pI 6.5)
Query 1 KHFDLRPKG 9 KHFDLRPKG Sbjct 334 KHFDLRPKG 342	9/9 (100%)	1.9	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 11 (48.0 kDa; pI 6.8)
Query 1 KYLVNPTGRF 10 KYLVNPTGRF Sbjct 224 KYLVNPTGRF 233	10/10 (100%)	0.2	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 11 (48.0 kDa; pI 6.8)
Query 1 KTAAYGHFGRD 11 KTAAYGHFGRD Sbjct 356 KTAAYGHFGRD 366	11/11 (100%)	0.046	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 11 (48.0 kDa; pI 6.8)
Query 1 RFVVGGPQGDCGLTGRK 17 RFVVGGPQGDCGLTGRK Sbjct 232 RFVVGGPQGDCGLTGRK 248	17/17 (100%)	2e-07	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 11 (48.0 kDa; pI 6.8)
Query 1 RKIIVDTYGGAAPHGGGAFSGKD 23 RKIIVDTYGGAAPHGGGAFSGKD Sbjct 247 RKIIVDTYGGAAPHGGGAFSGKD 269	23/23 (100%)	2e-12	S-adenosylmethionine synthetase (42.0 kDa; pI 5.6)	*"Dechloromonas aromatica"* RCB Spot 11 (48.0 kDa; pI 6.8)
Query 2 VLDGVVSLDIYR 13 VL+G VSLD YR Sbjct 274 VLEGYVSLDSYR 285	9/12 (75%)	154	3-polyprenyl-4-hydroxybenzoate decarboxylase, UbiD (56.0 kDa; pI 6.6)	*Wolbachia endosymbiont strain* TRS of *Brugia malayi* Spot 12 (48.0 kDa; pI 6.7)
Query 1 RLLNEPTAAAIAYGLDKA 18 RLLNEPTAAAIAYGLDKA Sbjct 180 RLLNEPTAAAIAYGLDKA 197	18/18 (100%)	4e-08	Fe-S protein assembly chaperone HscA (IMG 632157740) (65.5 kDa; pI 4.7)	*Polaromonas naphthalenivorans* CJ2 Spot 15 (48.0 kDa; pI 6.4)
Query 1 INAVSPSIALHEFLK 15 INAVSPSIALHEFLK Sbjct 194 INAVSPSIALHEFLK 208	15/15 (100%)	1e-05	Putative reductase or dehydrogenase protein ("short chain" dehydrogenase) (27.9 kDa; pI 6.4)	*Pseudoalteromonas haloplanktis* TAC125 Spot 19 (23.0 kDa; pI 7.2)

continued on next page

Table 5 continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 EGGKAM 6 EGGKAM Sbjct 31 EGGKAM 36	6/6 (100%)	2078	Methyltransferase (IMG 632021240) (38.1 kDa; pI 5.7)	*Syntrophus aciditrophicus* SB Spot 20 (24.0 kDa; pI 7.3)
Query 1 DNLDAMKTDNVATGKLPGLQALGITPASLEAIGPTYLGAR 40 DNLDAMKTDNVATGKLPGLQALGITPASLEAIGPTYLGAR Sbjct 263 DNLDAMKTDNVATGKLPGLQALGITPASLEAIGPTYLGAR 302	40/40 (100%)	2e-28	Nucleoside-diphosphate-sugar epimerases (EC 1.6.5.3 1.6.99.3) KO: K00329 NADH dehydrogenase KO: K00356 NADH dehydrogenase (33.9 kDa; pI 9.3)	*Polaromonas naphthalenivorans* CJ2 Spot 21 (25.0 kDa; pI 7.4)
Query 2 SLLLIEDDEAIR 13 SL+L+EDDE IR Sbjct 200 SLILVEDDEVIR 211	9/12 (75%)	3.5	3-ketoacyl-CoA thiolase; (thiolase I, acetyl-CoA transferase), in complex with FadB catalyzes (EC 2.3.1.16) (40.9 kDa; pI 6.1)	*Pseudoalteromonas haloplanktis* TAC125 Spot 23 (39.0 kDa; pI 7.2)
Query 3 LDIKATDSSAL 13 LD K TDSS L Sbjct 45 LDFKVTDSSEL 55	8/11 (72%)	562	4-diphosphocytidyl-2C-methyl-D-erythritol kinase (EC 2.7.1.148) (30.8 kDa; pI 6.5)	*Shewanella putrefaciens* CN-32 Spot 26 (36.0 kDa; pI 6.2)

* Peptide sequences which were not assigned to protein hits by the Mascot program were manually compared with the database at the NCBI (search for short, nearly exact matches; substitution matrix PAM30) using the BLAST program (Altschul et al., 1997). The percentage of identity was defined as the percentage of amino acids that are identical in two proteins. The line between the sequences shows identical and similar (+) amino acids whereas gaps are assign by a null (-). The lower the E value (expectation value), the more significant the score. The higher the score the better the alignment. The score for an alignment is calculated by summing the scores for each aligned position and the negative scores for gaps. Additional IMG numbers refer to defined genes or if paralogs of a gene are present in one genome.

† No data available.

‡ Molecular weight was determined from the *Escherichia coli* protein (Baker et al., 1992).

3.3 Characterization of strain FS^T

3.3.1 Enrichment and isolation

Enrichment cultures inoculated with 5 ml anoxic digested sludge from a municipal wastewater treatment plant contained 1 mM estradiol and 5 mM nitrate. Increase in turbidity and nitrate depletion without any nitrite accumulation began after one week in all bottles. New nitrate was added in portions of 5 mM whenever the residual electron acceptor was consumed. In contrast to the initial enrichment culture, subcultures exibited a slower reduction of 5 mM nitrate within 4 weeks of incubation. Subcultures of the controls without estradiol stopped nitrate reduction and growth.

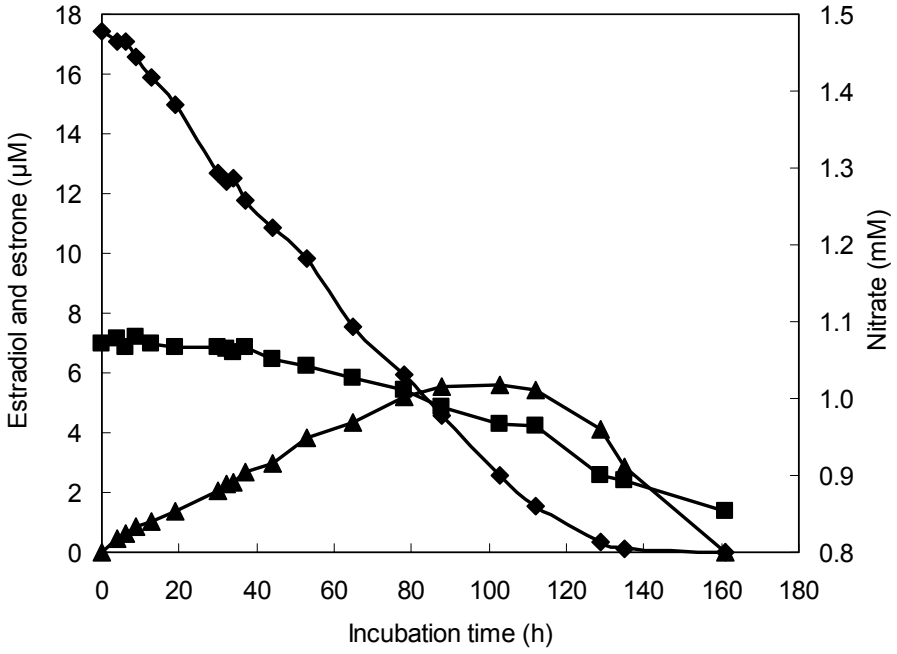


Fig. 9. Estradiol and estrone turnover with corresponding nitrate reduction during growth of the enrichment culture of strain FS^T. The data points are the average of duplicate determinations (Table 8A, Appendix): ♦, estradiol; ▲, estrone; ■, nitrate.

A preliminary time course experiment was carried out with the enrichment culture using estradiol as electron donor and 1070 ± 8.8 µM (mean ± standard deviation, n=2) nitrate. Estradiol could be dissolved during autoclaving to a concentration of 17.4 ± 0.21 µM (n=2). The decrease of estradiol and the turnover of intermediary estrone was monitored together with the reduction of nitrate. The results are summarized in Fig. 9. Degradation of estradiol is

directly accompanied by the formation of estrone (up to 5.6 ± 0.03 µM, n=2). This indicates that estrone is an intermediate of the estradiol degradation pathway. With estradiol amounts falling below 6 µM, estrone degradation starts, resulting in the complete consumption of estrone. 220 µM nitrate was consumed totally, no nitrite formation was observed.

A pure culture, designated strain FS^T, was obtained within one year performing four serial dilution series with either estradiol or testosterone (another selective growth substrate), followed by two subsequent agar shake dilutions with testosterone dissolved in DMSO and with nitrate as the electron acceptor. After 4-6 weeks gas production starts and small (approx. 1 mm in diameter) yellow-brown, disc-shaped colonies of strain FS^T appeared in the agar medium and were picked and transferred into liquid mineral medium.

3.3.2 Physiological, cytological, and morphological properties

Cells of strain FS^T showing the location of insertion of its flagellum are shown in Fig. 10. They were slightly curved rods with rounded ends, 0.3-0.5 x 0.6-1.6 µm in size. The main characteristics are listed in Table 6 and in the species description (Chapter 4.3).

Note, that the data about the phenotypic properties of the related taxa were taken from the descriptions of the respective type strains (Table 6). The phylogenetic analysis (Chapter 3.3.4, Fig. 13) was partly conducted with 16S rRNA gene sequences obtained from other isolates which were not designated as the type strains of the respective genera. No data could be obtained in the case of *Panacagrimonas perspica* (AB257720).

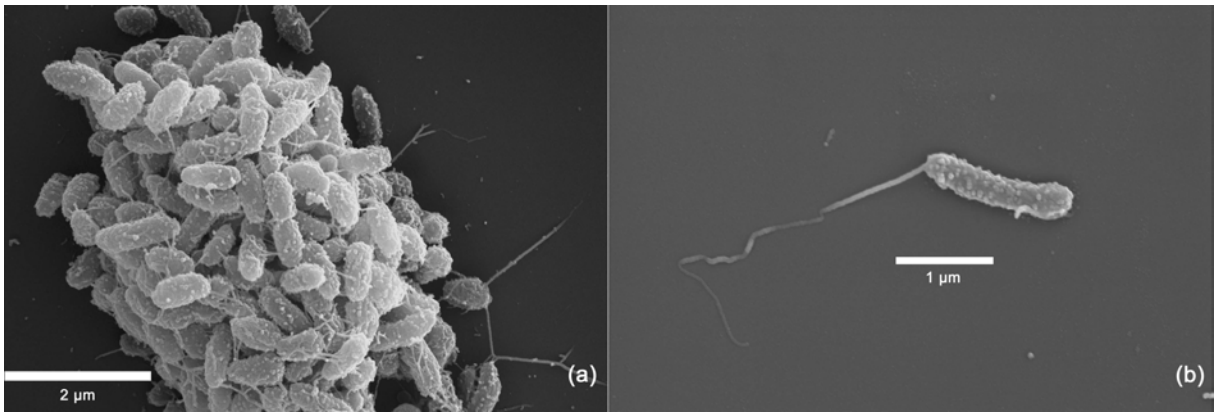


Fig. 10. Scanning electron micrographs of cells of strain FS^T grown on 1 mM testosterone and 5 mM nitrate. The magnification is 15000x (a) and 20000x (b), respectively.

Table 6. Physiological, cytological and morphological properties of strain FS^T in comparison with type strains of related genera belonging to the *Gammaproteobacteria*

Strains: 1, FS^T; 2, *Nevskia ramosa* Soe1^T (= DSM 11499^T); 3, *Hydrocarboniphaga effusa* AP103^T (= DSM 16095^T); 4, *Ectothiorhodospira shaposhnikovii* Kondratieva N1^T (= DSM 243^T); 5, *Nitrosococcus oceani* C-107^T (= ATCC 19707^T).

Symbols: +, growth; (+), faint or slow growth; -, no growth; ND, not determined or no data available. All type strains of the genera listed are negative for Gram stain and are motile by a single flagellum of polar insertion with the exception of *Ectothiorhodospira shaposhnikovii* and *Nitrosococcus oceani* bearing a tuft of polar flagella. Data for the genera were taken from Stürmeyer et al. (1998), Palleroni et al. (2004), Ventura et al. (2000), Imhoff & Süling (1996) and Watson (1965).

Character	**1**	**2**	**3**	**4**	**5**
Cell morphology	Curved rod	Bent Rod	Rod	Bent Rod	Spherical to ellipsoidal
Special morphological features	No	Rosette-like formation of microcolonies	Colonies on agar media are of irregular shape with the occurrence of swarming cells	Internal photosynthetic membranes (lamellar stacks). Gas vesicles	Arrangement of intracyoplasmatic membranes (central stacks of vesicles)
Cell dimensions (width x length) (µm)	0.3-0.5 x 0.6-1.6	0.7-1.1 x 1.5-2.3	0.75-0.85 x 1.5-2.0	0.8-1.5 x 1.5-4.0*	1.5-1.8 x 1.7-2.5
DNA G+C content (mol%)	61.9	67.8	60.0	61.4-63.6	50.0-51.0
Catalase	Positive	Weak activity	ND	ND	ND
Cytochrome oxidase	Positive	ND	ND	ND	ND
Major quinone system	Q-8	ND	ND	Q-7 and MK-7	ND
Aerobic metabolism	Yes§	Yes	Yes	No†	Yes
Chemoheterotrophic growth	Yes	Yes	Yes	Yes	No
Photoautotrophic or photoheterotrophic growth	No	ND	ND	Yes	ND
Chemoautotrophic growth	No	ND	ND	Yes	Yes‡
Growth on complex media	No	Yes	Yes	ND	ND
Denitrification	Yes	No	No	No	No
Nutritional spectrum of organic or anorganic substrates	Narrow	Wide	Wide	Wide	Unknown
Utilization of:					
Fatty acids	+	+	(+)	+	ND
Aliphatic hydrocarbons	-	ND	+	ND	ND
Estradiol	+	ND	ND	ND	ND
Testosterone	+	ND	ND	ND	ND
Benzoate	-	+	-	-	ND
Ethanol	-	+	+	-	ND
DL-lactate	-	+	+	+	ND
Glucose	-	+	+	ND	ND
Glutamate	+	+	+	ND	ND

* Cell size after growth with propionate as carbon source.

† Chemoautotrophic or chemoheterotrophic growth is possible under microaerobic conditions.

‡ Oxidation of ammonia.

§ With testosterone or 4-androstene-3,17-dione as electron donor.

3.3.3 Quantification of steroid hormone degradation and denitrification

Degradation of estradiol. Growth on estradiol with nitrate as the electron acceptor is shown in Fig. 11. Increase in biomass occurred simultaneously with dinitrogen monoxide formation and resulted in a complete depletion of nitrate after 14 days. Dinitrogen levels remained unchanged. Nitrite was not detected during growth. To show the increase in cell dry mass, the unit mg dl^{-1} was used. Cell dry mass increased with higher amounts of substrate dissimilated (Table 7). Electron recoveries, as well as the appropriate growth yields did not show any relevant trend of decrease concomitant to increasing amounts of provided estradiol. This indicates an almost complete oxidation of estradiol to carbon dioxide and water over a broad range of substrate amounts. Nitrate reduction resulted in dinitrogen monoxide formation, without any further reduction to dinitrogen and without an intermediate accumulation of nitrite. The assays 1-5 displayed an average of 98.2 ± 0.97 % of nitrate reduction to dinitrogen monoxide (Table 2A, Appendix) and, therefore, an assimilation of nitrate could be ruled out. A concentration of 0.25 g NH_4Cl l^{-1} was present in the medium.

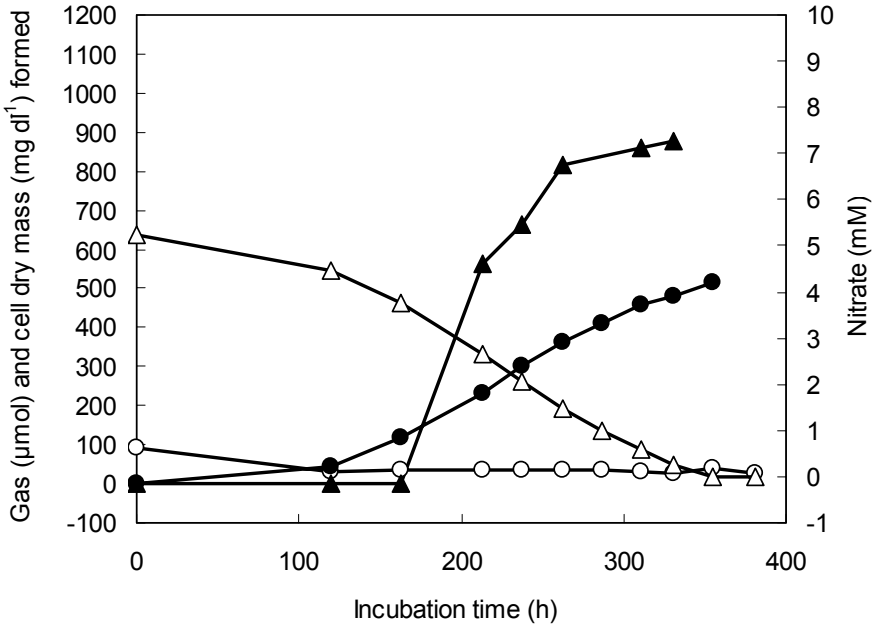


Fig. 11. Growth of *Steroidobacter denitrificans* FS^T on 1 mM estradiol with 5 mM nitrate (200 ml mineral medium). Growth could be assayed as increase in protein content assuming that 50 % of the dry cell weight is protein. Symbols of dinitrogen monoxide and dinitrogen are the average of duplicate determinations (Table 5A, Appendix): ▲, cell dry mass; △, nitrate; ●, dinitrogen monoxide; ○, dinitrogen.

Table 7. Quantification of estradiol oxidation and nitrate reduction by strain FS^T with different amounts of substrate provided. The total amount of nitrate in each infusion bottle was 500 µmol (100 ml mineral medium)

Estradiol provided [µmol]	Estradiol consumed totally [µmol]	Nitrate consumed* [µmol]	N_2O formed* [µmol]	Cell dry mass formed† [mg]	Estradiol dissimilated‡ [µmol]	Estradiol assimilated‡ [µmol]	Electron recovery [%]§	Growth yield Y_E [g/mol]¦
10.8	10.3	188.5	91.8	2.2	6.5	3.8	116.5	307.7
28.9	23.0	319.7	157.7	3.5	16.4	6.7	89.4	214.1
36.3	31.2	347.0	172.9	4.5	22.7	8.6	75.8	198.7
53.3	30.4	449.3	219.0	5.1	20.7	9.7	96.1	246.3
123.2	44.1	484.9	237.1	6.8	31.2	12.9	77.1	218.8

* Difference between compound measured at the beginning and at the end of incubation.

† Calculated via protein content after growth assuming that 50 % of the dry cell weight (X) was protein.

‡ Difference between estradiol consumed totally (ΔS) and estradiol assimilated (ΔS_B). The assimilated amount of estradiol was calculated by the equation (Chapter 8.2, Appendix):

$9\ C_{18}H_{24}O_2 + 46\ NH_4^+ + 22\ CO_2 + 30\ H_2O \rightarrow 46\ <C_4H_9O_2N> + 46\ H^+$

Thus, 1 mg of cell dry mass requires 0.0019 mmol estradiol. Dissimilated estradiol (ΔS_E) was calculated according to $\Delta S = \Delta S_E + \Delta S_B$.

