*First publ. in: Archives of Microbiology 185 (2006), 3, pp.192-201*

**Bodo Philipp · Henrike Erdbrink · Marc J. -F. Suter**
**Bernhard Schink**

# Degradation of and sensitivity to cholate in *Pseudomonas* sp. strain Chol1

Received: 22 June 2005 / Revised: 6 December 2005 / Accepted: 5 January 2006 / Published online: 24 January 2006


**Abstract** A facultative anaerobic bacterium, *Pseudomonas* sp. strain Chol1, degrading cholate and other bile acids was isolated from soil. We investigated how strain Chol1 grew with cholate and whether growth was affected by the toxicity of this compound. Under anoxic conditions with nitrate as electron acceptor, strain Chol1 grew by transformation of cholate to 7,12-dihydroxy-1,4-androstadiene-3,17-dione (DHADD) as end product. Under oxic conditions, strain Chol1 grew by transformation of cholate to 3,7,12-trihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (THSATD), which accumulated in the culture supernatant before its further oxidation to $CO_2$. Strain Chol1 converted DHADD into THSATD by an oxygenase-dependent reaction. Addition of cholate (≥10 mM) to cell suspensions of strain Chol1 caused a decrease of optical density and viable counts but aerobic growth with these toxic cholate concentrations was possible. Addition of CCCP or EDTA strongly increased the sensitivity of the cells to 10 mM cholate. EDTA also increased the sensitivity of the cells to DHADD and THSATD ( ≤ 1.7 mM). The toxicity of cholate and its degradation intermediates with a steroid structure indicates that strain Chol1 requires a strategy to minimize these toxic effects during growth with cholate. Apparently, the proton motive force and the outer membrane are necessary for protection against these toxic effects.

B. Philipp (✉) · H. Erdbrink · B. Schink
Universität Konstanz, Fachbereich Biologie, Mikrobielle Ökologie, Fach M654, 78457 Konstanz, Germany
E-mail: bodo.philipp@uni-konstanz.de
Tel.: +49-7531-884541
Fax: +49-7531-884047

*Present address:*H. Erdbrink
FH Furtwangen, 78054 Schwenningen, Germany

M. J. -F. Suter
Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Duebendorf, Switzerland

**Keywords** *Pseudomonas* · Bile acids · Cholate · Degradation · Steroids · Androstandienedione · Sensitivity

## Introduction

Bile acids are surface-active steroid compounds with a $C_5$ acyl side chain attached to C17 of the steroid skeleton. Their function is to support the digestion of lipophilic nutrients through solubilization and absorption in the gut of mammals. Bile acids are synthesized in the liver via stepwise modifications of cholesterol to cholate and chenodeoxycholate. These so-called primary bile acids are conjugated to glycine or taurine, stored in the gall bladder, and eventually secreted into the duodenum. In the intestine, bile acids are subject to bacterial transformations. The most common reactions are deconjugation and 7α- or 12α-dehydroxylation, leading to the secondary bile acids deoxycholate or lithocholate, respectively (Bortolini et al. 1997; Hylemon and Harder 1998). In humans, most of the untransformed and transformed bile acids are re-used in the enterohepatic cycle, but a considerable fraction of bile acids is released into the environment by either fecal (0.37–0.6 g $day^{-1}$, Hylemon and Harder 1998; Hayakawa 1982) or urinary excretion (4 mg $day^{-1}$, Hayakawa 1982). Degradation of bile acids by different bacteria isolated from various environments has been documented, and a unifying degradation scheme has been proposed (Hayakawa 1982). The metabolic pathway is initiated by oxidation of the 3-hydroxyl group, followed by desaturation of the A-ring. Then, the acyl side chain is shortened, presumably through β-oxidation, and finally cleaved off, leaving an oxo group at C17. The products of these reactions are androstene-3,17-diones. Further degradation of these C19 steroid compounds proceeds via hydroxylation at C9 leading to 9,10-secosteroids with a phenolic A-ring (Park et al. 1986), which are converted to intermediates of the central metabolism and oxidized completely. Several studies addressing aerobic metabolism of bile

acids by different *Pseudomonas* strains were published about 20 years ago (e.g. Tenneson et al. 1979; Leppik et al. 1982; Smith and Park 1984). Most of these reports were driven by the idea to produce commercially useful steroid products from bile acids and dealt almost exclusively with the chemical identification of degradation products or the optimization of fermentation processes while physiological aspects of bile acid metabolism were mainly neglected. However, degradation of bile acids calls attention to general questions of bacterial ecophysiology.