§ The values were calculated from electrons recovered as cell dry mass or consumed by nitrate reduction (reduction of NO_3^- to N_2O, requirement of 4 mol electrons per mol gas formed) in relation to the total amount of electrons (corresponding to 100 %) released by a complete oxidation of totally consumed estradiol.

¦ The molar growth yield Y_E was calculated by taking into account estradiol consumption for cell synthesis ($Y_E = \Delta X / \Delta S_E$).

Degradation of testosterone. A degradation time course with testosterone is shown in Fig. 12. Nitrate was completely reduced to dinitrogen monoxide after 200 h without the intermediate accumulation of nitrite. Levels of dinitrogen remained constant.

Cell dry mass increased with higher amounts of substrate dissimilated. Electron recoveries were between 80 % and 100 %, except for the assay in line 5 with the highest amount of substrate provided (Table 8). In the assays in line 4 and 5, nearly the same amount of cell dry mass was produced although more testosterone was totally consumed in the latter one. At the end of growth, the total amount of nitrate was converted nearly stoichiometrically to dinitrogen monoxide. The assays 1-5 (Table 8) displayed an average of 105.8 ± 4.3 % of nitrate reduced to dinitrogen monoxide (Table 3A, Appendix). Therefore, nitrate was not used for the assimilation of nitrogen.

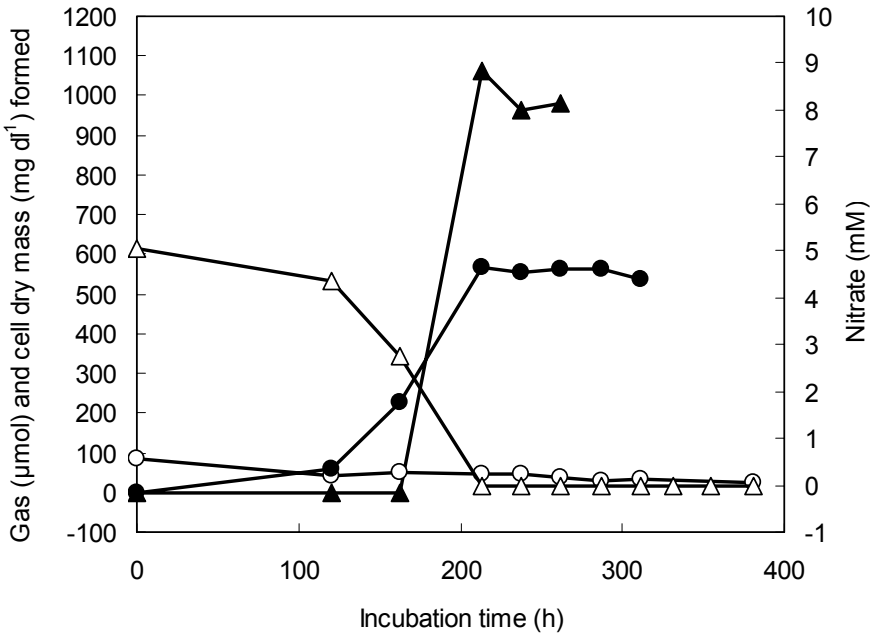


Fig. 12. Growth of *Steroidobacter denitrificans* FS^T on 1 mM testosterone with 5 mM nitrate (200 ml mineral medium). Growth could be assayed as increase in protein content assuming that 50 % of the dry cell weight is protein. Symbols of dinitrogen monoxide and dinitrogen are the average of duplicate determinations (Table 6A, Appendix): ▲, cell dry mass; △, nitrate; ●, dinitrogen monoxide; ○, dinitrogen.

Table 8. Quantification of testosterone oxidation and nitrate reduction by strain FS^T with different amounts of substrate provided. The total amount of nitrate in each infusion bottle was 500 µmol (100 ml mineral medium)

Testosterone provided [µmol]	Testosterone consumed totally [µmol]	Nitrate consumed* [µmol]	N_2O formed* [µmol]	Cell dry mass formed† [mg]	Testosterone dissimilated‡ [µmol]	Testosterone assimilated‡ [µmol]	Electron recovery [%]§	Growth yield Y_E [g/mol]¦
11.2	11.2	165.2	83.7	2.8	6.3	4.9	102.8	444.4
24.1	24.1	363.7	189.1	5.4	14.7	9.5	99.6	368.6
32.1	32.1	382.3	215.5	6.1	21.4	10.7	80.9	284.7
48.3	48.3	537.1	281.1	9.8	31.2	17.2	80.0	314.6
97.2	92.0	529.6	282.0	10.3	74.0	18.0	42.6	139.2

* Difference between compound measured at the beginning and at the end of incubation.

† Calculated via protein content after growth assuming that 50 % of the dry cell weight (X) was protein.

‡ Difference between testosterone consumed totally (ΔS) and testosterone assimilated (ΔS_B). The assimilated amount of testosterone was calculated by the equation (Chapter 8.2, Appendix):

$9\ C_{19}H_{28}O_2 + 50\ NH_4^+ + 29\ CO_2 + 24\ H_2O \rightarrow 50\ <C_4H_9O_2N> + 50\ H^+$

Thus, 1 mg of cell dry mass requires 0.00175 mmol testosterone. Dissimilated testosterone (ΔS_E) was calculated according to $\Delta S = \Delta S_E + \Delta S_B$.

§ The values were calculated from electrons recovered as cell dry mass or consumed by nitrate reduction (reduction of NO_3^- to N_2O, requirement of 4 mol electrons per mol gas formed) in relation to the total amount of electrons (corresponding to 100 %) released by a complete oxidation of totally consumed testosterone.

¦ The molar growth yield Y_E was calculated by taking into account testosterone consumption for cell synthesis ($Y_E = \Delta X / \Delta S_E$).

3.3.4 Phylogenetic analysis and chemotaxonomy

Comparative 16S rRNA gene sequence analysis revealed that strain FS^T represents a separate line of descent within the *Gammaproteobacteria* (Fig. 13). This class contains the photosynthetic purple sulfur bacteria (*Chromatiaceae* and *Ectothiorhodospiraceae*) together with a great number of familiar chemoorganotrophic bacterial groups, such as the *Enterobacteriaceae*, *Legionellaceae*, *Pasteurellaceae*, *Pseudomonadaceae*, *Vibrionaceae*, and also some chemolithotrophic mostly sulfur- or iron-oxidizing prokaryotes. A distant relationship of strain FS^T exists to the species *Nevskia ramosa* (87.7 % sequence similarity), *Hydrocarboniphaga effusa* (87.1 % sequence similarity), *Panacagrimonas perspica* (87.8 % sequence similarity), *Nitrosococcus oceani* strain C-107 (88.1 % sequence similarity) and *Ectothiorhodospira shaposhnikovii* strain Z1 (85.0 % sequence similarity). Strain FS^T is closer related to several environmental clone sequences, consisting of so far uncultivated bacteria, many of which were obtained from soil or microbial rhizosphere communities and in some cases from uranium mines and deposits.

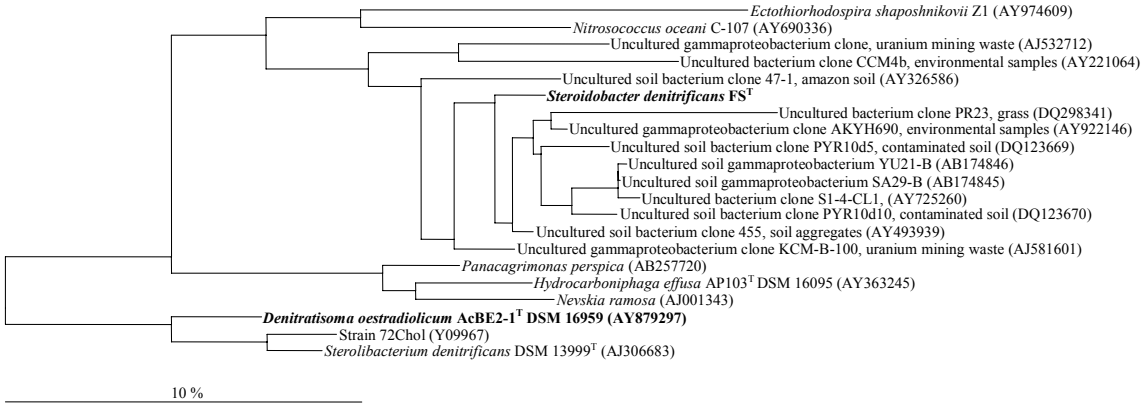


Fig. 13. Phylogenetic tree showing the affiliation of the 16S rRNA gene sequence from *Steroidobacter denitrificans* FS^T to selected reference sequences of members of the *Gammaproteobacteria*. In addition, clone sequences of hitherto uncultivated bacteria were added. The tree was calculated by maximum-likelihood analysis without using any filters. Only sequences of at least 1300 nt were used for tree calculations. The sequences of the steroid-degrading *Betaproteobacteria Denitratisoma oestradiolicum* AcBE2-1^T, *Sterolibacterium denitrificans*, and strain 72Chol were used as the outgroup. The accession numbers of sequences used are given in parentheses. The sequence of *Steroidobacter denitrificans* FS^T has not been deposited at GenBank/EMBL/DDBJ yet. Bar, 10 % estimated sequence divergence.

The quinone system consisted exclusively of ubiquinone Q-8. The fatty acid profile of strain FS^T mainly consists of $C_{15:0}$ (16.5 %), $C_{16:0}$ (7.0 %), $C_{17:0}$ (6.2 %), $C_{17:1}\omega8c$ (27.1 %), summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 8.3 %), and $C_{16:0}$ 10-methyl (7.0 %). The fatty acids $C_{9:0}$ (0.6 %), $C_{10:0}$ (1.3 %); $C_{11:0}$ (2.7 %), $C_{12:0}$ (1.8 %), $C_{13:0}$ (4.2 %), $C_{14:0}$ (0.9 %), $C_{15:1}\omega8c$ (2.5 %), $C_{16:1}\omega9c$ (0.9 %), $C_{17:1}\omega6c$ (3.0 %), $C_{18:1}\omega7c$ (4.2 %) were present in smaller amounts. The only hydroxylated fatty acids were $C_{11:0}$ 3-OH (1.2 %) and $C_{12:0}$ 3-OH (1.3 %). Additionally, two other branched fatty acids iso-$C_{16:1}$ (0.8 %) and $C_{17:0}$ 10-methyl (2.4 %) were also present.

4 Discussion

4.1 Description of strain AcBE2-1^T as *Denitratisoma oestradiolicum* gen. nov., sp. nov.

4.1.1 Physiology

Degradation experiments with the enrichment culture. The final concentrations of nitrate and nitrite indicate a complete oxidation of estradiol to carbon dioxide and water after an initial oxidation beyond estrone (Fig. 3). It was impossible to determine a cell dry mass increase since the substrate concentration was very low at 16.6 µM of estradiol. Therefore, assimilated estradiol could only be estimated from the results of the electron balances shown in Table 3. Approximately 60 % (10 µM) of the provided estradiol (16.6 µM) has been dissimilated. The dissimilated estradiol (10 µM) agrees well with the totally consumed nitrate (335 µM) and the amount of produced nitrite (130 µM). The residual nitrate (205 µM) was assumed to be reduced to a mixture of dinitrogen monoxide and dinitrogen. The formation of estrone is the first step in estradiol degradation. This initial oxidation is widespread in bacteria (Hanselman et al., 2003) and is mediated by an inducible 17*β*-hydroxysteroid dehydrogenase as found in *Comamonas testosteroni* (Benach et al., 2002) or possibly quite simply by unspecific alcohol dehydrogenases. The proteomic analysis of estradiol-induced soluble proteins in this bacterium revealed several dehydrogenases that might be involved in this initial estradiol degradation step (Chapter 4.2).

Quantification of estradiol oxidation and denitrification. A complete oxidation of estradiol to carbon dioxide and water could be assumed by the amounts of gases formed in cultures where a complete nitrate and nitrite consumption appeared (e.g., by oxidation of 25.4 µmol estradiol dissimilated, 91.6 µmol dinitrogen monoxide and 158.4 µmol dinitrogen were formed from 506.7 µmol nitrate, Table 3 and Table 1A, Appendix). The degradation equation is as follows:

$$C_{18}H_{24}O_2 + 20\ NO_3^- + 20\ H^+ \rightarrow 18\ CO_2 + 4\ N_2O + 6\ N_2 + 22\ H_2O$$

In carbon-limited cultures, higher amounts of nitrogen gases were found as expected from residual nitrate and nitrite (Table 1A, Appendix). The assays 1-5 displayed an average of 123.6 ± 26.2 % of nitrate reduced to dinitrogen monoxide and dinitrogen. This could be explained by the difficulty of making precise measurements of the gas production in cultures

with little substrate conversion (assays 1 and 2), especially of dinitrogen. Further evidence for a complete oxidation of estradiol could be obtained from produced carbon dioxide. However, the quantitative experiments were conducted in a bicarbonate-buffered mineral medium with a He/CO_2 atmosphere (80:20, v/v). Therefore a sensitive determination of the expected carbon dioxide from estradiol oxidation by gas chromatography was not feasible. A dependence of estradiol degradation on carbon dioxide in the form of bicarbonate could not be ruled out, since no growth with estradiol was observed in potassium phosphate-buffered medium. Radio-labeled [^{14}C]estradiol should be used in future studies for the detection of carbon dioxide as an end product of anaerobic estradiol degradation in strain AcBE2-1^T.

4.1.2 Taxonomy

Comparative 16S rRNA gene sequence analysis showed that strain AcBE2-1^T belongs to the order *Rhodocyclales* within the *Betaproteobacteria* (Fig. 6). Members of this order comprise a physiologically versatile assemblage of bacteria, many of them responsible for the removal of anthropogenic compounds in the environment or in biotechnological systems (e.g., wastewater treatment plants).

Most known species belonging to the genera *Thauera* and *Azoarcus* and couple anaerobic degradation of aromatic or aliphatic hydrocarbons with dissimilatory reduction of nitrate (Anders et al., 1995; Mechichi et al., 2002). Other *Azoarcus* species together with members of the genera *Azovibrio*, *Azospira*, and *Azonexus* are associated with grass roots, where they fix dinitrogen (Reinhold-Hurek & Hurek, 2000). Furthermore, it has been recognized that the genera *Dechloromonas* and *Azospira* (formerly *Dechlorosoma*) harbor the dominant (per)chlorate-reducing bacteria in the environment (Achenbach et al., 2001; Coates et al., 1999). Another important bioremediation process which exploits so far uncultured bacteria of this order is enhanced biological phosphorus removal in sewage treatment (Crocetti et al., 2000). These bacteria were provisionally named "*Candidatus* Accumulibacter phosphatis" (Hesselmann et al., 1999). Other uncultivated representatives of the *Rhodocyclales* to date are might be the numerically dominant bacteria in activated sludge from a nitrifying-denitrifying wastewater treatment plant and a denitrifying quinoline-removal bioreactor, where they presumably contribute to denitrification and removal of organic compounds (Juretschko et al., 2002; Loy et al., 2005; Liu et al., 2005).

Highest sequence similarity of strain AcBE2-1^T was to the cholesterol-degrading, denitrifying bacteria *Sterolibacterium denitrificans* DSM 13999^T (Tarlera & Denner, 2003) and the partially described strain 72Chol (Harder & Probian, 1997), with similarity values below 93.9 %. Strain AcBE2-1^T is positioned in a separate cluster distinct from known lineages of the order *Rhodocyclales* (Appendix Fig. 1A). 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 (Appendix Fig. 1A), and information regarding their physiological properties is lacking. Several clone sequences within this cluster belong to the *Betaproteobacteria* and probably contribute to wastewater treatment (Juretschko et al., 2002; Loy et al., 2005; Liu 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.
Ubiquinone Q-8 was detected by reversed-phase TLC. Ubiquinone Q-8 is a characteristic feature of the *Betaproteobacteria* (Collins & Jones, 1981; Yokota et al., 1992). The fatty acid profile of AcBE2-1^T is clearly different from that of *Sterolibacterium denitrificans*, for which $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso $C_{15:0}$ 2-OH) , and $C_{18:1}\omega7c$ 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 creating a new genus for strain AcBE2-1^T was the high sequence divergence from related genera and phenotypic features that were characteristically different from those of the related taxa. In contrast to its nearest relatives *Sterolibacterium denitrificans* and 72Chol, strain AcBE2-1^T was able to use estradiol, estrone, short-chain fatty acids and several citric acid cycle intermediates, but not cholesterol or long-chain fatty acids. On the basis of the 16S rDNA gene sequence in combination with chemotaxonomic and physiological data, strain AcBE2-1^T is considered to be the type strain of a novel species and in a new genus, for which the name *Denitratisoma oestradiolicum* gen., nov. is proposed (Fahrbach et al., 2006).

Description of *Denitratisoma* gen. nov.

Denitratisoma (De.ni.tra.ti.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 x 0.8-2.0 µm in size) with rounded ends, and occurred 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 µm). Metabolism is strictly oxidative. Nitrate is reduced to a mixture of N_2O and N_2, nitrite accumulates intermediately. Oxidase-positive and catalase-negative. Anaerobic oxidation of estradiol to carbon dioxide and water 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 are not reduced. Optimal

growth occurs at 28 to 30 °C and pH 7.0-7.2. Salinity range is 0-1.0 % (NaCl, w/v). Ubiquinone Q-8 is the single quinone. Major fatty acids are $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH and $C_{16:0}$. Minor components are $C_{18:1}\omega7c$ 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* estradiol; L. neut. suff. *-icum* belonging to; N.L. neut. adj. *oestradiolicum* belonging to estradiol, referring to estradiol utilization).

Exhibits the following properties in addition to those given in the genus description. The fatty acid profile comprises $C_{14:0}$ (0.6 %), $C_{16:0}$ (29.8 %), $C_{8:0}$ 3-OH (2.3 %), sum in feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 53.4 %), $C_{16:1}\omega5c$ (1.5 %), $C_{18:1}\omega5c$ (0.3 %), $C_{18:1}\omega7c$ (11.8 %), and $C_{18:0}$ (0.3 %). Electron donors used with nitrate as the electron acceptor are listed in Table 2. No growth with following substrates (not listed in Table 2): formate, laureate, isovalerate, oleate, adipate, pimelate, primary aliphatic alcohols (C_1 to C_4), 2-propanol, 1,2-propandiol, cyclohexanol, cyclopentanone, acetone, 2-butanone, citrate, L(+)-tartrate, ascorbate, L-cysteine, glutamate, D(+)-glucose, D(-)-fructose, D(+)-galactose, D(+)-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 estradiol or estrone in liquid or solid media. Substrates used with oxygen as the electron acceptor are acetate, pyruvate or fumarate. On exposure of cells to air, an expanded lag phase occurs, even after several transfers. Formed small colonies on R2A agar after a prolonged incubation time. 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-Soers (Germany).

4.2 Proteomic analysis of *Denitratisoma oestradiolicum* AcBE2-1^T

Application of two-dimensional gel electrophoresis allowed to demonstrate the presence of estradiol-induced soluble proteins in *Denitratisoma oestradiolicum* AcBE2-1^T. Since the anaerobic degradation of estradiol is completely unknown until now, the analysis of these estradiol-induced proteins by a proteomic approach will for the first time give insights into such a complex metabolic pathway and its regulation.

Cell-free extracts from estradiol grown cells were found to contain several proteins that were absent in extracts from acetate grown cells (Fig. 7 and 8). On the other hand, several proteins were obviously repressed upon estradiol presence or were expressed in lower amounts under these growth conditions (Fig. 7 and 8). Carrying out two-dimensional gel electrophoresis, proteins were separated using either IPG strips of pH ranges of 3-10 (Fig. 7) or 5-8 (Fig. 8) in the first dimension. Protein separation in the first dimension with a pH range from 3-10 revealed that most proteins were found between pH 5-8. Although the proteins were better separated by using IPG strips of a pH range of 5-8, some gel spots might represent a mixture of several proteins. These proteins have not been resolved completely due to similar molecular weights and isoelectric points. Indeed, several protein hits were obtained in one spot (Table 4, 5, 11A, 13A and 14A).

Estradiol-induced proteins were manually excised from the gels. Tryptic digest of these proteins and separation of peptide fragments by nano-HPLC in combination with tandem mass spectrometry (LC-ESI-Q-TOF-MS) revealed sufficient sequence information to query databases. It is noteworthy that most of the hits found in databases rely on DNA sequences obtained from whole sequenced prokaryotic genomes which were translated to amino acid sequences. Therefore, the obtained MS/MS data were also compared to amino acid sequences that derived originally from nucleotide sequences. Additionally, most of these protein sequences were aligned in the databases together with information about theoretical molecular masses and isoelectric points (Integrated Microbial Genomes (IMG) system at http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

Among 45 investigated gel spots, approximately 25 estradiol-induced proteins were found to be of high identity to known prokaryotic proteins (Table 4 and 5). Sequence identities of peptides to known proteins are given in Table 4, 5, 11A, 12A, 13A and 14A. Note, that the term “identity” was here defined not only as the percentage of amino acids that are identical in two proteins. The term “identity” was used with regard to molecular mass, isoelectric point, degree of sequence identity, and function. For some of the estradiol-induced proteins, database searches resulted in less identity (Table 11A and 13A, Appendix). Also sequence similarities to eukaryotic proteins were found (Table 12A and 14A, Appendix). Nevertheless,

they were selected and the most interesting protein hits are introduced here due to their putative role in anaerobic estradiol degradation by *Denitratisoma oestradiolicum*.

Five of the obtained peptide sequences of spot 11 in Fig. 8 (Table 5, IPG pH 5-8) were found to be identical to S-adenosylmethionine synthetase of "*Dechloromonas aromatica*" RCB. The position of spot 11 in Fig. 8 corresponds with the position of spot 9 in Fig. 7 (Table 4, IPG pH range of 3-10). Spot 9 in Fig. 7 revealed the same results with six peptide sequences to be identical to this protein. The theoretical molecular mass of S-adenosylmethionine synthetase differed only slightly with the position on the gel (Fig. 7 and 8) but the protein migrated to a higher pH as S-adenosylmethionine synthetase possessing a theoretical isoelectric point of 5.6. However, the most reliable protein identifications are those in which several different sequences match to the same protein. S-adenosylmethionine synthetase (EC 2.5.1.6) is the enzyme that catalyzes the formation of S-adenosylmethionine (AdoMet) from methionine and ATP. The sequence of AdoMet synthetase is highly conserved throughout isozymes and species. In bacteria there is a single isoform of AdoMet synthetase.

AdoMet is an important methyl donor for transmethylation and it is obviously needed in high amounts during growth on estradiol, indicating a putative methylation reaction in the degradation pathway. A methyltransferase (*ubiE*/COQ5 methyltransferase family) was found in spot 20 (Table 5, IPG pH 5-8), but the position on the gel did not correspond with the theoretical isoelectric point and molecular weight of the protein of *Syntrophus aciditrophicus*. A number of methyltransferases have been shown to share regions of similarities (Lee et al., 1997). Apart from the ubiquinone/menaquinone biosynthesis methyltransferases (for example, the *C*-methyltransferase from the *ubiE* gene of *Escherichia coli*), this family also includes methyltransferases involved in biotin and sterol biosynthesis and in phosphatidylethanolamine methylation. The *C*-methyltransferase encoded by the *ubiE* gene is AdoMet-dependent and methylates the aromatic ring at C-3 of 2-octaprenyl-6-methoxy-1,4-benzoquinol (Meganathan, 2001).

Database searches of one peptide sequence obtained from spot 12 (Table 5, IPG pH 5-8) revealed 75 % sequence identity to 3-octaprenyl-4-hydroxybenzoate decarboxylase. This enzyme, studied in detail in *E. coli*, catalyses the third reaction in ubiquinone biosynthesis, namely, decarboxylation of 3-octaprenyl-4-hydroxybenzoate to 2-octaprenylphenol. Breinig et al. (2000) found sequence similarities to this enzyme in phenol-induced cells of the denitrifying bacterium *Thauera aromatica*. It is thought to catalyze the reverse reaction, a carboxylation of phenylphosphate to 4-hydroxybenzoate under release of phosphate.

The database search found 80 % of the amino acids of spot 4 (Table 5, IPG pH 5-8) to be identical to 3-demethylubiquinone-9 3-methyltransferase in *Rhodoferax ferrireducens* DSM 15236. This unspecific enzyme is AdoMet dependent and catalyzes the methylation of hydroxy groups in quinone biosynthesis (Meganathan, 2001).

From spot 9 in Fig. 8 (Table 5, IPG pH 5-8), three different peptide sequences were obtained which correspond to the enzymes UDP-galactose 4-epimerase (EC 5.1.3.2) of *Geobacter metallireducens* GS-15, to a putative 20*β*-hydroxysteroid dehydrogenase of *Sulfitobacter* sp. NAS-14.1, and to a putative quinone oxidoreductase of *Corynebacterium diphtheriae* NCTC 13129. These enzymes belong to the FAD/NAD(P)-binding Rossmann fold superfamily clan which contains the NAD dependent epimerase/dehydratase family, the 3*β*-hydroxysteroid dehydrogenase/isomerase family, the zinc-containing dehydrogenases, and the “short-chain” dehydrogenase family. Within recent years, it has become apparent that the enzyme UDP-galactose 4-epimerase belongs to the the latter family. The “short-chain” dehydrogenase family is a very large family of enzymes, most of which are known to be NAD- or NADP-dependent oxidoreductases. Other enzymes in this family include 20*β*-hydroxysteroid dehydrogenase, 7*α*-hydroxysteroid dehydrogenase, 17*β*-hydroxysteroid dehydrogenase, and dihydropteridine reductase.

Spot 17 (Table 11A, IPG pH 3-10) contains a cyclohexanol/cyclopentanol dehydrogenase from *Comamonas testosteroni* which corresponds to a “short-chain” dehydrogenase found in spot 19 and spot 21 (Table 5, IPG pH 5-8). All three spots possess nearly the same molecular weight and isoelectric point. Additionally, one obtained peptide sequence in spot 21 (Table 5, IPG pH 5-8) showed 100 % sequence identity to a nucleoside-diphosphate-sugar epimerase of *Polaromonas naphthalenivorans* CJ2, which belongs to the 3*β*-hydroxysteroid dehydrogenase/isomerase family. The enzyme 3*β*-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase catalyzes the oxidation and isomerisation of 5-ene-3 beta-hydroxypregnene and 5-ene-hydroxyandrostene steroid precursors into the corresponding 4-ene-ketosteroids necessary for the formation of all classes of steroid hormones.

Spot 2 (Table 5, IPG pH 5-8) had an overall sequence identity of 100 % to an iron-containing alcohol dehydrogenase (*adhB*) in *Azoarcus* EbN1 (Rabus et al., 2005). Currently three, structurally and catalytically different types of alcohol dehydrogenases are known. These are zinc-containing “long-chain” alcohol dehydrogenases, “short-chain” alcohol dehydrogenases, and iron-containing alcohol dehydrogenases. The latter have been found in yeast and *Zymomonas mobilis* and were closely related to propanediol oxidoreductase (*Escherichia coli*), NADPH- and NADH-dependent butanol dehydrogenases in *Clostridium acetobutylicum*, bacterial glycerol dehydrogenases, NAD-dependent 4-hydroxybutyrate dehydrogenase (*Clostridium kluyveri*), and 1,3-propanediol dehydrogenases of *Citrobacter freundii* and *Klebsiella pneumoniae*. Another iron-dependent enzyme in *E. coli* harbor three different activities. Alcohol dehydrogenase activity, acetaldehyde dehydrogenase activity (acetylating), and pyruvate-formate-lyase deactivase activity. The putative function in *Denitratisoma oestradiolicum* during estradiol degradation could be an oxidation/reduction of hydroxylated

C-atoms of the steroid skeleton, although no iron-containing hydroxysteroid dehydrogenase has been described so far. As discussed above, hydroxysteroid dehydrogenases belong to the “short-chain” alcohol dehydrogenases.

The theoretical molecular mass of aldehyde dehydrogenase of *Bacillus subtilis* agreed well with the position of spot 6 on the gel (Table 5, IPG pH 5-8), but another peptide sequence obtained from this spot was identical with an enzyme involved in isoprenoid biosynthesis (see below). In addition, spot 8 (Table 5, IPG pH 5-8) was identified as an aldehyde dehydrogenase from *Burkholderia xenovorans* LB400. Aldehyde dehydrogenases are enzymes which oxidizes a wide variety of aliphatic and aromatic aldehydes using NAD(P) as a cofactor and possibly also functions in the degradation of estradiol. The aldehyde dehydrogenase family includes succinate-semialdehyde dehydrogenase (EC 1.2.1.16), lactaldehyde dehydrogenase (EC 1.2.1.22), benzaldehyde dehydrogenase (EC 1.2.1.28), methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9), delta-1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12), acetaldehyde dehydrogenase (EC 1.2.1.10), and glutamate-5-semialdehyde dehydrogenase (EC 1.2.1.41).

Spot 7 (Table 5, IPG pH 5-8) was identified as an acetyl-CoA acetyltransferase of *Pseudomonas fluorescens* (synonym acetoacetyl-CoA thiolase EC 2.3.1.9). This enzyme is specific for the thiolysis of acetoacetyl-CoA and is involved in biosynthetic pathways such as poly-beta-hydroxybutyrate synthesis or steroid biogenesis. Two different types of thiolases are found both in eukaryotic and in prokaryotic cells. Acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase (EC 2.3.1.16). 3-ketoacyl-CoA thiolase (also called thiolase I) has a broad chain-length specificity for its substrates and is also involved in degradative pathways such as fatty acid beta-oxidation. The molecular mass of 3-ketoacyl-CoA thiolase of *Pseudoalteromonas haloplanktis* TAC125 agreed well with that of spot 23 (Table 5, IPG pH 5-8). 3-ketoacyl-CoA thiolase is also involved in bile acid biosynthesis and also possibly in the estradiol metabolism of *Denitratisoma oestradiolicum*.

Interestingly, mammalian nonspecific lipid-transfer protein (nsL-TP; also known as sterol carrier protein 2) is a protein which seems to exist in two different forms. A 14 kDa protein (SCP-2) and a larger 58 kDa protein (SCP-x). The former is found in the cytoplasm or the mitochondria and is involved in lipid transport, the latter is found in peroxisomes. The C-terminal part of SCP-x is identical to SCP-2 while the N-terminal portion is evolutionary related to thiolases (Baker et al., 1991).

Spot 3 (Table 5, IPG pH 5-8) showed 100 % sequence identity to the enzyme 2-hydroxychromene-2-carboxylate isomerase (*nahD*) in the genome of *Rubrivivax gelatinosus* PM1. The enzyme belongs to the thioredoxin family containing a diverse set of proteins with a thioredoxin-like structure. 2-hydroxychromene-2-carboxylate isomerase

enzyme catalyzes one step in prokaryotic polyaromatic hydrocarbon degradation pathways (Eaton, 1994). The first three steps of the aerobic naphthalene degradation pathway are similar to those involved in the degradation of monoaromatic compounds such as toluene or benzene (Lengeler et al., 1999). Subsequent reactions differ because of the instability of the 1,2-dihydroxynaphthalene ring cleavage product leads to 2-hydroxychromene-2-carboxylate which is converted to *trans*-*o*-hydroxybenzylidenepyruvate catalyzed by 2-hydroxychromene-2-carboxylate isomerase. This sequence of reactions could also be possible in a hypothetical estradiol pathway if molecular oxygen is present and similar ring cleavage products would be produced by oxygenases. However, no aerobic growth on estradiol by *Denitratisoma oestradiolicum* has been observed.

One peptide sequence of spot 15 (Table 5, IPG pH 5-8) has 100 % identity to Fe-S protein assembly chaperone HscA of *Polaromonas naphthalenivorans* CJ2. These protein is related to Hsp70 chaperones that are known to help to fold many proteins. Hsp70 assisted folding involves repeated cycles of substrate binding and release and have an average molecular weight of 70 kDa. Hsp70 activity is ATP dependent. Hsp70 proteins are made up of two regions. The amino terminus is the ATPase domain and the carboxyl terminus is the substrate binding region. HscA appears to be involved in the assembly of Fe-S proteins (Fe-S cluster formation).

One obtained peptide sequence of spot 19 (Table 5, IPG pH 5-8) revealed a putative reductase or dehydrogenase protein *Pseudoalteromonas haloplanktis* TAC125 that seems to be related to “short chain” alcohol dehydrogenases. Such proteins could be involved in steroid oxidation/reduction and were discussed above for the results of spot 2 (Table 5, IPG pH 5-8), 9 (Table 5, IPG pH 5-8), 21 (Table 5, IPG pH 5-8) and 17 (Table 11A, IPG pH 3-10).

Spot 26 (Table 5, IPG pH 5-8) was identified as 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase (EC 2.7.1.148) of *Shewanella putrefaciens* CN-32. This ATP-dependent enzyme participates in the steroid biosynthesis to isopentenyl phosphate and dimethylallyl diphosphate. These isomers are the universal five-carbon precursors of isoprenoids. In *Archaea* and some bacteria the biosynthesis of these precursors is through the mevalonate pathway. In most bacteria, isopentenyl phosphate and dimethylallyl diphosphate synthesis is accomplished by seven enzymes in a pathway called methylerythritol phosphate pathway. Stage four, the only ATP-dependent step is catalyzed by 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase. Another protein of the nonmevalonate pathway was found in spot 6 (Table 5, IPG pH 5-8), the 1-hydoxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase, a [4Fe-4S] protein (Seemann et al., 2001). This protein was also found in spot 11 (Table 4, IPG pH 3-10). The enzyme catalyzes the ring opening and reduction of 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate to yield 1-hydoxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (Hecht et al., 2001).

One peptide sequence of spot 5 (Table 4, IPG pH 3-10) has 97 % sequence identity to Enoyl-CoA hydratase/isomerase of *Desulfitobacterium hafniense* DCB-2. This enzyme belongs to a protein family which contains a diverse set of enzymes including napthoate synthase, 3-hydoxybutyryl-CoA dehydratase (crotonase), carnitate racemase, and dodecanoyl-CoA delta-isomerase. Napthoate synthase with a pedicted size of 28.5 kDa is involved in the biosynthesis of menaquinone and converts *O*-succinyl-benzoyl-CoA to 1,4-dihydroxy-2-naphthoic acid (Driscoll & Taber, 1992).

Two different peptide sequences were received from spot 14 (Table 4, IPG pH 3-10). One showed moderate sequence identity to 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase of *Brucella melitensis* 16M, an enzyme involved in aerobic toluene or ethylbenzene degradation (Yamada et al., 1998). The other peptide showed 100 % sequence identity to an esterase/lipase-like protein (carboxylesterase) from *Burkholderia* sp. 383 which is involved in alkaloid biosynthesis.

Although aerobic testosterone degradation in *Comamonas testosteroni* is known to be catalyzed by steroid-inducible enzymes, only limited information is available about the regulatory mechanisms governing steroid-inducible gene expression (Marcus & Talalay, 1956, Möbus et al., 1997; Xiong & Maser, 2001; Xiong et al., 2003; Pruneda-Paz et al., 2004; Horinouchi et al., 2001; Thomas et al., 1989). Here, the question still remains whether steroid-dependent gene induction in prokaryotes resembles that found in vertebrates, where steroid hormones act by binding to specific nuclear receptors, which leads to transcriptional regulation of different gene products and the desired physiological response. No relationships with the steroid/steroid receptor system known from vertebrates could be inferred in prokaryotes. An exception exists for the NodD protein receptor, a ligand-dependent transcriptional activator which binds flavonoids of herbal origin for the initiation and maintenance of nitrogen-fixing symbiosis between *Sinorhizobium meliloti* and *Medicago sativa* (Fox, 2004). The regulation in rhizobia-legume symbiosis has functional similarities with that of steroid-mediated actions in vertebrates. No significant nucleotide level homology between these two proteins exists, but both receptors recognize and respond to a shared group of organic compounds (e.g., phytoestrogens). Recent evolutionary phylogenetic data have shown that the estrogen receptor is the ancestral receptor from which all other steroid receptors have evolved (Thornton, 2001).

Another hint for the possibility of transcriptional regulation by a steroid/steroid receptor system in prokaryotes could be the finding of peptide sequences in *Denitratisoma oestradiolicum* which have similarties with vertebrate steroid receptors (Table 12A and 14A, Appendix). Some of these amino acid sequences were found in both gels nearly at the same gel positions, although the isoelectric points are slightly different. Spot 9 and 14 in Fig. 7 (Table 12A, IPG pH 3-10) correspond to spot 13 and spot 26 in Fig. 8 (Table 14A, IPG

pH 5-8), respectively. However, no prokaryotic steroid receptor has yet been described and further studies are required to prove its occurrence in *Denitratisoma oestradiolicum*.

Proteomics technologies help to advance the understanding of physiological and cellular processes in prokaryotes. In the present study, application of two-dimensional gel electrophoresis in combination with tandem mass spectrometry allowed to demonstrate the substrate inducibility and identification of estradiol specific proteins. Proteins were detected with the intent of focusing on the major enzymes involved in the anaerobic estradiol degradation pathway of the newly isolated denitrifying bacterium *Denitratisoma oestradiolicum* AcBE2-1^T. Nevertheless, several results are questionable and further experiments with this proteomic approach are required (e.g., application of narrower pH ranges in the first dimension; two-dimensional gel electrophoresis of the membrane fraction of crude extracts).

In conclusion, information about amino acid sequence data of estradiol-induced proteins obtained in this initial proteomic approach could partly be used to construct degenerate oligonucleotides for cloning and sequencing the respective genes coding for these enzymes. The complete nucleotide sequence information of the putative enzymes will allow to search for related proteins and to make functional and structural comparisons with other metabolic enzymes. These adjacent molecular methods together with the identification of intermediate compounds and enzymatic studies represent a prerequisite for a detailed characterization and elucidation of the anaerobic estradiol degradation pathway by this novel denitrifying bacterium.

4.3 Description of strain FS^T as *Steroidobacter denitrificans* gen. nov., sp. nov.

4.3.1 Physiology

Degradation experiments with the enrichment culture. A complete oxidation of 17.4 µM estradiol to carbon dioxide and water would have required 281 µM nitrate, assuming that approximately 70 % (12.2 µM) of the total estradiol (17.4 µM) has been dissimilated and nitrate was reduced to dinitrogen monoxide. The relation of dissimilated and assimilated substrate was adopted from the results given in Table 7. Dinitrogen monoxide was later detected as the final product of denitrification by strain FS^T (see below). Since only 220 µM nitrate was reduced by this enrichment culture, it could be supposed that further unknown intermediates were not yet consumed after estrone has been completely degraded (Fig. 9).

Quantification of steroid hormone oxidation and denitrification. Data provided by growth experiments and electron balances clearly demonstrated the oxidation of estradiol or testosterone and their utilization as growth substrates, concomitant with the reduction of nitrate to dinitrogen monoxide. Produced cell dry mass increased with the amount of steroid provided (refer to Table 7 and 8). Amounts of consumed nitrate are in good agreement with the amounts of dinitrogen monoxide formed (refer to Table 2A and 3A, Appendix), indicating that nitrate was used for respiration and energy generation, but not for assimilation.