First, regarding the amount of bile acids released by animals, these compounds should constitute a significant resource of water-soluble steroid compounds in the environment, and their metabolic fate should therefore be of ecological interest. We could not find any information about environmental concentrations or bioavailability of bile acids. Steroids are comparably stable compounds, and their environmental fate should be influenced by the availability of oxygen. Anaerobic metabolism of bile acids has been addressed only in a few studies (Barnes et al. 1975; Tenneson et al. 1977; Owen and Bilton 1983) and, to our knowledge, a quantitative analysis of growth has never been performed. For example, *Pseudomonas* sp. NCIB 10590 showed anaerobic nitrate-dependent cholate degradation in dense cell suspensions whereas it could not grow with cholate under theses conditions (Owen and Bilton 1983). Thus, our first goal was to investigate whether growth with bile acids is possible under anoxic conditions.

Second, bile acids are generally toxic for bacteria. As detergents, they interfere with biological membranes and may cause cell lysis (Helenius and Simons 1975). This toxic effect is exploited for selective cultivation of enteric bacteria on McConkey agar that contains about 2 mM deoxycholate. Bacteria living in the duodenum and the small intestine face bile acids at concentrations up to 20 mM (Thanassi et al. 1997). Enteric bacteria have resistance mechanisms (Gunn 2000) by which they protect themselves from the toxic effects, e.g. the outer membrane as a diffusion barrier (Nikaido 2003) and efflux pumps that remove bile acids from the cytoplasm (Poole 2004). Efflux pumps of the resistance-cell-division-nodulation superfamily employ the proton motive force to pump toxic compounds from the cytoplasm through the inner and outer membranes into the surrounding medium. All these protection mechanisms require energy, and the intestinal environment provides sufficient nutrients to cover this extra energetic investment. The situation is fundamentally different for bacteria that face bile acids in oligotrophic environments like soil. There, degradation of bile acids should be a major challenge for bacteria. Bacteria using these toxic compounds for growth have to invest part of their energy into protection while taking an increased risk of damage because they have to take up bile acids in order to metabolize them. The toxic effects of bile acids have not been considered in any of the aforementioned publications dealing with the degradation of these compounds. Thus, our second goal was to investigate whether the toxicity of bile acids affects growth with these compounds.

In the first part of this study, we characterize cholate metabolism of a newly isolated denitrifying bacterium growing with bile acids as carbon and energy source under anaerobic and aerobic conditions. In the second part, we study the sensitivity of this strain towards cholate.

## Materials and methods

### Media and cultivation

For enrichment and isolation of anaerobic bile acid-degrading bacteria a non-reduced bicarbonate-buffered mineral medium with vitamins (Widdel and Pfennig 1981), selenite–tungstate solution, and trace element solution SL10 (Widdel et al. 1983) was used. This medium contained Na-deoxycholate (1 mM) as the sole source of carbon and energy and $NaNO_3$ (8 mM) as electron acceptor. A pure culture of a deoxycholate-degrading bacterium, *Pseudomonas* sp. strain Chol1, was obtained by serial dilution in an agar-containing medium. For aerobic and anaerobic cultivation of *Pseudomonas* sp. strain Chol1, a phosphate-buffered (pH 7.0–7.1) mineral medium containing the following ingredients (final concentration in mM) was used: $K_2HPO_4$ (35), $NaH_2PO_4$ (15), $NH_4Cl$ (20), $MgSO_4$ (1.5), $CaCl_2$ (0.01), and trace element solution SL10. This medium was named MMChol. Bile acids were added as substrates from sterile stock solutions. For anaerobic cultivation, MMChol was cooled and dispensed into infusion bottles (sealed with butyl rubber septa) under $N_2$ atmosphere. A headspace of ca. 25% volume filled with $N_2$ was left. Anoxic MMChol contained nitrate (10 mM) as electron acceptor, and substrates were added from anoxic stock solutions. Strain Chol1 was maintained and transferred weekly on solid MMChol medium (1.5% agar) with cholate (2 mM) as substrate. Stock cultures were kept in Luria-Bertani (LB) medium with 40% (w/v) glycerol in liquid $N_2$.