A comparison between growth on estradiol and growth on testosterone revealed that the latter was a slightly better substrate for growth (Fig. 11 and 12). The doubling times were 27 h for estradiol and 25 h for testosterone, respectively. Higher growth yields were obtained with testosterone (Table 8) than with estradiol (Table 7) under carbon limitation and larger amounts of cell dry mass were produced with testosterone as the electron donor. This can be explained by the C-19 methyl group, providing additional reducing equivalents. The assimilation equations shown in Table 7 and 8 already indicate higher yields of biomass, with 4 mol of the empirical formula $<C_4H_9O_2N>$ of cell dry mass more to be gained with testosterone than with estradiol (Chapter 8.2, Appendix).

The assays with estradiol (Table 7) were stopped and analyzed before all the nitrate provided was completely consumed in the assays with excess amounts of substrate (assays in line 3, 4 and 5). Nearly the same amount of estradiol was consumed in the assays in line 3 and 4, but in the latter one, more nitrate was reduced and larger amounts of cell dry mass were produced. In this case, both assays are not comparable to each other in this growth phase. Differences during growth cannot be excluded since the end of the exponential growth phase was not reached. The assay in line 3 might be stopped at a stage where the

bacteria still degrade an intermediate. Electron recoveries of assays in line 3 and 5 indicate an incomplete oxidation of estradiol. This could explain why the bacteria of assay 3 has consumed less nitrate, while having consumed the same amount of estradiol compared to assay 4.

Nevertheless, electron balances of other assays indicate a complete oxidation of estradiol to carbon dioxide and water. Stoichiometric equations for steroid degradation and denitrification were established on the basis of raw data. Using the data of the assay in line 4 (Table 7), the molar ratios between estradiol dissimilated and dinitrogen monoxide formed from nitrate reduction agree well with the following equation:

$$C_{18}H_{24}O_2 + 23\ NO_3^- + 23\ H^+ \rightarrow 18\ CO_2 + 11.5\ N_2O + 23.5\ H_2O$$

Such an equation for the oxidation of testosterone could only be set up with data from carbon-limited cultures. Hence, the principle degradation equation of testosterone is as follows (data were taken from assay in line 2, Table 8):

$$C_{19}H_{28}O_2 + 25\ NO_3^- + 25\ H^+ \rightarrow 19\ CO_2 + 12.5\ N_2O + 26.5\ H_2O$$

Assays with excess testosterone suggest an incomplete oxidation. This is reflected in the decreasing electron recoveries and growth yields. Their sharp drop of about 50 % in transition between the assays in line 4 and 5 (Table 8) indicates assay in line 3 to represent the final amount of steroid, in relation to the supplied amount of nitrate, that allows for a complete oxidation of testosterone. This notion is supported by the accumulation of 4-androstene-3,17-dione (< 0.1 mg l^{-1}) in the last assay of Table 8, while none of the assays with estradiol displayed any intermediates detectable by the applied HPLC method.

In contrast, estradiol seems to be used more effectively over a broad range of substrate amounts, as electron recoveries and growth yields remain at a high level, even at excess amounts of substrate. This is even the case when amounts of substrate exceed the border of substrate limitation, located around 50 µmol in assay in line 4 (Table 7). The reason for this might be based on two features. First, as already mentioned above, testosterone supplies more carbon and energy per mol of substrate, making an incomplete degradation possible. The second point could be that, in order to gain energy from estradiol, the bacteria have to go through a certain preliminary phase to activate the molecule in a first step, before the degradation of the steroid nucleus in a second phase. This hypothesis was confirmed by a lower growth rate on estradiol. This growth limiting step could be associated to the presence of the aromatic A-ring.

Incomplete oxidation of the substrate, as observed with testosterone, was also found with strain 72Chol, during degradation of excess amounts of cholesterol (Harder & Probian, 1997). The authors proposed the formation of an intercellular storage compound.
The most interesting result certainly lies in the capacity of strain FS^T to degrade both testosterone or estradiol as substrate, which up to now, has not been described in any of the other steroid degrading-bacteria, neither under oxic nor under anoxic conditions. Growth on testosterone and 4-androstene-3,17-dione was also tested with *Denitratisoma oestradiolicum*, but finally cell lysis repeatedly occurred after a second transfer into fresh medium. These findings might indicate that growth was affected by the toxicity of these C-19 steroids by interfering with the cell membrane causing cell lysis.

Aspects of denitrification properties. Most known facultatively anaerobic, heterotrophic bacteria produce dinitrogen or nitrite as the major end products of nitrate respiration. Already, there are some bacteria that lack the last step of denitrification. Strain FS^T produced dinitrogen monoxide as the sole end product of denitrification. At the end of growth, the total amount of nitrate was converted nearly stoichiometrically to dinitrogen monoxide (Table 2A and 3A). The previously characterized denitrifiers that are known to produce dinitrogen monoxide were isolated from activated sludge, ammonia-supplied biofilters, soil, natural freshwater, solar salterns or hot springs. Examples include representatives from the *Betaproteobacteria* [*Chromobacterium violaceum* (Bazylinski et al., 1986) and *Ottowia thiooydans* (Spring et al., 2004)], *Firmicutes* (*Bacillus halodenitrificans*; Denariaz et al., 1989) and *Actinobacteria* (*Streptomyces nitrosporeus*; Wenzhofer et al., 1997). Especially within the *Gammaproteobacteria*, denitrification with dinitrogen monoxide formation was observed in *Pseudomonas chlororaphis* (Christensen & Tiedje, 1988), *Luteimonas mephitis* (Thierry et al., 2004), *Stenotrophomonas nitritireducens* (Finkmann et al., 2000), and several species of the genus *Pseudoxanthomonas* (Finkmann et al., 2000; Chen et al., 2002; Thierry et al., 2004). Note, that the mentioned species of the latter three genera possess the ability to reduce nitrite but not nitrate with the production of dinitrogen monoxide. The reduction of nitrite to dinitrogen monoxide by *Pseudoxanthomonas* species is not a detoxification process or serves as an electron sink. It is thought to be coupled to energy production (Finkmann et al., 2000; Thierry et al., 2004).
It is known that dinitrogen monoxide is emitted by bacteria in various environments. Unlike the other nitrogen oxides, dinitrogen monoxide is a greenhouse gas. Per unit of weight, dinitrogen monoxide as the third most important gas after methane and carbon dioxide has 296 times the effect of the latter gas for producing global warming. The formation of dinitrogen monoxide as an end product of denitrification also distinguishes strain FS^T from the other known denitrifying, steroid-degrading bacteria that have been isolated so far. These

bacteria, *Sterolibacterium denitrificans* and 72Chol, produce dinitrogen as the final product of denitrification. *Denitratisoma oestradiolicum* displays an intermediary accumulation of nitrite, and forms a mixture of dinitrogen monoxide and dinitrogen.

4.3.2 Taxonomy

The phylogenetic analysis showed that strain FS^T has no known close relatives and represents a separate line of descent within the *Gammaproteobacteria* (Fig. 13). Beside a distant relationship (16S rRNA gene sequence similarity of about 87.1 %) to *Nevskia ramosa* (Glöckner et al., 1998; Stürmeyer et al., 1998), *Hydrocarboniphaga effusa* (Palleroni et al., 2004), *Panacagrimonas perspica*, *Nitrosococcus oceani* C-107 (Watson, 1965), and *Ectothiorhodospira shaposhnikovii* Z1, none of these bacteria are known to perform an anaerobic metabolism with such complex organic compounds like C-18 or C-19 steroids, while using nitrate as the electron acceptor which is then reduced to dinitrogen monoxide as the final end product of denitrification (Table 6).

Anoxygenic photosynthetic purple sulfur bacteria of the genus *Ectothiorhodospira* accumulate elemental sulfur outside their cells (Madigan et al., 2003). These prokaryotes are mainly found in illuminated anoxic zones of lakes or sulfur springs where hydrogen sulfide accumulates and can trigger massive blooms of purple sulfur bacteria. Hydrogen sulfide is used as electron donor for assimilation of carbon dioxide. All cultured strains of *Nitrosococcus oceani* originate from a variety of marine environments and, together with *N. halophilus*, comprises the only two species of ammonia-oxidizing *Gammaproteobacteria* (Ward & O'Mullan, 2002). The 16S rRNA gene sequences of *Nevskia ramosa* and *Hydrocarboniphaga effusa* cluster together and represent a deeply branching lineage in the *Xanthomonas* group. *Nevskia ramosa*, discovered 1892 by Famintzin in the surface of a freshwater aquarium, has a strictly aerobic metabolism and grows with a broad range of organic compounds including monomeric sugars, sucrose, starch, cellulose, ethanol, organic acids, amino acids, benzoate, and Tween 20 or Tween 80 (Stürmeyer et al., 1998). This bacterium is a conspicuous inhabitant of the air-water interface of calm freshwater bodies. The interface harbors a microbial community referred to as the neuston (Naumann, 1917). 16S rRNA gene sequences of *Nevskia ramosa* strains showed a moderate but distant relationship to the strictly aerobic *Hydrocarboniphaga effusa*, an alkane-degrading bacterium isolated from oil-contaminated soil (Palleroni et al., 2004). This bacterium shows an unusual appearance of colonies on various solid media and grows on aliphatic hydrocarbons (C_6-C_{19}), amino acids, sugars, propionate, ethanol, fumarate, DL-lactate, LB medium, nutrient agar, and TSA agar. Growth on mid-chain fatty acids is slow and very poor. *Hydrocarboniphaga*

effusa can be distinguished from *Nevskia ramosa* due to the property of the latter to form rosette-like colonies at air-water interfaces.

Strain FS^T is closer related to several environmental clone sequences, consisting of so far uncultivated bacteria, many of which were obtained from soil or microbial rhizosphere communities and in some cases from uranium mines and deposits (Fig. 13). Though culture-independend techniques have improved, they still provide only limited insights into the physiology of prokaryotes. Therefore, the role of not-yet-cultured prokaryotes in the environment cannot be fully appreciated until these bacteria are available for a detailed physiological characterization and taxonomy.

On the other hand, the existence and physiological role of strain FS^T in anoxic digested sludge is far from clear and the most related 16S rRNA gene sequences belong to so far uncultivated bacteria which were almost obtained from soil samples of different origin. Some knowledge exists about the extent and ecological characteristics of the denitrification capacity in anoxic sludge digestors (Kaspar & Tiedje, 1981; Etchebehere et al., 2001). Investigations about the diversity of denitrifying bacteria in anoxic sludge are lacking but a high denitrification capacity in anoxic, nitrate-free habitats was observed and appears due to conventional denitrifiers (Tiedje et al., 1982). Many denitrifiers and, as well strain FS^T, could be discharged into nitrate-free anoxic sludge digestors via excess sewage sludge from biological treatment.

In fact, on the basis of the physiological and morphological properties given in Table 6 and in the species description, it is obvious that strain FS^T differs substantially from the above mentioned bacteria. From the results presented, it can be concluded that strain FS^T represents a novel taxon within the *Gammaproteobacteria*, for which the name *Steroidobacter denitrificans* gen. nov., sp. nov. is proposed. This conclusion is based on 16S rRNA gene sequence analysis in combination with chemotaxonomic and physiological data (Fahrbach et al., in preparation).

Description of *Steroidobacter* gen. nov.

Steroidobacter (Ste.ro.i.do.bac'ter. Gr. n. *Steroides* the steroid; N.L. n. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod or staff; N.L. masc. n. *Steroidobacter* a bacterium that degrades steroids).

Gram-negative rod-shaped cells, slightly curved, 0.3-0.5 µm wide and 0.6-1.6 µm long, with rounded ends, occuring singly or in pairs, non-spore-forming, motile. Selective enrichment and isolation with estradiol or testosterone. Colonies in agar dilution series with testosterone and nitrate are small (diameter approx. 1 mm) yellow-brown, disc-shaped. Chemoorganotroph, non-fermentative, metabolism respiratory, reduction of nitrate to nitrous oxide as the sole end product of denitrification without any intermediate accumulation of nitrite. Ability to utilize both C-18 or C-19 steroids as sole source of carbon and energy under denitrifying conditions, but only C-19 steroids under air. Oxidation of only a narrow number of organic substrates. Electron acceptors nitrate, nitrite or oxygen are used; sulfate, sulfite, (per)chlorate, or fumarate are not reduced. Optimal growth conditions are at 28-30 °C and at pH 7.2-7.4 under denitrifying conditions. The quinone system consists of Q-8. The major fatty acids are $C_{17:1}\omega8c$, $C_{15:0}$, $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH, $C_{16:0}$, $C_{16:0}$ 10-methyl and $C_{17:0}$. Minor components are $C_{11:0}$, $C_{13:0}$, $C_{15:1}\omega8c$, $C_{18:1}\omega7c$ and $C_{17:0}$ 10-methyl. The DNA G+C content of the type species is 61.9 mol%. The type species is *Steroidobacter denitrificans*.

Description of *Steroidobacter denitrificans* sp. nov.

Steroidobacter denitrificans (de.ni.tri'fi.cans. L. prep. *de* away from; L. n. *nitrum* soda; N.L. n. *nitras* nitrate; N.L. v. *denitrifico* to denitrify; N.L. part. adj. *denitrificans* denitrifying).

Exhibits the following properties in addition to those given in the genus description. Only a few electron donors can be used with nitrate as the electron acceptor: estradiol (1 mM), estrone (1 mM), testosterone (1 mM), 4-androstene-3,17-dione (1 mM), acetate (2.5 mM), propionate (2.5 mM), isobutyrate (2.5 mM), valerate (2.5 mM), caproate (2.5 mM), heptanoate (2.5 mM), or glutamate (2.5 mM). Does not utilize the following electron donors with nitrate as the electron acceptor: 19-nor-testosterone, ethinyl estradiol, acetone, ascorbate, primary aliphatic alcohols (C_1-C_4), cyclohexanol, formate, butyrate, palmitate, stearate, crotonate, adipate, DL-lactate, succinate, pyruvate, fumarate, citrate, glycerol, pentane, benzoate, D(+)-glucose, D(+)-galactose, sucrose, yeast extract, ammonium, or thiosulfate. No aerobic growth on R2A or CASO agar as well as on CASO-Bouillon or Nutrient Broth. No aerobic growth with testosterone, 4-androstene-3,17-dione, estradiol or estrone. The fatty acid profile comprises $C_{9:0}$ (0.6 %), $C_{10:0}$ (1.3 %); $C_{11:0}$ (2.7 %), $C_{12:0}$ (1.8 %),

$C_{13:0}$ (4.2 %), $C_{14:0}$ (0.9 %), $C_{15:0}$ (16.5 %), $C_{16:0}$ (7.0 %), $C_{17:0}$ (6.2 %), $C_{15:1}\omega 8c$ (2.5 %), $C_{16:1}\omega 9c$ (0.9 %),$C_{17:1}\omega 8c$ (27.1 %), $C_{17:1}\omega 6c$ (3.0 %), $C_{18:1}\omega 7c$ (4.2 %), $C_{11:0}$ 3-OH (1.2 %), $C_{12:0}$ 3-OH (1.3 %), summed feature 3 ($C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH; 8.3 %), iso-$C_{16:1}$ H (0.8 %), $C_{16:0}$ 10-methyl (7.0 %) and and $C_{17:0}$ 10-methyl (2.4 %). Ubiquinone Q-8 is the only quinone. The DNA G+C content for the type strain is 61.9 mol%. The type strain, FS^T (the bacterium has not been deposited at DSMZ and JCM yet), was isolated from an enrichment culture inoculated with anoxic digested sludge from a municipal wastewater treatment plant in Aachen-Soers (Germany).

4.4 General discussion

Fate of steroid hormones in the environment. Steroidal hormones in the environment might pose serious risks to human and wildlife health (Sumpter & Johnson, 2005). Analysis of steroid estrogens in both water and sewage sludge showed that estradiol and estrone were mainly degraded in the denitrification step during wastewater treatment (Andersen et al., 2003). The treatment plant investigated by Andersen et al. (2003) consisted of an activated sludge system with two denitrification tanks and a nitrification step between the first and second clarifier. In the second denitrification tank and the following aerated reactor, the natural estrogens were degraded so that a total of more than 98 % was eliminated, mainly in the denitrifying step. Layton et al. (2000) investigated the degradability of estradiol, estrone, ethinyl estradiol, and testosterone in wastewater treatment systems under oxic conditions, performing laboratory mineralization assays with radio-labeled substrates. With the exception of ethinyl estradiol (removal 20 %), all other steroid hormones were almost completely oxidized to carbon dioxide and water within 24 h (removal >90 %).

Although removal efficiencies of municipal sewage treatment plants reach >90 % with regard to elimination of such compounds (Layton et al., 2000; Andersen et al., 2003; Joss et al., 2004), observed estrogenic effects in fish downstream of sewage treatment plants can be mainly attributed to the remaining steroid hormones in the discharged effluents due to incomplete removal (Pickering & Sumpter, 2003; Vethaak et al., 2005). Management strategies aiming at improving the elimination efficiencies of the sludge treatment process will have to be based on in depth understanding of the biological processes governing the degradation of steroid hormones.

The isolation of two novel *Proteobacteria* from sewage sludge that mineralize both estradiol or testosterone with nitrate as the electron acceptor indicate, that denitrifying bacteria play an important role in steroid hormone degradation. Approximately 6 x 10^3 estradiol-oxidizing, nitrate-reducing bacteria g^{-1} sludge dry weight were present in cycloheximide-treated activated sludge of the municipal wastewater treatment plant in Aachen-Soers. This simple estimation was conducted with the MPN method. Another MPN dilution series was inoculated with untreated activated sludge but no growth could be detected. This striking difference between cycloheximide-treated and untreated sludge might be explained by the low numbers of estradiol-degrading prokaryotes present and a high rate of protozoan grazing during incubation. Furthermore, the enrichment culture of *Denitratisoma oestradiolicum* could only be obtained by using cycloheximide-treated activated sludge. The applied MPN method is not sufficiently accurate to detect and to quantify particular bacteria within a such complex microbial community. In combination with the traditional MPN method, molecular techniques (e.g., quantitative fluorescence *in situ* hybridization, FISH) should be applied to estimate the

occurrence and abundance of described steroid hormone-degrading bacteria in sewage sludge.

It might be questionable that the denitrifying bacteria isolated in this work significantly contribute to the elimination of steroid hormones in the environment (especially if molecular oxygen is limited), although the narrow substrate utilization patterns of the two isolates investigated in this study may point towards a substrate specialization. Nevertheless, it is questionable whether these bacteria are responsible for the main estrogen elimination since steroid hormone concentrations are normally in the nanogram per liter range and whether these isolates are the numerically dominant and/or functionally significant species during wastewater treatment.

However, the phylogenetic relationship of *Denitratisoma oestradiolicum* to so far uncultivated but abundant representatives of the betaproteobacterial population in wastewater treatment plants (Fig. 1A, Appendix) suggests an important function. The physiological role of *Steroidobacter denitrificans* in anoxic sewage treatment is unclear. This subject has been already discussed in Chapter 4.3.

Ecophysiological studies concerning the functional significance of *Denitratisoma oestradiolicum* and *Steroidobacter denitrificans* in the environment and the elucidation of the anaerobic degradation pathways of estradiol and testosterone may contribute to a better understanding of the fate of steroid hormones during wastewater treatment and also in natural habitats. Fluorescence *in situ* hybridization (FISH) with rRNA-targeted nucleic acid probes could be used for direct identification and quantification of these steroid hormone-degrading isolates in complex samples like sewage sludge, sediment or soil (Wagner et al., 2003).

While the enrichment and isolation of anaerobic bacteria that are degrading natural steroid hormones was successful in this study, attempts to enrich bacteria using ethinyl estradiol as electron donor still remained in vain. This, even though various inocula were used in this study with either nitrate or sulfate as electron acceptors (Table 10A, Appendix). No reports about anaerobic bacteria degrading ethinyl estradiol have emerged so far. As reported in several studies (Andersen et al., 2003; Joss et al., 2004; Ternes et al. 1999a; Ternes et al. 1999b; Layton et al., 2000), ethinyl estradiol represents the most recalcitrant unconjugated steroid hormone. Still, ethinyl estradiol degradation was reported by Andersen et al. (2003) and Layton et al. (2000), which must be due mainly to aerobic degradation. The activated sludge system of the wastewater treatment plant investigated by Andersen et al. (2003) had a sludge retention time of 11-13 d, which might allow of degrading ethinyl estradiol.

Aerobic enrichment cultures were also prepared in this work with ethinyl estradiol (Table 10A, Appendix), but no growth could be detected. Aerobic degradation of ethinyl estradiol was so far only observed by members of the genus *Rhodococcus* (Yoshimoto et al., 2004).

Co-metabolic transformations, in which nitrifying bacteria hydroxylate natural and synthetic steroid hormones have been described (Vader et al., 2000; Shi et al., 2004). A significant contribution to an elimination of steroid hormones through such unspecific enzyme reactions remains questionable, if one imagine how complex such an environment as activated sludge is and how many other transformation reactions of organic compounds might occur during wastewater treatment.

Steroids as growth substrates and the isolation of so far uncultivated bacteria. In recent years, several studies applying cultivation-independent techniques have demonstrated that bacteria isolated using standard cultivation methods (e.g., agar plates with nutrient-rich media, oxic conditions and moderate temperatures) are rarely numerically dominant in microbial habitats (e.g., activated sludge) from which they were obtained. These prokaryotes are estimated to constitute less than 1 % of all microbial species and is called the “great plate-count anomaly” (Staley & Konopka, 1985). Isolation of pure cultures marked the beginning of scientific microbiology, and studies with pure cultures provided the basis of our knowledge about *Bacteria* and *Archaea*. Yet, after more than 100 years, probably only a few percent of the extant bacterial species have been isolated and studied.

The isolation and characterization of all members of the microbial world is an impossible task. A much more realistic goal is to classify those prokaryotes that are abundant in their ecosystem, biotechnological promising, or of medical or agricultural significance. Although much can now be done with cultivation-independent methods of molecular biology there is a continued need for the skilled application of old methods and the development of new cultivation methods for prokaryotes. Even the most powerful technologies cannot substitute for research on pure cultures. Clearly, future research on microbial diversity will largely benefit from both approaches.

The selective enrichment and isolation of so far uncultivated bacteria like *Denitratisoma oestradiolicum* AcBE2-1^T and *Steroidobacter denitrificans* FS^T seems to be favoured due to the chemical properties of steroid hormones (e.g., low solubility in water, complex structure with a low number of functional groups). In the natural environment, nonpolar organic carbon substrates occur in the adsorbed state. In the adsorbed state, these substrates are often not directly available and rates of desorption control the rate of degradation. Consequently, enrichment strategies, in which especially nonpolar substrates are offered in the adsorbed state or compounds that are anyway weakly water soluble (e.g., polycyclic aromatic hydrocarbons or steroid hormones) may provide a more relevant low-bioavailability environment, and hence lead to the isolation of novel types of prokaryotes (Tang et al., 1998; Grosser et al., 2000). As mentioned above, steroid hormones occur already in relatively low concentrations (nanogram per liter range) in the environment. However, microbial

degradation seems only to be possible where the bacteria, which are able to degrade such complex trace compounds, have further substrates (e.g., fatty acids) as carbon sources available.

Future outlook. This work has shown that newly isolated denitrifying bacteria have the capacity to degrade natural steroid hormones in the absence of molecular oxygen. Currently nothing is known about the anaerobic degradation pathways of these compounds. This is a critical issue, since anoxic conditions can be found in river sediments or occur during the denitrification step in wastewater treatment.

Both bacteria are proposed to be used as model organisms to investigate the anaerobic degradation pathways of estradiol and testosterone in future studies. Since previously known aerobic pathways contain oxygen-dependent steps, these two bacteria must perform novel, yet unknown biotransformations and contain alternative oxygen-independent enzymes to circumvent these steps. In particular, the degradation steps, mostly concerned with reactions of quaternary C-atoms, will likely be the reactions with the highest degree of novelty. Further investigations promise to yield new insights into the biochemistry of steroid hormones, as well as potential new tools for biotechnological applications.

In order to investigate the metabolic pathways, experiments with suspensions of steroid-induced whole cells for the identification of intermediate compounds could be performed. Radio-labeled substrates could be used for detection of metabolites in trace amounts and tandem mass spectrometry for their identification. The obtained knowledge about metabolic products formed by whole cells could be used for enzymatic studies of the respective enzymes involved. On the basis of amino acid sequence information of steroid-induced proteins obtained from the proteomic approach conducted with *Denitratisoma oestradiolicum*, genetic tools could be employed for identifying, cloning, and sequencing the genes encoding the key enzymes. *Steroidobacter denitrificans* will also be used to compare the protein patterns after anaerobic growth on estradiol and testosterone. Additionally, insights into the degradation pathways of natural steroid hormones are might be also important for the assessment of the degradability of synthetic steroid hormones (e.g., ethinyl estradiol **6**, Fig. 1) and of their high persistence under various redox conditions.

5 Summary

During the last decade, the endocrine disrupting activity of steroid hormones both of natural and of anthropogenic origin was recognized as a potential risk to the fertility of wildlife populations in various ecosystems and the human population. Concerns about potential negative ecological effects of steroid hormones have resulted in an increased interest with regard to the elimination of these compounds in soil, sediments, and during wastewater treatment. Analysis of steroid hormones showed that denitrification significantly contributes to the overall elimination of estradiol in the sewage sludge process. However, nothing is known about the bacteria responsible for this estradiol degradation. Also no information exists about the degradation of testosterone under anoxic conditions.

This work gives first data about the anaerobic degradation of both estradiol or testosterone by novel denitrifying bacteria. These bacteria (strain AcBE2-1^T and strain FS^T) were isolated in this work from activated sludge and anoxic digested sludge of a municipal wastewater treatment plant with estradiol or testosterone as the sole source of carbon and energy and nitrate as the electron acceptor.

Strain AcBE2-1^T was isolated from activated sludge with estradiol and nitrate. This bacterium oxidized estradiol beyond estrone 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 into account assimilated estradiol. With nitrate as the electron acceptor, the bacterium also grew on estrone, fatty acids (C_2 to C_6), isobutyrate, crotonate, DL-lactate, or several citric acid cycle intermediates. 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*). Together with several clone sequences obtained from hitherto uncultivated bacteria, strain AcBE2-1^T forms a novel cluster distinct from known lineages. 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 is clearly different from *Sterolibacterium denitrificans*, 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.

Strain FS^T was isolated from anoxic digested sludge on estradiol and testosterone with nitrate as the electron acceptor. Strain FS^T represents the first bacterium that anaerobically mineralizes both estradiol (C-18) or testosterone (C-19). The degradation balance measured in testosterone-limited cultures indicated a complete oxidation to carbon dioxide and water. Estradiol oxidation was almost complete even in nitrate-limited cultures. Estrone and 4-androstene-3,17-dione were identified as intermediates of estradiol and testosterone degradation, respectively. Dinitrogen monoxide was the sole end product of denitrification, without intermediate nitrite formation. Beside estradiol and testosterone, only a narrow spectrum of organic substrates were used as electron donors under denitrifying conditions including estrone, 4-androstene-3,17-dione, acetate, propionate, isobutyrate, valerate, caproate, heptanoate, or glutamate. Comparative 16S rRNA gene sequence analysis revealed that strain FS^T has no known close relatives and represents a separate line of descent within the *Gammaproteobacteria* positioned among clone sequences of so far uncultivated bacteria. A distant relationship of about 87.1 % 16S rRNA gene sequence similarity exists to the genera *Nevskia*, *Hydrocarboniphaga*, *Nitrosococcus*, and *Ectothiorhodospira*, all of which exhibit different physiological properties in comparison with strain FS^T. The G+C content of the DNA was 61.9 mol%. Based on the high 16S rRNA gene sequence divergence and different physiological properties to previously described bacteria in combination with chemotaxonomic data, strain FS^T was indicated as the type strain of a type species placed in a new genus *Steroidobacter* gen. nov., for which the name *Steroidobacter denitrificans* gen. nov., sp. nov. is proposed.

A proteomic approach with *Denitratisoma oestradiolicum* allowed to demonstrate the induction of estradiol-specific proteins and resulted in the identification of several enzymes for example S-adenosylmethionine synthase, S-adenosylmethionine-dependent methyltransferases, 3-octaprenyl-4-hydroxybenzoate decarboxylase, and several hydroxysteroid dehydrogenases that might be involved in anaerobic estradiol degradation. These initial results represent a prerequisite for a detailed characterization and elucidation of the estradiol degradation pathway in this novel denitrifying bacterium. The obtained amino acid sequence data could be used in future studies to construct degenerated oligonucleotides for cloning and sequencing the respective genes coding for these proteins. The complete nucleotide sequence information will then allow to search for related proteins and to make functional and structural comparisons.

6 Zusammenfassung

Während des letzten Jahrzehnts wurde die Wirkung von Steroidhormonen aus natürlichen und anthropogenen Quellen auf Mensch und Tier als ein Risiko für deren Fortpflanzungsfähigkeit erkannt. Diese negativen ökologischen Effekte haben zu einem vermehrten Interesse an der Abbaubarkeit dieser Verbindungen im Boden, in Sedimenten und während des Abwasserreinigungsprozesses geführt. Analysen von Steroidhormonen haben gezeigt, dass die Denitrifikation signifikant zur Gesamtelimination von Estradiol im Abwasserreinigungsprozess beitragen kann. Jedoch ist nichts über die Bakterien bekannt, welche für diesen Estradiolabbau verantwortlich sind. Auch über den Abbau von Testosteron unter anoxischen Bedingungen liegen bisher keine Informationen vor.

Diese Arbeit liefert erste Daten über den anaeroben Abbau von Estradiol und Testosteron durch neuartige denitrifizierende Bakterien. Es wurden zwei neuartige denitrifizierende Bakterien (Stamm AcBE2-1^T und Stamm FS^T) mit Estradiol oder Testosteron als einziger Energie- und Kohlenstoffquelle aus Belebt- und Faulschlamm einer kommunalen Kläranlage isoliert.

Stamm AcBE2-1^T wurde mit Estradiol und Nitrat aus Belebtschlamm isoliert. Dieses Bakterium oxidierte Estradiol über Estron vollständig zu Kohlendioxid und Wasser und reduzierte Nitrat zu einer Mischung aus Distickstoffmonoxid und molekularem Stickstoff. Während des Wachstums wurde Nitrit akkumuliert. Unter Berücksichtigung von assimiliertem Estradiol lag die Elektronenbilanz zwischen 90 und 100 %. Das Bakterium konnte mit Nitrat als Elektronenakzeptor auch Estron, Fettsäuren (C_2 bis C_6), Isobutyrat, Crotonat, DL-Lactat oder mehrere Intermediate des Tricarbonsäurezyklus als Energiequelle nutzen. Eine phylogenetische Analyse der Gensequenz der 16S rRNA ergab, dass Stamm AcBE2-1^T eine eigene Abstammungslinie in der Familie *Rhodocyclaceae* innerhalb der *Betaproteobacteria* darstellt. Stamm AcBE2-1^T bildet zusammen mit mehreren Klonsequenzen von bisher nicht kultivierten Bakterien ein neues Cluster neben den bekannten Abstammungslinien. Die nächsten Verwandten mit einer Sequenzähnlichkeit von <93,9 % sind die Cholesterol-abbauenden Denitrifizierer *Sterolibacterium denitrificans* DSM 13999^T und Stamm 72Chol (= DSM 12783). Der G+C Gehalt der DNA betrug 61,4 mol%. Der Nachweis von Ubichinon Q-8 und ein Fettsäuremuster, welches sich deutlich von dem von *Sterolibacterium denitrificans* unterschied, untermauern die Ergebnisse der phylogenetischen Analyse. Aufgrund der Sequenzdaten des 16S rRNA Gens, gemeinsam mit chemotaxonomischen und physiologischen Daten, lässt sich Stamm AcBE2-1^T (= DSM 16959^T = JCM 12830^T) in einer neuen Gattung *Denitratisoma* gen. nov. als der Typstamm der Typspezies *Denitratisoma oestradiolicum* gen. nov., sp. nov. einordnen.

Der denitrifizierende Stamm FS^T wurde mit Estradiol und Testosteron aus Faulschlamm isoliert. Stamm FS^T ist das erste Bakterium, welches sowohl Estradiol (C-18) als auch Testosteron (C-19) anaerob mineralisieren kann. Die Abbaubilanz zeigte in Testosteron-limitierten Kulturen eine vollständige Oxidation zu Kohlendioxid und Wasser. Eine nahezu vollständige Oxidation von Estradiol wurde auch in Nitrat-limitierten Kulturen festgestellt. Estron und 4-Androsten-3,17-dion wurden als Zwischenprodukte des Estradiol- bzw. Testosteronabbaus identifiziert. Distickstoffmonoxid ist das einzige Endprodukt der Denitrifikation. Es wurde keine Nitritakkumulation während des Wachstums festgestellt. Neben Estradiol und Testosteron wurde nur ein enges Spektrum organischer Substrate als Elektronendonoren unter denitrifizierenden Bedingungen genutzt. Das Bakterium konnte mit Nitrat als Elektronenakzeptor auch Estron, 4-Androsten-3,17-dion, Acetat, Propionat, Isobutyrat, Valerat, Caproat, Heptanoat oder Glutamat als Substrate nutzen. Vergleiche der Gensequenz der 16S rRNA ergaben, dass Stamm FS^T keine unmittelbaren Verwandten besitzt und somit eine eigene Abstammungslinie zwischen Klonsequenzen von bisher nicht kultivierten Bakterien innerhalb der *Gammaproteobacteria* darstellt. Eine entfernte Verwandtschaft von Stamm FS^T besteht zu den Gattungen *Nevskia*, *Hydrocarboniphaga*, *Nitrosococcus*, und *Ectothiorhodospira* (etwa 87,1 % Sequenzähnlichkeit des 16S rRNA Gens). Der G+C Gehalt der DNA betrug 61,9 mol%. Aufgrund des hohen Sequenzunterschiedes des 16S rRNA Gens und der verschiedenartigen physiologischen Eigenschaften zu bisher beschriebenen Arten, wurde gemeinsam mit chemotaxonomischen Daten gezeigt, dass Stamm FS^T als Typstamm einer Typspezies in einer neuen Gattung *Steroidobacter* gen. nov. eingeordnet werden kann, für welche der Name *Steroidobacter denitrificans* gen. nov., sp. nov. vorgeschlagen wurde.

Eine Proteomanalyse von *Denitratisoma oestradiolicum* zeigte die Induzierbarkeit Estradiol-spezifischer Proteine und führte zur Identifizierung mehrerer Enzyme wie zum Beispiel der S-Adenosylmethionin Synthase, S-Adenosylmethionin-abhängiger Methyltransferasen, der 3-Polyprenyl-4-hydroxybenzoat Carboxylase und mehrerer Hydroxysteroid-Dehydrogenasen, welche am anaeroben Estradiolabbau beteiligt sein könnten. Diese ersten Ergebnisse stellen die Vorraussetzung für eine detaillierte Charakterisierung und Aufklärung des Abbauweges von Estradiol durch dieses neuartige denitrifizierende Bakterium dar. Die erhaltenen Aminosäuresequenzen könnten in zukünftigen Untersuchungen verwendet werden, um degenerierte Oligonucleotide zur Klonierung und Sequenzierung der jeweiligen codierenden Gene herzustellen. Die kompletten Gensequenzen erlauben dann funktionelle und strukturelle Vergleiche zu verwandten Proteinen.

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8 Appendix

8.1 Phylogenetic analysis of *Denitratisoma oestradiolicum*

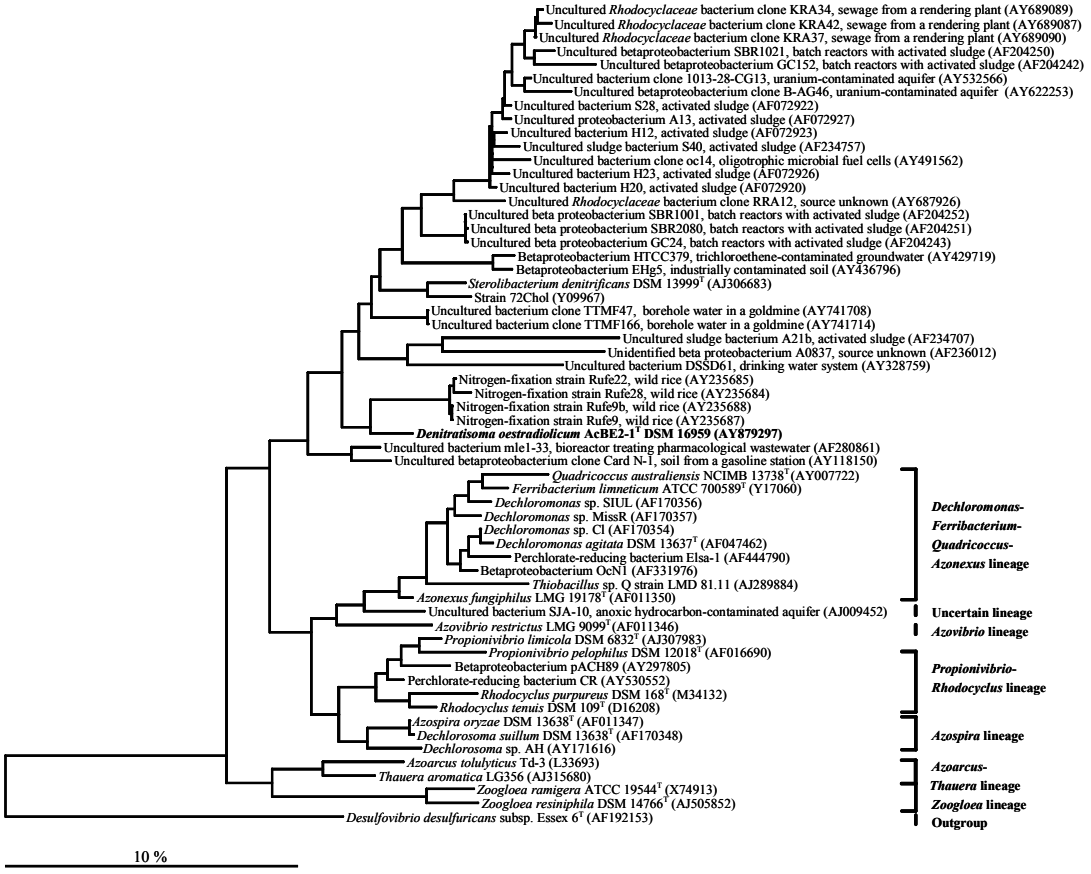

Fig. 1A. Phylogenetic tree showing the affiliation of the 16S rRNA gene sequence from *Denitratisoma oestradiolicum* AcBE2-1^T to selected reference sequences within the *Betaproteobacteria*. The tree was calculated by maximum-likelihood analysis and corrected with a 50 % filter and a termini filter. The tree topology was nearly identical by using the neighbour-joining and maximum-parsimony analysis (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.