### Growth experiments

Growth was followed by measuring the optical density at 600 nm ($OD_{600}$) in a spectrophotometer. All growth experiments were performed at 30°C. For aerobic growth experiments, 10 ml test tubes with 2.5 ml of MMChol containing the respective bile acids were seeded with strain Chol1 from an agar plate and incubated on a rotatory mixer (CVM, Fröbel) at 80 rpm for 8–14 h. With these pre-cultures, 100 ml MMChol in 500 ml Erlenmeyer flasks with baffles were inoculated to $OD_{600}$ of 0.01. Before inoculation, the cells were centrifuged and resuspended in fresh medium to avoid transfer of supernatant from the pre-cultures. The main

cultures were incubated on a shaker (Certomat, Braun) at 180 rpm. Immediately after inoculation and at intervals thereafter, samples were withdrawn to measure $OD_{600}$ and metabolite concentrations. For that, the samples were centrifuged (10 min at 13,200 rpm in an Eppendorf microfuge), and the supernatant was stored at −20°C until HPLC analysis. For anaerobic growth experiments, cells from an agar plate were suspended in 1.5 ml MMChol without substrate and transferred to 100 ml anoxic MMChol with substrates using a sterile syringe. After 2 days of static incubation at 30°C, 1 or 10 ml of this anaerobic pre-culture were used to inoculate 100 ml or 1 l, respectively, of MMChol containing bile acids. Samples were taken with syringes and processed for measurements as described above. For determination of dry mass, cells were collected in weight-constant test tubes by centrifugation of defined volumes of outgrown cultures. The cell pellets were washed twice with distilled water and dried at 70°C overnight. Alternatively, defined volumes of anaerobic cultures were collected on weight-constant polycarbonate filters with 0.2 µm pore size (Schleicher and Schuell). After cooling the tubes or the filters in a desiccator, the dry weights of the collected cells were determined. For yield calculations, the empirical formula $<C_4H_7O_3>$ for biomass was used.

## Sensitivity tests with cell suspensions

Cells were grown aerobically as described above and harvested aseptically in mid-exponential growth phase by centrifugation in 50 ml plastic tubes at 10,000×*g* for 10 min at room temperature. Cells were washed once in the same volume of MMChol without carbon source and adjusted to obtain suspensions of $OD_{600} = 1$. These suspensions were kept at 4°C and used for experiments as soon as possible. One millilitre of a cell suspension in half-micro plastic cuvettes was supplied with different concentrations (0–40 mM) of cholate to a final volume of 1.2 ml. These cell suspensions were incubated at room temperature, and $OD_{600}$ was measured at regular intervals after inverting the sample twice. The number of surviving cells after exposure towards cholate was quantified by counting colony forming units (CFU). Twenty microlitre of cholate solutions with different concentrations (0–40 mM) was filled into 96 well microtiter plates (Nunclon Surface, Nunc), mixed with 180 µl of cell suspensions ($OD_{600} = 1$), and incubated at room temperature. After 20 min, cell suspensions were decimally diluted in MMChol without carbon source. Three aliquots (15 µl) of appropriate dilution steps were used to determine CFU $ml^{-1}$ by the drop and plate method (Hoben and Somasegaran 1982). For inhibitor studies, 0.5 or 1 mM CCCP (carbonyl cyanide chlorophenylhydrazone) and 1 or 2 mM EDTA (stock solution 100 mM in water) were applied. To test the sensitivity of strain Chol1 to degradation intermediates, cells were harvested and washed as described above, but finally resuspended in filter-sterilized culture supernatants. Supernatants from completely grown anaerobic cultures were used directly (to test for sensitivity to DHADD) or after aerobic conversion of DHADD to THSATD (see below). Survival of cells after a 20 min exposure to DHADD- or THSATD-containing supernatants was determined by CFU counts. All sensitivity tests were reproduced in at least three independent experiments.

## Detection of DHADD monooxygenase activity

Cells were harvested as described above, except that the temperature was at 4°C, and washed with 50 mM potassium phosphate buffer (pH 7.1). Pellets of washed cells resulting from 600 ml culture were resuspended in 1–3 ml potassium phosphate buffer and broken by 3–5 passages through a cooled French press at 138 MPa. Homogenates were centrifuged at 17,900×*g* for 10 min at 4°C to separate the cell-free extract from cell debris. Cytosol and membrane fractions were separated by ultracentrifugation at 100,000×*g* for 60 min at 4°C. Assays to determine the conversion of DHADD to THSATD contained 50 mM Tris/HCl buffer (pH 8.0), cytosol fraction (about 1 mg protein), 0.3 mM NADH, and were started by the addition of purified DHADD (70 µM) from aqueous stock solutions. The preparation of DHADD is described below. Immediately after starting the reaction and at regular intervals thereafter, samples (100 µl) were withdrawn and stopped by addition of 10 µl HCl (1 M). After a centrifugation step, the supernatant of these samples was stored at −20°C before HPLC analysis. Protein concentration was determined by the BCA assay (Pierce, USA) with bovine serum albumin as standard.