8.2 Assimilation equations of estradiol and testosterone

Assimilation of estradiol. The assimilated amount of estradiol was calculated by applying the following equation:

$C_{18}H_{24}O_2 \rightarrow 18\ CO_2 + 92\ [H]$ x18

$4\ CO_2 + 18\ [H] \rightarrow <C_4H_9O_2N>$ x92

$18\ C_{18}H_{24}O_2 \rightarrow 324\ CO_2 + 1656\ [H]$

$368\ CO_2 + 1656\ [H] \rightarrow 92\ <C_4H_9O_2N>$

$18\ C_{18}H_{24}O_2 + 44\ CO_2 \rightarrow 92\ <C_4H_9O_2N>$:2

$9\ C_{18}H_{24}O_2 + 22\ CO_2 \rightarrow 46\ <C_4H_9O_2N>$

The equation was expanded to account for assimilation of ammonium:

$9\ C_{18}H_{24}O_2 + 46\ NH_4^+ + 22\ CO_2 + 30\ H_2O \rightarrow 46\ <C_4H_9O_2N> + 46\ H^+$

The molecular weight of cell mass with the empirical formula $<C_4H_9O_2N>$ is 103 g mol^{-1}. Therefore, 9 mol estradiol per 46 mol cell mass correspond to 1.9 mmol estradiol per g cell mass.

Assimilation of testosterone. The assimilated amount of testosterone was calculated by applying the following equation:

$C_{19}H_{28}O_2 \rightarrow 19\ CO_2 + 100\ [H]$ x18

$4\ CO_2 + 18\ [H] \rightarrow <C_4H_9O_2N>$ x100

$18\ C_{19}H_{28}O_2 \rightarrow 342\ CO_2 + 1800\ [H]$

$400\ CO_2 + 1800\ [H] \rightarrow 100\ <C_4H_9O_2N>$

$18\ C_{19}H_{28}O_2 + 58\ CO_2 \rightarrow 100\ <C_4H_9O_2N>$:2

$9\ C_{19}H_{28}O_2 + 29\ CO_2 \rightarrow 50\ <C_4H_9O_2N>$

The equation was expanded to account for assimilation of ammonium:

$9\ C_{19}H_{28}O_2 + 50\ NH_4^+ + 29\ CO_2 + 24\ H_2O \rightarrow 50\ <C_4H_9O_2N> + 50\ H^+$

The molecular weight of cell mass with the empirical formula $<C_4H_9O_2N>$ is 103 g mol^{-1}. Therefore, 9 mol testosterone per 50 mol cell mass correspond to 1.7 mmol testosterone per g cell mass.

8.3 Quantification of steroid hormone degradation and denitrification

Table 1A. N-ratios of reduced nitrate, formed nitrite, dinitrogen monoxide and dinitrogen during estradiol oxidation in quantitative growth experiments with *Denitratisoma oestradiolicum* AcBE2-1^T (refer to Table 3)

Nitrate consumed [µmol]	Nitrite formed [µmol]	Dinitrogen monoxide formed [µmol]	Dinitrogen formed [µmol]	Ratio [%]
207.8	130.7	43.2	19.1	161.6
377.0	213.7	76.8	36.3	138.5
487.6	235.0	90.9	54.6	115.2
506.7	0	91.6	158.4	98.7
501.3	0	143.8	116.6	103.9

Table 2A. N-ratios of reduced nitrate and formed dinitrogen monoxide during estradiol oxidation in quantitative growth experiments with *Steroidobacter denitrificans* FS^T (refer to Table 7)

Nitrate consumed [µmol]	Dinitrogen monoxide formed [µmol]	Ratio [%]
188.5	91.8	97.4
319.7	157.7	98.7
347.0	172.9	99.7
449.3	219.0	97.5
484.9	237.1	97.8

Table 3A. N-ratios of reduced nitrate and formed dinitrogen monoxide during testosterone oxidation in quantitative growth experiments with *Steroidobacter denitrificans* FS^T (refer to Table 8)

Nitrate consumed [µmol]	Dinitrogen monoxide formed [µmol]	Ratio [%]
165.2	83.7	101.3
363.7	189.1	104.0
382.3	215.5	112.7
537.1	281.1	104.7
529.6	282.0	106.5

8.4 Complete datasets of time course experiments

Table 4A. Time course experiment with *Denitratisoma oestradiolicum* AcBE2-1^T (refer to Chapter 3.1.4). These data were used for Fig. 5

Dinitrogen monoxide					
Time [h]	1 [µmol]	2 [µmol]	3 [µmol]	Mean [µmol]	Standard diviation [± µmol]
0	0	0	0	0	0
48	0	0	0	0	0
66	0	0	0	0	0
87	18	19	24	20.3	0.003
97	46	46	56	49.4	0.006
109	89	98	112	99.7	0.012
120	132	138	140	136.6	0.004
136	169	180	180	176.4	0.006
144	185	192	189	188.5	0.003
160	177	178	197	183.9	0.011
Dinitrogen					
0	5	0	18	7.7	0.009
48	0	0	18	6.0	0.010
66	9	5	16	10.2	0.006
87	20	17	37	24.7	0.010
97	34	27	49	36.4	0.011
109	49	47	63	52.7	0.009
120	63	60	73	65.2	0.007
136	75	72	85	77.5	0.006
144	77	79	88	81.5	0.005
160	95	96	101	97.0	0.003
Nitrate	1 [mM]	2 [mM]	3 [mM]	Mean [mM]	Standard diviation [± mM]
0	5.2	5.4	5.5	5.4	0.140
48	5.3	5.4	5.2	5.3	0.113
66	4.4	4.8	4.8	4.7	0.201
87	2.0	2.1	2.1	2.1	0.066
97	1.2	1.2	1.1	1.1	0.025
109	< LOQ	< LOQ	< LOQ	-	-
Nitrite					
0	0.4	0.4	0.5	0.4	0.024
48	0.6	0.5	0.5	0.5	0.043
66	1.1	0.8	0.8	0.9	0.156
87	3.0	2.9	3.0	3.0	0.072
97	3.6	3.5	3.5	3.5	0.086
109	2.8	2.5	2.8	2.7	0.156
120	< LOQ	< LOQ	< LOQ	-	-

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Table 4A continued

Cell dry mass					
Time [h]	1 [mg l^{-1}]	2 [mg l^{-1}]	3 [mg l^{-1}]	Mean [mg l^{-1}]	Standard diviation [± mg l^{-1}]
0	4.2	4.2	3.2	3.9	0.608
48	5.3	0.6	1.2	2.4	2.539
66	ND*	ND	ND	ND	ND
87	27.4	22.1	24.2	24.6	2.652
97	ND	ND	ND	ND	ND
109	48.5	49.5	49.5	49.2	0.608
120	85.1	68.5	69.6	74.4	9.264
136	70.6	62.2	67.5	66.8	4.259

* Not determined.

Table 5A. Time course experiment with *Steroidobacter denitrificans* FS^T grown on estradiol and nitrate (refer to Chapter 3.3.3). These data were used for Fig. 11

Dinitrogen monoxide				
Time [h]	1 [µmol]	2 [µmol]	Mean [µmol]	Standard diviation [± µmol]
0	0	0	0	0
120	30	57	43.8	0.019
163	81	158	119.5	0.055
213.5	178	284	230.8	0.075
237.5	255	352	303.5	0.068
262.5	307	414	360.4	0.075
287.5	358	466	412.2	0.077
311.5	410	509	459.5	0.070
331.5	443	514	478.6	0.050
355.5	502	526	514.3	0.017
381.5	548	526	537.1	0.016
Dinitrogen				
0	123	64	93.7	0.041
120	34	25	29.2	0.006
163	39	32	35.7	0.005
213.5	40	33	36.4	0.005
237.5	39	31	35.3	0.006
262.5	38	31	34.8	0.005
287.5	36	30	33.2	0.004
311.5	35	28	31.9	0.005
331.5	29	26	27.5	0.002
355.5	37	40	38.4	0.002
381.5	24	26	24.8	0.001

Table 6A. Time course experiment with *Steroidobacter denitrificans* FS^T grown on testosterone and nitrate (refer to Chapter 3.3.3). These data were used for Fig. 12

Dinitrogen monoxide				
Time [h]	1 [µmol]	2 [µmol]	Mean [µmol]	Standard diviation [± µmol]
0	0	0	0	0
120	59	50	54.5	0.006
163	229	210	219.5	0.013
213.5	567	550	558.6	0.012
237.5	555	550	552.6	0.004
262.5	561	530	545.7	0.022
287.5	564	550	557.1	0.010
311.5	536	520	527.8	0.011
331.5	ND*	ND	ND	ND
355.5	ND	ND	ND	ND
381.5	550	530	539.7	0.014
Dinitrogen				
0	30	144	87.2	0.081
120	3	77	40	0.052
163	20	79	49.6	0.041
213.5	19	78	48.3	0.042
237.5	17	74	45.3	0.041
262.5	17	61	39.3	0.031
287.5	0	60	30.	0.042
311.5	0	68	33.9	0.048
331.5	ND*	ND	ND	ND
355.5	ND	ND	ND	ND
381.5	0	52	26.2	0.037

* Not determined.

Table 7A. Time course experiment with the enrichment culture of *Denitratisoma oestradiolicum* AcBE2-1^T (refer to Chapter 3.1.1). These data were used for Fig. 3

Estradiol				
Time [h]	1 [µM]	2 [µM]	Mean [µM]	Standard diviation [± µM]
0	16.8	16.4	16.6	0.312
1	16.1	16.2	16.1	0.052
2	15.5	15.5	15.5	0.052
3	15.2	14.7	14.9	0.363
4	14.5	14.5	14.5	0
5	13.8	14.5	14.2	0.493
7	12.7	12.9	12.8	0.130
9.5	12.2	12.2	12.2	0.000
11.5	10.6	10.8	10.7	0.104
14.5	8.7	8.7	8.7	0.000
17.5	6.4	6.5	6.4	0.130
22.75	1.3	1.5	1.4	0.104
23.75	0.5	0.7	0.6	0.104
24.75	< LOD	< LOD	-	-
25.75	< LOD	< LOD	-	-
28.5	< LOD	< LOD	-	-
51.5	< LOD	< LOD	-	-
77.5	< LOD	< LOD	-	-
Estrone				
0	0	0	0	0
1	0.5	0.4	0.5	0.021
2	0.6	0.6	0.6	0.008
3	0.7	0.8	0.7	0.016
4	0.8	0.9	0.9	0.039
5	1.0	0.9	1	0.024
7	1.1	1.1	1.1	0.037
9.5	1.3	1.3	1.3	0.016
11.5	1.4	1.3	1.3	0.044
14.5	1.2	1.3	1.3	0.034
17.5	1.2	1.2	1.2	0.029
22.75	0.5	0.5	0.5	0.024
23.75	< LOD	1.0	1.0	-
24.75	< LOD	< LOD	-	-
25.75	< LOD	< LOD	-	-
28.5	< LOD	< LOD	-	-
51.5	< LOD	< LOD	-	-
77.5	< LOD	< LOD	-	-

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Table 7A continued

Nitrate				
Time [h]	1 [µM]	2 [µM]	Mean [µM]	Standard diviation [± µM]
0	1121	1187	1154	47
1	1153	1189	1171	25
2	1195	1179	1187	11
3	1206	1192	1199	10
4	1173	1176	1174	2
5	1174	1174	1174	0
7	1135	1158	1147	16
9.5	1192	1155	1173	26
11.5	956	1169	1063	151
14.5	1021	1021	1021	0
17.5	1006	1037	1022	22
22.75	908	911	910	2
23.75	885	890	888	3
24.75	856	874	865	13
25.75	874	853	864	15
28.5	840	798	819	30
51.5	890	857	874	23
77.5	853	873	863	14
Nitrite				
0	< LOQ	< LOQ	-	-
1	< LOQ	< LOQ	-	-
2	< LOQ	< LOQ	-	-
3	< LOQ	< LOQ	-	-
4	< LOQ	< LOQ	-	-
5	< LOQ	< LOQ	-	-
7	< LOQ	< LOQ	-	-
9.5	< LOQ	< LOQ	-	-
11.5	< LOQ	< LOQ	-	-
14.5	< LOQ	< LOQ	-	-
17.5	< LOQ	< LOQ	-	-
22.75	107.0	85.0	96.0	15.5
23.75	97.6	99.3	98.5	1.2
24.75	127.6	128.5	128.0	0.6
25.75	124.3	130.4	127.4	4.3
28.5	121.5	122.0	121.7	0.3
51.5	111.5	119.6	115.5	5.7
77.5	100.9	113.5	107.2	8.9

Table 8A. Time course experiment with the enrichment culture of *Steroidobacter denitrificans* FS^T grown on testosterone and nitrate (refer to Chapter 3.3.1). These data were used for Fig. 9

Estradiol				
Time [h]	1 [µM]	2 [µM]	Mean [µM]	Standard diviation [± µM]
0	17.3	17.6	17.4	0.208
4.5	16.9	17.3	17.1	0.260
6.5	17.0	17.3	17.1	0.208
9.5	16.4	16.7	16.6	0.156
13.5	15.8	16.0	15.9	0.130
19	14.9	15.1	15.0	0.104
30.25	12.6	12.8	12.7	0.104
32.25	12.0	12.7	12.4	0.519
34	12.1	12.9	12.5	0.597
37.75	11.6	12.0	11.8	0.312
44.25	10.5	11.2	10.8	0.519
53.75	9.5	10.1	9.8	0.389
65.25	7.0	8.1	7.6	0.831
78.75	5.5	6.4	6.0	0.597
88.25	4.1	5.1	4.6	0.701
103.75	2.0	3.1	2.6	0.794
112.25	1.0	2.0	1.5	0.711
129	0.1	0.5	0.3	0.322
135.75	< LOD	0.2	0.2	-
161.5	< LOD	< LOD	-	-
Estrone				
0	0	0	0	0
4.5	0.5	0.5	0.5	0.005
6.5	0.6	0.6	0.6	0.034
9.5	0.9	0.8	0.8	0.050
13.5	1.1	0.9	1.0	0.107
19	1.5	1.3	1.4	0.141
30.25	2.2	2.0	2.1	0.136
32.25	2.3	2.3	2.3	0.013
34	2.3	2.4	2.4	0.042
37.75	2.8	2.6	2.7	0.136
44.25	3.1	2.9	3.0	0.175
53.75	4.0	3.7	3.8	0.183
65.25	4.3	4.4	4.3	0.016
78.75	5.4	5.1	5.2	0.209
88.25	5.8	5.4	5.6	0.340
103.75	5.7	5.6	5.6	0.026
112.25	5.2	5.7	5.4	0.392
129	3.4	4.8	4.1	0.968
135.75	2.0	3.7	2.8	1.190
161.5	< LOD	< LOD	-	-

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Table 8A continued

Nitrate				
Time [h]	1 [µM]	2 [µM]	Mean [µM]	Standard diviation [± µM]
0	1076	1064	1070	8.782
4.5	1088	1070	1079	12.545
6.5	1055	1077	1066	15.248
9.5	1078	1084	1081	4.106
13.5	1078	1064	1071	10.264
19	1068	ND*	1068	ND
30.25	1065	1068	1066	2.281
32.25	1071	1056	1064	10.264
34	1051	1067	1059	11.816
37.75	1084	1050	1067	24.635
44.25	1043	1058	1051	10.607
53.75	1047	1037	1042	6.729
65.25	1024	1029	1027	3.421
78.75	1013	1010	1012	2.623
88.25	983	997	990	9,352
103.75	969	967	968	1.140
112.25	949	978	963	20.187
129	892	908	900	11.405
135.75	879	907	893	19.731
161.5	849	860	854	7.755

* Not determined.

8.5 Enrichment cultures

Table 9A. Enrichment cultures with estradiol (E2) as sole source of carbon and energy

Designation	Electron acceptor	Inoculum	Result
AcE2BS	Nitrate	Activated sludge from the WWTP Aachen-Soers	Isolation of *Denitratisoma oestradiolicum*
AcE2FS	Nitrate	Anoxic sewage sludge from the WWTP Aachen-Soers	Isolation of *Steroidobacter denitrificans*
E2FS-Sulfat	Sulfate*	Anoxic sewage sludge from the WWTP Aachen-Soers	No growth observed†
Wurm-E2	Sulfate*	Anoxic sediment from the Wurm river (Aachen-Soers)	No growth observed
Bergkamen-E2	Nitrate	Industrial activated sludge from the ITP Bergkamen	No growth observed
Bergkamen-E2	Sulfate*	Industrial activated sludge from the ITP Bergkamen	No growth observed
E2BS-Sulfat	Sulfate*	Activated sludge from the WWTP Aachen-Soers	No growth observed
Graben-E2	Nitrate	Water-mud mixture sampled from a trench near Aachen	No growth observed
Graben-E2	Sulfate*	Water-mud mixture sampled from a trench near Aachen	No growth observed
Mist-E2	Nitrate	Manure from a farm near Aachen	No growth observed
Mist-E2	Sulfate*	Manure from a farm near Aachen	No growth observed
Kaarst-E2	Nitrate	Activated sludge from the WWTP Kaarst	No growth observed
Kaarst-E2	Sulfate*	Activated sludge from the WWTP Kaarst	No growth observed

* 1 mM ferrous sulfide was used as reductant.

† Even after 2-3 years of incubation.

Table 10A. Enrichment cultures with ethinyl estradiol (EE2) and nitrate as the electron acceptor

Designation	Electron acceptor	Inoculum	Result
EE2-O2	Oxygen	Activated sludge from the WWTP Aachen-Soers	No growth observed‡
EE2-O2	Oxygen	Industrial activated sludge from the ITP Bergkamen	No growth observed‡
EE2FS-Sulfat	Sulfate*	Anoxic sewage sludge from the WWTP Aachen-Soers	No growth observed†
Wurm-EE2	Sulfate*	Anoxic sediment from the Wurm river (Aachen-Soers)	No growth observed
Bergkamen-EE2	Nitrate	Industrial activated sludge from the ITP Bergkamen	No growth observed
Bergkamen-EE2	Sulfate*	Industrial activated sludge from the ITP Bergkamen	No growth observed
EE2BS-Sulfat	Sulfate*	Activated sludge from the WWTP Aachen-Soers	No growth observed
Graben-EE2	Nitrate	Water-mud mixture sampled from a trench near Aachen	No growth observed
Graben-EE2	Sulfate*	Water-mud mixture sampled from a trench near Aachen	No growth observed
Mist-EE2	Nitrate	Manure from a farm near Aachen	No growth observed
Mist-EE2	Sulfate*	Manure from a farm near Aachen	No growth observed
Kaarst-EE2	Nitrate	Activated sludge from the WWTP Kaarst	No growth observed
Kaarst-EE2	Sulfate*	Activated sludge from the WWTP Kaarst	No growth observed

* 1 mM ferrous sulfide was used as reductant.

† Even after 2-3 years of incubation.

‡ Even after 3 month of incubation in potassium phosphate-buffered medium.