## Preparation of degradation intermediates and products

DHADD was obtained from cultures grown anaerobically with cholate. For production of THSATD, washed cholate-grown cells were resuspended in DHADD-containing culture supernatants and incubated on a rotary mixer for 4–5 h at 30°C. Conversion of DHADD to THSATD was confirmed by HPLC analysis (see below). DHADD- and THSTAD-containing supernatants were centrifuged and, if necessary, filtered (0.2 µm pore size). Then, the supernatants were extracted twice with half their volume of dichloromethane (DCM). The DCM phase was evaporated and the solid matter was dissolved in a small volume of bidistilled water, and the desired peak was purified by HPLC (see below). HPLC fractions containing DHADD or THSATD were extracted twice with DCM. After evaporation of DCM, the compounds were dissolved in a liquid appropriate for further analysis (water for LC–MS; chloroform for IR).

### HPLC

Cholate and its degradation intermediates and products were analysed with an HPLC system consisting of two high-pressure pumps (Shimadzu LC-10ATvp), an autoinjector (Gilson 234), a $C_{18}$-reversed phase column (Grom-Sil 120 ODS, 5 μm, 150×4.6 mm; Grom), and a UV–VIS diode array detector (Shimadzu SPD-M 10) with a scanning range from 190 to 500 nm. As eluents, K/Na-phosphate buffer (10 mM, pH 7.1; eluent A) and acetonitrile (eluent B) were used, at a flow rate of 1 ml $min^{-1}$. Analysis of supernatants from growth and degradation experiments was performed isocratically (30% B). Cholate eluted after 5–5.8 min and was quantified at 210 nm with external standards (1, 5, and 10 mM). For preparation of degradation intermediates and products, a gradient method was applied, starting with 20% B for 2 min, rising to 90% B within 9 min, and returning to 20% B within 1 min, followed by 4 min equilibration.

Nitrate and nitrite were analysed with an anion-exchange column (A06, 3×120 $mm^2$, Sykam) attached to the HPLC system described above. A 40 mM NaCl solution was used as eluent at a flow rate of 1 ml $min^{-1}$. Nitrate eluted after 5.6 min and nitrite after 3.1 min. Both compounds were detected at 210 nm and quantified with external standards (0.1, 0.5, and 1 mM).

### Spectroscopical methods

LC–MS was performed on an API4000 LC/MS/MS (Applied Biosystems) using atmospheric pressure chemical ionization (APCI) in the negative ion mode. Chromatography was performed on an Agilent HP 1100 Series system using a Macherey & Nagel CC 125/2 Nucleosil 120-3 C18 column. The following isocratic elution was used: 70% ammonium acetate (10 mM, pH 7), 30% acetonitrile. Thirty microlitres were injected. Samples were analysed either in full scan mode (200–450 Da) or MS/MS mode (collision energy −30V). UV/VIS spectra were acquired either with a diode-array detector during HPLC analysis (see above) or with a double-beam spectrophotometer (Uvikon 930, Kontron Instruments). DHADD dissolved in methanol was quantified by its extinction coefficient of $\varepsilon = 14{,}860\ M^{-1}\ cm^{-1}$ (Tenneson et al. 1979). Infrared spectra were acquired with a Fourier-transform infrared spectrometer (FTS 70, BioRad) with a resolution of one wave number. Samples were dissolved in chloroform and measured in a $CaF_2$ cuvette ($d = 0.1$ mm).

### Chemicals

Sodium cholate was purchased from Fluka; all other bile acids and steroid compounds were from Sigma.

### Phylogenetic analysis

Phylogenetic analysis of strain Chol1 was performed as described earlier (Schmitt-Wagner et al. 2003).