8.6 Proteomic analysis of *Denitratisoma oestradiolicum* AcBE2-1^T

Table 11A. Estradiol-induced soluble proteins in *Denitratisoma oestradiolicum* AcBE2-1^T with less significant identity to known proteins with regard to molecular mass, isoelectric point, degree of sequence identity, or function (gel with linear pH 3-10 IPG strip)*

AcBE2-1^T protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 QSALDFHQFPVPGK 14 QSALDFHQFPVPGK Sbjct 7 QSALDFHQFPVPGK 20	14/14 (100%)	4e-05	Phosphate acetyltransferase (77.4 kDa; pI 5.0)	*Yersinia pestis* KIM Spot 1 (15.0 kDa; pI 4.3)
Query 1 QLLQEQES 8 QLLQEQES Sbjct 130 QLLQEQES 137	8/8 (100%)	30	4-hydroxybenzoyl-CoA thioesterase (25.1 kDa; pI 8.7)	*Desulfuromonas acetoxidans* DSM 684 Spot 3 (30.0 kDa; pI 5.2)
Query 1 FPLPVEVIPMAR 12 FPLPVEVIPMAR Sbjct 129 FPLPVEVIPMAR 140	12/12 (100%)	0.001	Ribose 5-phosphate isomerase (24.5 kDa; pI 5.3)	*Pseudoalteromonas atlantica* T6c Spot 5 (28.0 kDa; pI 6.0)
Query 1 TIAREAFEDVDSDELEPVR 19 TIAREAFEDVDSDELEPVR Sbjct 562 TIAREAFEDVDSDELEPVR 580	19/19 (100%)	7e-10	Acyl-CoA dehydrogenase (64.1 kDa; pI 4.4)	*Haloarcula marismortui* ATCC 43049 Spot 5 (28.0 kDa; pI 6.0)
Query 1 SKTAELFGTACAVGAIIAEQVDHISK 26 SKTAELFGTACAVGAIIAEQVDHISK Sbjct 215 SKTAELFGTACAVGAIIAEQVDHISK 240	26/26 (100%)	2e-15	Octaprenyl-diphosphate synthase (42.1 kDa; pI 8.3)	*Rickettsia conorii* str. Malish 7 Spot 5 (28.0 kDa; pI 6.0)
Query 1 TAPFKSQNMSLNSYVTPDGNEIGR 24 TAPFKSQNMSLNSYVTPDGNEIGR Sbjct 36 TAPFKSQNMSLNSYVTPDGNEIGR 59	24/24 (100%)	1e-14	Cobyric acid synthase (55.3 kDa; pI 6.8)	*Geobacillus kaustophilus* HTA426 Spot 7 (125.0 kDa; pI 5.9)
Query 1 QLAIANKLDA 10 QLAIANK+ A Sbjct 93 QLAIANKIKA 102	8/10 (80%)	424	Acetyl-CoA:acetoacetyl-CoA transferase (56.0 kDa; pI 5.8)	*Bacillus halodurans* C-125 Spot 7 (125.0 kDa; pI 5.9)
Query 1 QTLASEGLLR 10 QTLA +GLLR Sbjct 282 QTLAGDGLLR 291	8/10 (80%)	98	Probable permease component of an ABC-transporter (68.5 kDa; pI 8.1)	*Rhodopirellula baltica* SH 1 Spot 8 (87.5 kDa; pI 5.8)
Query 1 ATQLLKDAGLK 11 ATQLLKDAGLK Sbjct 291 ATQLLKDAGLK 301	11/11 (100%)	0.27	N-acetyltransferase, GNAT family (69.1 kDa; pI 9.6)	*Pyrococcus abyssi* GE5 Spot 8 (87.5 kDa; pI 5.8)

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Table 11A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 AIGDIANA 8 AIGDIA A Sbjct 311 AIGDIAGA 318	7/8 (87%)	2232	Probable dihydrolipoamide dehydrogenase (49.7 kDa; pI 7.0)	*Rhodobacter sphaeroides* 2.4.1 Spot 8 (87.5 kDa; pI 5.8)
Query 1 AADEPQTL 8 A DEPQTL Sbjct 165 APDEPQTL 172	7/8 (87%)	513	Similar to ketosteroid isomerase-related protein (32.1 kDa; pI 4.5)	*Azotobacter vinelandii* AvOP Spot 8 (87.5 kDa; pI 5.8)
Query 1 QTLASEGLLR 10 QTLA +GLLR Sbjct 282 QTLAGDGLLR 291	8/10 (80%)	98	Probable permease component of an ABC-transporter (68.5 kDa; pI 8.1)	*Rhodopirellula baltica* SH 1 Spot 8 (87.5 kDa; pI 5.8)
Query 1 SAAFFALGIAK 11 SAAFFALGIAK Sbjct 52 SAAFFALGIAK 62	11/11 (100%)	0.27	2-oxoglutarate decarboxylase (EC 4.1.1.71) (62.5 kDa; pI 6.0)	*Lactococcus lactis* subsp. *lactis* Il1403 Spot 9 (50.0 kDa; pI 6.8)
Query 1 NTDSLGVQVS 10 NT+ LGV+VS Sbjct 389 NTEGLGVEVS 398	7/10 (70%)	762	Acetyl-CoA decarbonylase/synthase complex gamma subunit (cdhE) (52.5 kDa; pI 4.9)	*Archaeoglobus fulgidus* DSM 4304 Spot 10 (50.0 kDa; pI 6.2)
Query 1 RATAAVCR 8 RATA VCR Sbjct 207 RATAMVCR 214	7/8 (87%)	934	Cyclohexa-1.5-diene-1-carboxyl-CoA hydratase (NA† kDa; pI NA†)	Uncultured bacterium Spot 10 (50.0 kDa; pI 6.2)
Query 3 RAEVAILHADVVHNAR 18 RAEV L ADVVHN R Sbjct 60 RAEVEAL-ADVVHNVR 74	12/16 (75%)	1.6	3-octaprenyl-4-hydroxybenzoate carboxy-lyase (21.7 kDa; pI NA†)	*Ralstonia metallidurans* CH34 Spot 10 (50.0 kDa; pI 6.2)
Query 1 MPF-GGVPV 8 MPF GGVPV Sbjct 203 MPFGGGVPV 211	8/9 (88%)	253	Molybdenum cofactor biosynthesis protein A (36.0 kDa; pI 8.4)	*Anaeromyxobacter dehalogenans* 2CP-C Spot 10 (50.0 kDa; pI 6.2)
Query 1 QALAARGLAS 10 Q LAARGLAS Sbjct 211 QVLAARGLAS 220	9/10 (90%)	54	Thioesterase (90.5 kDa; pI 6.9)	*Rhodopseudomonas palustris* BisB18 Spot 10 (50.0 kDa; pI 6.2)

continued on next page

Table 11A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 ELMAVAGEAL 10 ELM VAGE L Sbjct 145 ELMDVAGEQL 154	8/10 (80%)	235	Probable 2-hydroxyacid dehydrogenase (43.7 kDa; pI 6.6)	*Rhodopirellula baltica* SH 1 Spot 11 (70.0 kDa; pI 6.2)
Query 1 VLLELMASR 9 VL+ LMASR Sbjct 71 VLIGLMASR 79	7/9 (77%)	1239	COG0043: 3-polyprenyl-4-hydroxybenzoate decarboxylase and related decarboxylases (54.2 kDa; pI NA†)	*Oenococcus oeni* PSU-1 Spot 11 (70.0 kDa; pI 6.2)
Query 1 SSSWESRI----AAGLA--VEAI 17 SSSWE R+ A GL+ VEA+ Sbjct 126 SSSWEQRLAVIRAEGLSAIVEAV 148	12/23 (52%)	71	3-oxoadipate enol-lactone hydrolase/4-carboxymuconolactone decarboxylase (41.8 kDa; pI 5.4)	*Caulobacter crescentus* CB15 Spot 11 (70.0 kDa; pI 6.2)
Query 1 GLTDTM---LR 8 GLTDTM LR Sbjct 159 GLTDTMRLELR 169	8/11 (72%)	612	Short-chain dehydrogenase/reductase (30.8 kDa; pI 9.8)	*Francisella tularensis* SCHU S4 Spot 11 (70.0 kDa; pI 6.2)
Query 1 FPILGATAGVIASIQVAEVVK 21 FPILGATAGVI SIQV EVVK Sbjct 182 FPILGATAGVIGSIQVTEVVK 202	19/21 (90%)	0.052	moeB/thiF molybdopterin or thiamine synthase protein (25.5 kDa; pI 5.7)	*Pyrococcus abyssi* GE5 Spot 11 (70.0 kDa; pI 6.2)
Query 2 ASGGKSEAK 10 A GGKSEAK Sbjct 112 AMGGKSEAK 120	8/9 (88%)	1377	3-methylcrotonyl-CoA carboxylase alpha subunit (71.1 kDa; pI 5.3)	*Agrobacterium tumefaciens* str. C58 Spot 12 (87.5 kDa; pI 6.3)
Query 1 SREVAAEFAAR 11 SREVAAEFAAR Sbjct 228 SREVAAEFAAR 238	11/11 (100%)	0.15	Carboxylesterase (55.1 kDa; pI 8.8)	*Mycobacterium tuberculosis* CDC1551 Spot 12 (87.5 kDa; pI 6.3)
Query 1 TVAVYSEADASAR 13 TVAVYSEADASAR Sbjct 28 TVAVYSEADASAR 40	13/13 (100%)	0.003	Putative acyl-CoA carboxylase alpha chain protein (72.2 kDa; pI 5.9)	*Azoarcus* sp. EbN1 Spot 14 (35.0 kDa; pI 6.6)
Query 1 APLTTMGPLIEKDER 15 APLTTMGPLIEKDER Sbjct 179 APLTTMGPLIEKDER 193	15/15 (100%)	4e-06	Diaminopimelate epimerase (NA† kDa; pI NA†)	*Bacillus licheniformis* ATCC 14580 Spot 14 (35.0 kDa; pI 6.6)

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Table 11A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 THNSKELTK 9 T N KELTK Sbjct 192 TQNMKELTK 200	7/9 (77%)	1240	Bifunctional protein: methylenetetrahydrofolate dehydrogenase; methenyltetrahydrofolate cyclohydrolase (31.1 kDa; pI 6.0)	*Bacillus thuringiensis* serovar *konkukian* str. 97-27 Spot 15 (32.0 kDa; pI 6.6)
Query 3 NANPTAENIK 12 N NPTA+ IK Sbjct 118 NPNPTADEIK 127	7/10 (70%)	567	4-hydroxybenzoyl-CoA reductase alpha/gamma subunit (hcrC/hcrA) (NA† kDa; pI NA†)	Uncultured bacterium Spot 17 (28.0 kDa; pI 7.3)
Query 1 LDGVVNNAGIL 11 LD +VNNAGIL Sbjct 84 LDALVNNAGIL 94	9/11 (81%)	19	cyclohexanol dehydrogenase or cyclopentanol dehydrogenase (NA† kDa; pI NA†)	*Comamonas testosteroni* or *Comamonas* sp. NCIMB 9872 Spot 17 (28.0 kDa; pI 7.3)
Query 1 GRAEAIAMVTVR 12 GR EAIAMVTVR Sbjct 731 GRVEAIAMVTVR 742	11/12 (91%)	0.084	Formate dehydrogenase, alpha subunit, anaerobic (IMG 401035720) (90.3 kDa; pI 7.5)	*Desulfovibrio desulfuricans* G20 Spot 17 (28.0 kDa; pI 7.3)
Query 1 AALGGYL 7 AALGGYL Sbjct 225 AALGGYL 231	7/7 (100%)	924	Phenazine biosynthesis family protein (29.1 kDa; pI 4.7)	*Pseudomonas syringae* pv. *tomato* str. DC3000 Spot 17 (28.0 kDa; pI 7.3)
Query 1 EQAPLALKHSGIACIVAK 18 EQAPLALKHSGIACIVAK Sbjct 70 EQAPLALKHSGIACIVAK 87	18/18 (100%)	3e-08	3-isopropylmalate dehydratase (18.0 kDa; pI 8.1)	*Methanosarcina acetivorans* C2A Spot 17 (28.0 kDa; pI 7.3)

* Peptide sequences which were not assigned to protein hits by the Mascot program were manually compared with the database at the NCBI (search for short, nearly exact matches; substitution matrix PAM30) using the BLAST program (Altschul et al., 1997). The percentage of identity was defined as the percentage of amino acids that are identical in two proteins. The line between the sequences shows identical and similar (+) amino acids whereas gaps are assign by a null (-). The lower the E value (expectation value), the more significant the score. The higher the score the better the alignment. The score for an alignment is calculated by summing the scores for each aligned position and the negative scores for gaps. Additional IMG numbers refer to defined genes or if paralogs of a gene are present in one genome.

† No data available.

Table 12A. Estradiol-induced soluble proteins in *Denitratisoma oestradiolicum* AcBE2-1^T with partial significant sequence identity to known eukaryotic proteins (gel with linear pH 3-10 IPG strip)*

AcBE2-1^T protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 TSGPSVLNGPAVGPLNTPQPPALTSK 26 TSGPSVLNGPAVGPLNTPQPPALTSK Sbjct 250 TSGPSVLNGPAVGPLNTPQPPALTSK 275	26/26 (100%)	5 x 10^{-15}	Estrogen receptor type beta (NA† kDa; pI NA†)	*Sparus aurata* Spot 9 (50.0 kDa; pI 6.8)
Query 1 EAERPLPWA 9 E+ERPLP A Sbjct 940 ESERPLPRA 948	7/9 (77%)	360	Sterol regulatory element binding transcription factor 1 isoform a (NA† kDa; pI NA†)	*Homo sapiens* Spot 9 (50.0 kDa; pI 6.8)
Query 1 MTTEQQTLR 9 +TT+QQTLR Sbjct 368 LTTQQQTLR 376	7/9 (77%)	134	Estrogen receptor beta2 (NA† kDa; pI NA†)	*Sparus aurata* and *Micropogonias undulatus* Spot 14 (35.0 kDa; pI 6.6)
Query 1 LNKPEK 6 LNKPEK Sbjct 263 LNKPEK 268	6/6 (100%)	1260	Type II estrogen receptor (NA† kDa; pI NA†)	*Oreochromis niloticus* Spot 14 (35.0 kDa; pI 6.6)

* Peptide sequences which were not assigned to protein hits by the Mascot program were manually compared with the database at the NCBI (search for short, nearly exact matches; substitution matrix PAM30) using the BLAST program (Altschul et al., 1997). The percentage of identity was defined as the percentage of amino acids that are identical in two proteins. The line between the sequences shows identical and similar (+) amino acids whereas gaps are assign by a null (-). The lower the E value (expectation value), the more significant the score. The higher the score the better the alignment. The score for an alignment is calculated by summing the scores for each aligned position and the negative scores for gaps.

† No data available.

Table 13A. Estradiol-induced soluble proteins in *Denitratisoma oestradiolicum* AcBE2-1^T with less significant identity to known proteins with regard to molecular mass, isoelectric point, degree of sequence identity, or function (gel with linear pH 5-8 IPG strip)*

AcBE2-1^T protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 VPVFADENASQLASR 15 VPVFADENASQLASR Sbjct 161 VPVFADENASQLASR 175	15/15 (100%)	2e-05	Phosphoribosylglycinamide formyltransferase (24.1 kDa; pI 5.8)	*Pseudoalteromonas atlantica* T6c Spot 4 (40.0 kDa; pI 6.0)
Query 1 SLVPKNELR 9 SLVPKN LR Sbjct 195 SLVPKNLLR 203	8/9 (88%)	698	Shikimate 5-dehydrogenase (30.0 kDa; pI NA†)	*Pyrococcus furiosus* DSM 3638 Spot 4 (40.0 kDa; pI 6.0)
Query 1 GEGAPVRRSGEGIK 14 G+GAP+RRS E IK Sbjct 456 GQGAPIRRSHEAIK 469	10/14 (71%)	11	Succinate dehydrogenase flavoprotein subunit (EC 1.3.99.1) KO: K00239 succinate dehydrogenase flavoprotein subunit (62.3 kDa; pI 7.5)	*Rhodobacter sphaeroides* ATCC 17025 Spot 4 (40.0 kDa; pI 6.0)
Query 1 LCAHAALR 8 LCAH ALR Sbjct 141 LCAH-ALR 147	7/8 (87%)	3276	2-aminobenzoate-CoA ligase (58.6 kDa; pI NA†)	*Azoarcus evansii* Spot 4 (40.0 kDa; pI 6.0)
Query 1 LARLRAVVLAHR 12 LARLRAVVLAHR Sbjct 39 LARLRAVVLAHR 50	12/12 (100%)	0.013	Aldehyde dehydrogenase (IMG 400300920) (53.1 kDa; pI 9.6)	*Burkholderia cenocepacia* HI2424 Spot 4 (40.0 kDa; pI 6.0)
Query 1 NLLISSCR 8 +LLI SCR Sbjct 93 DLLIESCR 100	6/8 (75%)	2442	Subunit of benzylsuccinate CoA-transferase (IMG 610562530) (45.4 kDa; pI 5.3)	*Azoarcus* sp. EbN1 Spot 4 (40.0 kDa; pI 6.0)
Query 1 AMSAAHVAFDRTLSSSLKLIR 21 AM+AAH AFDRTL SLK +R Sbjct 33 AMAAAHHAFDRTLQGSLKAMR 53	15/21 (71%)	0.003	Probable two-component transmembrane sensor Kinase transcription regulator protein (IMG 3513290) (50.9 kDa; pI 8.0)	*Ralstonia solanacearum* GMI1000 Spot 5 (48.0 kDa; pI 5.9)
Query 1 CDLCIGRENGPACVEVCPTEAL 22 CDLCI RE GPACVEVCPT AL Sbjct 131 CDLCINREKGPACVEVCPTAAL 152	19/22 (86%)	6e-10	4Fe-4S ferredoxin, iron-sulfur binding (IMG 635970090) (20.0 kDa; pI 7.1)	*Rhodopseudomonas palustris* BisB18 Spot 5 (48.0 kDa; pI 5.9)

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Table 13A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 RKILVDDRV 9 RKILVDDRV Sbjct 99 RKILVDDRV 107	9/9 (100%)	2.5	Probable oxidoreductase protein (NA† kDa; pI NA†)	*Rhodobacterales bacterium* HTCC2654 Spot 6 (48.0 kDa; pI 6.1)
Query 1 DEGLLDSR 8 DEGLLDSR Sbjct 333 DEGLLDSR 340	8/8 (100%)	39	COG0667: Predicted oxidoreductases (related to aryl-alcohol dehydrogenases) (NA† kDa; pI NA†)	*Burkholderia fungorum* LB400 Spot 6 (48.0 kDa; pI 6.1)
Query 1 LAVSEVDGI 9 L VSE+DGI Sbjct 132 LGVSEIDGI 140	7/9 (77%)	511	S-adenosylmethionine-dependent methyltransferase, putative (40.1 kDa; pI 6.9)	*Streptococcus thermophilus* CNRZ1066 Spot 6 (48.0 kDa; pI 6.1)
Query 2 SIEAMAK 8 SIEAMAK Sbjct 140 SIEAMAK 146	7/7 (100%)	231	3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase (87.4 kDa; pI 7.0)	*Bacillus halodurans* C-125 Spot 9 (31.0 kDa; pI 6.5)
Query 1 EMIQGTNVMK 10 EMIQ N++K Sbjct 288 EMIQSNNIIK 297	6/10 (60%)	1256	NADH dehydrogenase I, D subunit (47.6 kDa; pI 6.7)	*"Dechloromonas aromatica"* RCB Spot 9 (31.0 kDa; pI 6.5)
Query 1 ARRILVDGEIGDDGVPR 17 ARRILVDGEIGDDGVPR Sbjct 317 ARRILVDGEIGDDGVPR 333	17/17 (100%)	1e-07	4-hydroxyphenylpyruvate dioxygenase (42.1 kDa; pI 5.4)	*Burkholderia thailandensis* E264 Spot 9 (31.0 kDa; pI 6.5)
Query 1 NTVKPESIAVFCLTPGGVRLAR 22 NTVKPESIAVFCLTPGGVRLAR Sbjct 2 NTVKPESIAVFCLTPGGVRLAR 23	22/22 (100%)	3e-12	Cobalamin biosynthesis protein CbiG (NA† kDa; pI NA†)	*Yersinia mollaretii* ATCC 43969 Spot 9 (31.0 kDa; pI 6.5)
Query 1 NKVTVVMGEAK 11 NKVTVVMGEAK Sbjct 106 NKVTVVMGEAK 116	11/11 (100%)	0.056	Dihydrolipoamide dehydrogenase (51.8 kDa; pI 6.8)	*Rhodobacter sphaeroides* ATCC 17025 Spot 12 (48.0 kDa; pI 6.7)
Query 1 RIDMDKVRE 9 RIDMDKVRE Sbjct 155 RIDMDKVRE 163	9/9 (100%)	0.58	Glycine/serine hydroxymethyltransferase (45.7 kDa; pI 5.3)	*Brevibacterium linens* BL2 Spot 13 (48.0 kDa; pI 6.6)

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Table 13A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 RLCPADYHRY 10 RLCPADYHRY Sbjct 170 RLCPADYHRY 179	10/10 (100%)	0.025	Phosphatidylserine decarboxylase (33.9 kDa; pI 5.1)	*Desulfuromonas acetoxidans* DSM684 Spot 14 (48.0 kDa; pI 6.5)
Query 1 KISAALDDQGRI 12 KISAALDDQGRI Sbjct 442 KISAALDDQGRI 453	12/12 (100%)	0.014	Putative aldehyde dehydrogenase protein (80.1 kDa; pI 6.0)	*Sinorhizobium meliloti* 1021 Spot 14 (48.0 kDa; pI 6.5)
Query 1 MTVSPAGSPCRR 12 MTVSPAGSPCRR Sbjct 1 MTVSPAGSPCRR 12	12/12 (100%)	0.003	Putative mannose-1-phosphate guanylyltransferase (59.7 kDa; pI 6.6)	*Azoarcus* sp. EbN1 Spot 14 (48.0 kDa; pI 6.5)
Query 1 RGPTAIEAIRD 11 RGPTAIEAIRD Sbjct 419 RGPTAIEAIRD 429	11/11 (100%)	0.061	Fumarate hydratase, class I, putative (54.9 kDa; pI 5.4)	*Pseudomonas syringae* pv. tomato str. DC3000 Spot 15 (48.0 kDa; pI 6.4)
Query 1 FEQTYFSTFK 10 FEQTYFSTFK Sbjct 484 FEQTYFSTFK 493	10/10 (100%)	0.075	Acetyl-coenzyme A synthetase (72.1 kDa; pI 6.1)	*Yersinia pseudotuberculosis* IP 32953 Spot 15 (48.0 kDa; pI 6.4)
Query 1 IGAVHSVIFGGFSPEAVAGR 20 IGAVHSVIFGGFSPEAVAGR Sbjct 155 IGAVHSVIFGGFSPEAVAGR 174	20/20 (100%)	9e-10	Acetyl-coenzyme A synthetase (NA† kDa; pI NA†)	*Salmonella enterica* subsp. *enterica* serovar *choleraesuis* strain SC-B67 Spot 15 (48.0 kDa; pI 6.4)
Query 1 DAVAAFGEPGR 11 DAVAAFGEPGR Sbjct 743 DAVAAFGEPGR 753	11/11 (100%)	0.14	COG4631:Xanthine dehydrogenase, molybdopterin-binding subunit B (85.6 kDa; pI 5.3)	*Brevibacterium linens* BL2 Spot 18 (13.0 kDa; pI 6.5)
Query 1 LATVVTPR 8 LAT VTPR Sbjct 465 LATGVTPR 472	7/8 (87%)	1845	Putative 2,4-dienoyl-CoA reductase (72.7 kDa; pI 7.0)	*Burkholderia pseudomallei* K96243 Spot 18 (13.0 kDa; pI 6.5)
Query 1 KAISQATR 8 KAIS ATR Sbjct 11 KAISDATR 18	7/8 (87%)	1375	SAM-dependent methyltransferase (NA† kDa; pI NA†)	*Reinekea* sp. MED297 Spot 18 (13.0 kDa; pI 6.5)

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Table 13A continued

AcBE2-1^T protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 LDGGLAVSTAVR 12 LD GLAV T VR Sbjct 83 LDAGLAV-TDVR 93	9/12 (75%)	949	Carboxymuconolactone decarboxylase (IMG 500068030) (27.8 kDa; pI 6.2)	*Burkholderia ambifaria* AMMD Spot 18 (13.0 kDa; pI 6.5)
Query 2 QEIFAA 7 QEIFAA Sbjct 82 QEIFAA 87	6/6 (100%)	1845	5-carboxymethyl-2-hydroxymuconate isomerase (14.1 kDa; pI 6.7)	*Silicibacter* sp. TM1040 Spot 18 (13.0 kDa; pI 6.5)
Query 2 QDPLPR 7 QDPLPR Sbjct 436 QDPLPR 441	6/6 (100%)	884	Phosphoenolpyruvate carboxylase (EC 4.1.1.31) (98.3 kDa; pI 6.6)	*Xanthomonas axonopodis* pv. *citri* str. 306 Spot 19 (23.0 kDa; pI 7.2)
Query 1 LGSFLKLVEIKTK 13 LGSFLKLVEIKTK Sbjct 3 LGSFLKLVEIKTK 15	13/13 (100%)	0.002	UbiA prenyltransferase (36.4 kDa; pI 9.6)	*Clostridium thermocellum* ATCC 27405 Spot 19 (23.0 kDa; pI 7.2)
Query 1 LAEITPGDVEK 11 LAEITPGDVEK Sbjct 111 LAEITPGDVEK 121	11/11 (100%)	0.077	4-aminobutyrate aminotransferase (47.3 kDa; pI 5.2)	*Leifsonia xyli* subsp. *xyli* str. CTCB07 Spot 21 (25.0 kDa; pI 7.4)
Query 1 AYLEKHPEK 9 AYLEKHPEK Sbjct 146 AYLEKHPEK 154	9/9 (100%)	1.3	Phospholipid/glycerol acyltransferase (35.0 kDa; pI 10.0)	*Pseudomonas fluorescens* PfO-1 Spot 21 (25.0 kDa; pI 7.4)
Query 1 HAPDGETR 8 HAPD ETR Sbjct 240 HAPDNETR 247	7/8 (87%)	315	Radical SAM enzyme, Cfr family (41.0 kDa; pI 4.7)	*Salinibacter ruber* DSM 13855 Spot 21 (25.0 kDa; pI 7.4)
Query 2 ATPGMDTI-EK 11 ATP MDTI EK Sbjct 205 ATPMMDTIPEK 215	9/11 (81%)	293	Short-chain dehydrogenase/reductase SDR (27.6 kDa; pI 5.1)	*Psychrobacter cryohalolentis* K5 Spot 21 (25.0 kDa; pI 7.4)
Query 1 LGLGDTSPSS 10 +G+G+TSPSS Sbjct 181 MGIGNTSPSS 190	7/10 (70%)	1273	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (38.0 kDa; pI 4.5)	*Desulfuromonas acetoxidans* DSM 684 Spot 21 (25.0 kDa; pI 7.4)

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Table 13A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 ALTSCGTKIIAEVKK 15 ALTSCGTKIIAEVKK Sbjct 41 ALTSCGTKIIAEVKK 55	15/15 (100%)	3e-05	Indole-3-glycerol phosphate synthase (29.2 kDa; pI 4.9)	*Aquifex aeolicus* VF5 Spot 21 (25.0 kDa; pI 7.4)
Query 1 AAFDRSVAAVQGLVDACK 18 AAFDRSVAAVQGLVDACK Sbjct 296 AAFDRSVAAVQGLVDACK 313	18/18 (100%)	6e-08	Malate dehydrogenase (NA† kDa; pI NA†)	*Nitrobacter* sp. Nb-311A Spot 21 (25.0 kDa; pI 7.4)
Query 1 LHVPSGAVGGLDVLRALR 18 LHVPSGAVGGLDVLRALR Sbjct 122 LHVPSGAVGGLDVLRALR 139	18/18 (100%)	1e-07	Predicted dinucleotide-utilizing enzyme (28.2 kDa; pI 5.8)	*Methanopyrus kandleri* AV19 Spot 22 (26.5 kDa; pI 7.5)
Query 1 VLKPSEVR 8 VL+PSEVR Sbjct 42 VLRPSEVR 49	7/8 (87%)	316	Putative acetyltransferase (IMG 3798050) (20.2 kDa; pI 6.6)	*Bacteroides fragilis* YCH46 Spot 23 (39.0 kDa; pI 7.2)
Query 2 EVVSRPPPR 10 E V+RPPPR Sbjct 22 ETVNRPPPR 30	7/9 (77%)	294	Thioesterase superfamily (26.9 kDa; pI 10.9)	*Rhodopseudomonas palustris* BisB18 Spot 23 (39.0 kDa; pI 7.2)
Query 1 LSFSECTK 8 L+F ECTK Sbjct 53 LTFPECTK 60	6/8 (75%)	1375	2-methylcitrate dehydratase PrpD (53.2 kDa; pI 8.3)	*Geobacter metallireducens* GS-15 Spot 23 (39.0 kDa; pI 7.2)
Query 1 ALLPKVDALLVGGGMCFTFLAALGHPVGASLLESEMIDTCKDLLAEAGDR 50 ALLPKVDALLVGGGMCFTFLAALGHPVGASLLESEMIDTCKDLLAEAGDR Sbjct 217 ALLPKVDALLVGGGMCFTFLAALGHPVGASLLESEMIDTCKDLLAEAGDR 266	50/50 (100%)	5e-38	Phosphoglycerate kinase (41.1 kDa; pI 4.8)	*Frankia* sp. EAN1pec Spot 23 (39.0 kDa; pI 7.2)
Query 1 SHPAAAAPSPALP 13 S+P+AAAPSPA P Sbjct 4 SNPSAAAPSPAPP 16	10/13 (76%)	21	Short-chain dehydrogenase/reductase SDR (NA† kDa; pI NA†)	*Ralstonia eutropha* JMP134 Spot 23 (39.0 kDa; pI 7.2)
Query 1 VVLSVTPR 8 VVL VTPR Sbjct 319 VVLGVTPR 326	7/8 (87%)	569	Aldehyde dehydrogenase (NA† kDa; pI NA†)	*Ralstonia metallidurans* CH34 Spot 23 (39.0 kDa; pI 7.2)
Query 1 RSPFVTSGLRL 11 RSPFVTSGLRL Sbjct 354 RSPFVTSGLRL 364	11/11 (100%)	0.11	Serine hydroxymethyltransferase (44.9 kDa; pI 6.8)	*Xanthomonas oryzae* pv. *oryzae* KACC10331 Spot 24 (50.0 kDa; pI 7.3)

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Table 13A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 QYAELCELGGCR 12 QYAELCELGGCR Sbjct 525 QYAELCELGGCR 536	12/12 (100%)	0.001	Phospholipid/glycerol acyltransferase (65.7 kDa; pI 9.0)	*Thiomicrospira crunogena* XCL-2 Spot 25 (63.0 kDa; pI 5.3)
Query 1 FIAAYPAGR 9 FIA YPAGR Sbjct 24 FIAKYPAGR 32	8/9 (88%)	143	COG1905: NADH:ubiquinone oxidoreductase subunit (24.0 kDa; pI NA†)	*Magnetospirillum magnetotacticum* MS-1 Spot 26 (36.0 kDa; pI 6.2)
Query 1 QILFTTEDIDLPK 13 QILFTTEDIDLPK Sbjct 76 QILFTTEDIDLPK 88	13/13 (100%)	2e-04	Adenylyltransferase thiF thiamine biosynthesis protein ThiF (27.6 kDa; pI 4.3)	*Escherichia coli* CFT073 Spot 26 (36.0 kDa; pI 6.2)
Query 1 EHIALASSLGMK 12 EHIALASSLGMK Sbjct 242 EHIALASSLGMK 253	12/12 (100%)	0.010	Glycerophosphoryl diester phosphodiesterase (49.3 kDa; pI 4.6)	*Psychrobacter cryohalolentis* K5 Spot 26 (36.0 kDa; pI 6.2)
Query 1 AIYVNNT 7 AIYV+NT Sbjct 336 AIYVDNT 342	6/7 (85%)	741	Radical SAM (42.8 kDa; pI 7.7)	*Enterococcus faecium* DO Spot 26 (36.0 kDa; pI 6.2)
Query 1 SNLIGMGILPLEFPQGVTR 19 SNLIGMGILPLEFPQGVTR Sbjct 804 SNLIGMGILPLEFPQGVTR 822	19/19 (100%)	7e-10	Aconitate hydrase 1 (NA† kDa; pI NA†)	*Shigella flexneri* 2a str. 301 Spot 26 (36.0 kDa; pI 6.2)
Query 1 HAEPE--AAARR 10 HAEP+ AAARR Sbjct 26 HAEPDVAAAARR 37	9/12 (75%)	88	3-hydroxy-3-methylglutaryl-coenzyme A reductase (41.3 kDa; pI 4.1)	*Haloarcula marismortui* ATCC 43049 Spot 27 (42.0 kDa; pI 5.7)
Query 1 AAGGGSRGAK 10 AAGGG RG K Sbjct 160 AAGGGGRGMK 169	8/10 (80%)	2994	Acetyl-CoA carboxylase, biotin carboxylase (IMG 625811180) (48.8 kDa; pI 6.1)	*Bradyrhizobium* sp. BTAi1 Spot 27 (42.0 kDa; pI 5.7)

* Peptide sequences which were not assigned to protein hits by the Mascot program were manually compared with the database at the NCBI (search for short, nearly exact matches; substitution matrix PAM30) using the BLAST program (Altschul et al., 1997). The percentage of identity was defined as the percentage of amino acids that are identical in two proteins. The line between the sequences shows identical and similar (+) amino acids whereas gaps are assign by a null (-). The lower the E value (expectation value), the more significant the score. The higher the score the better the alignment. The score for an alignment is calculated by summing the scores for each aligned position and the negative scores for gaps. Additional IMG numbers refer to defined genes or if paralogs of a gene are present in one genome.

† No data available.

Table 14A. Estradiol-induced soluble proteins in *Denitratisoma oestradiolicum* AcBE2-1^T with partial significant sequence identity to known eukaryotic proteins (gel with linear pH 5-8 IPG strip)*

AcBE2-1^T protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 1 MRCGSCAVEPLSMNR 15 MRCGSCAVEPLSMNR Sbjct 164 MRCGSCAVEPLSMNR 178	15/15 (100%)	6e-07	ARNT protein (aryl hydrocarbon receptor nuclear translocator protein) (NA† kDa; pI NA†)	*Stenotomus chrysops* Spot 2 (25.0 kDa; pI 5.8)
Query 1 TYCKGKQC 8 TYC G+QC Sbjct 564 TYCDGQQC 571	6/8 (75%)	2685	Estrogen receptor BetaB (NA† kDa; pI NA†)	*Fundulus heteroclitus* Spot 7 (48.0 kDa; pI 6.2)
Query 1 NRDSR-----QESEADL 12 NR+ R QESEA+L Sbjct 26 NRNIRSIIVDQESEANL 42	9/17 (52%)	502	3-ketosteroid-delta-1-dehydrogenase, putative (61.6 kDa; pI 7.7)	*Aspergillus fumigatus* Af293 Spot 13 (48.0 kDa; pI 6.6)
Query 2 PRLSPR 7 PRLSPR Sbjct 35 PRLSPR 40	6/6 (100%)	1541	Estrogen receptor alpha (NA† kDa; pI NA†)	*Oryzias javanicus* Spot 13 (48.0 kDa; pI 6.6)
Query 1 SCATAEVHSRR 11 SCATAEVHSRR Sbjct 122 SCATAEVHSRR 132	11/11 (100%)	0.057	Androgen receptor (NA† kDa; pI NA†)	*Carassius auratus* Spot 21 (25.0 kDa; pI 7.4)
Query 2 GLDTTALGLIG 12 GLDTTA GL+G Sbjct 1856 GLDTTAPGLMG 1866	9/11 (81%)	291	Nuclear receptor coactivator (NA† kDa; pI NA†)	*Homo sapiens* Spot 21 (25.0 kDa; pI 7.4)
Query 1 LFTSWEEPK 9 LF SWEE K Sbjct 343 LF-SWEEAK 350	7/9 (77%)	1547	3-beta-hydroxysteroid dehydrogenase hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 2 3 beta-hydroxysteroid dehydrogenase/delta 5-->4-isomerase (3Beta-HSD) [Includes: 3-beta-hydroxy-delta(5)-steroid dehydrogenase] (3-beta-hydroxy-5-ene steroid dehydrogenase) (Progesterone reductase); Steroid delta-isomerase (Delta-5-3-ketosteroid isomerase) (NA† kDa; pI NA†)	*Canis familiaris* Spot 25 (63.0 kDa; pI 5.3)

continued on next page

Table 14A continued

AcBE2-1[T] protein, amino acid sequence and NCBI sequence	No. of amino acids (% identity)	E value	Protein (molecular weight; isoelectric point)	Organism and Spot number on the gel
Query 3 MLTS--VHRPDR 12 ML + VHRPDR Sbjct 505 MLDAHRVHRPDR 516	8/12 (66%)	512	Estrogen receptor alpha (NA† kDa; pI NA†)	*Acanthopagrus schlegelii* Spot 26 (36.0 kDa; pI 6.2)
Query 3 DAGMKK 8 DAGMKK Sbjct 73 DAGMKK 78	6/6 (100%)	2014	Nuclear receptor subfamily 1, group I, member 3 (NA† kDa; pI NA†)	*Bos taurus* Spot 26 (36.0 kDa; pI 6.2)
Query 2 PRLSPR 7 PRLSPR Sbjct 35 PRLSPR 40	6/6 (100%)	1567	Estrogen receptor alpha (NA† kDa; pI NA†)	*Oryzias javanicus* Spot 26 (36.0 kDa; pI 6.2)
Query 1 MS-VEDEY 7 MS VEDEY Sbjct 180 MSPVEDEY 187	7/8 (87%)	741	Nuclear hormone receptor family protein 35, isoform a oder steroid/thyroid/retinoic nuclear hormone receptor homolog nhr-35 (NA† kDa; pI NA†)	*Caenorhabditis elegans* Spot 26 (36.0 kDa; pI 6.2)
Query 1 EDFSVFVR 8 EDFS+F+R Sbjct 204 EDFSIFLR 211	6/8 (75%)	741	S-adenosylmethionine-dependent methyltransferase (NA† kDa; pI NA†)	*Arabidopsis thaliana* Spot 26 (36.0 kDa; pI 6.2)

* Peptide sequences which were not assigned to protein hits by the Mascot program were manually compared with the database at the NCBI (search for short, nearly exact matches; substitution matrix PAM30) using the BLAST program (Altschul et al., 1997). The percentage of identity was defined as the percentage of amino acids that are identical in two proteins. The line between the sequences shows identical and similar (+) amino acids whereas gaps are assign by a null (-). The lower the E value (expectation value), the more significant the score. The higher the score the better the alignment. The score for an alignment is calculated by summing the scores for each aligned position and the negative scores for gaps.

† No data available.

8.7 Table of Figures

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Wissenschaftlicher Bildungsgang

Michael Fahrbach

geboren am 28.01.1974 in Bad Mergentheim

1995	Abitur an der Justus-von-Liebig-Schule Aalen
1996 - 1999	Studium der Biologie an der Universität Konstanz
1999 - 2000	Studium der Biologie an der Universität Würzburg
2000 - 2002	Fortsetzung des Biologiestudiums an der Universität Konstanz. Mündliche Abschlussprüfung in den Fächern Pflanzenphysiologie und Mikrobiologie. Diplomarbeit am Lehrstuhl für Mikrobielle Ökologie bei Prof. Dr. Bernhard Schink. Arbeitstitel: Untersuchungen zum anaeroben Abbau von Thioetherverbindungen und Ethanolaminen.
2003 - 2006	Promotion am Institut für Hygiene und Umweltmedizin der RWTH Aachen.