## Results

### Isolation of *Pseudomonas* sp. strain Chol1

Anoxic enrichment cultures for nitrate-dependent degradation of deoxycholate were inoculated with soil from a meadow close to the University of Konstanz. This meadow was regularly grazed by sheep and has therefore likely been in contact with bile acids from their feces. We obtained one enrichment culture that grew slowly with deoxycholate. Upon subsequent transfers, this enrichment grew more rapidly and became a homogeneous culture of motile rod-shaped bacteria. Repeated isolation of single colonies resulted in a pure culture of a bacterium designated as strain Chol1 that grew with deoxycholate, cholate, chenodeoxycholate, or lithocholate as sole sources of carbon and energy under anoxic conditions with nitrate as electron acceptor. Strain Chol1 could also grow with the conjugated bile acids taurocholate and glycocholate. All bile acids were degraded aerobically as well. Sequence analysis of the 16S rDNA gene of strain Chol1 (gene bank accession number AY727863) showed a sequence identity of more than 99% to the species *Pseudomonas stutzeri*. A characteristic morphologic trait of strain Chol1 was the formation of rough, coherent colonies that have been described earlier for *P. stutzeri* (van Niel and Allen 1952; Palleroni et al. 1970). Therefore, strain Chol1 likely belonged to the species *P. stutzeri*.

### Anaerobic degradation

Anaerobic, nitrate-dependent growth of strain Chol1 with cholate occurred with a doubling time of ca. 11 h. Cholate was degraded concomitantly with growth and almost stoichiometric reduction of nitrate to nitrite, which accumulated before further reduction (Fig. 1). The molar growth yield with cholate was 53.9 g dry mass $(mol\ cholate)^{-1}$. When more nitrate (5 mM) was added to outgrown cultures, only a small amount of it was reduced to nitrite, and $OD_{600}$ as well as the growth yield did not increase further. Concomitant with cholate consumption, degradation products accumulated in the culture supernatant (Fig. 1). These degradation products showed very similar UV absorption spectra with maxima at 243–245 nm, which are characteristic for steroid compounds with a 3-keto-1,4-diene structure (Tenneson et al. 1979). The most prominent degradation product ($P_{6.0\ min}$) eluted after 6.0 min (Fig. 2) and had an absorption maximum at 245 nm. This product was purified from the culture supernatant and characterized

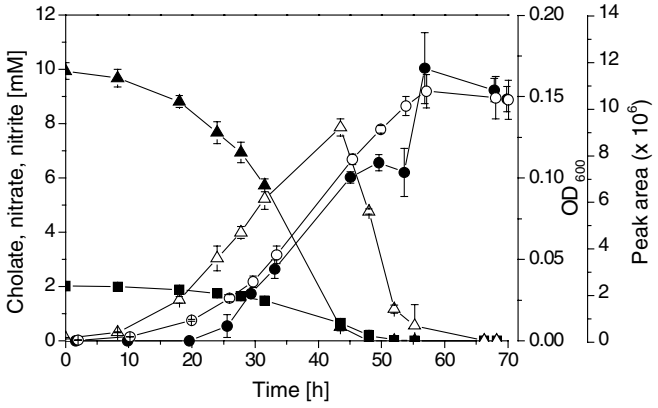


**Fig. 1** Anaerobic, nitrate-dependent growth of *Pseudomonas* sp. strain Chol1 with cholate: $OD_{600}$ (*filled circle*), cholate (*filled square*), nitrate (*filled triangle*), nitrite (*open triangle*), and DHADD (*open circle*)

further. LC–MS analysis of this compound showed a molecular ion at $m/z = 315$ ($M^-$). This mass corresponds to the formula $C_{19}H_{24}O_4$ of the uncharged molecule (M = 316 Da). A second ion of $P_{6.0\ min}$ at $m/z = 297$ indicated the loss of water and, therefore, the presence of a hydroxyl group. A third molecular ion of $P_{6.0\ min}$ with a mass of $m/z = 375$ was explainable by adduct formation with acetic acid. IR analysis of $P_{6.0\ min}$ yielded signals at wave numbers 1,660 and 1,736 $cm^{-1}$ that were indicative of the presence of two different keto groups. Comparing these IR spectra to those of the reference compounds 1,4-androstadiene-3,17-dione and testosterone showed that these keto groups have to be located at the C3 and C17 position, respectively. Taken together, the spectroscopic data suggest that $P_{6.0\ min}$ is 7,12-dihydroxy-1,4-androstadiene-3,17-dione (DHADD; Fig. 3). This conclusion is supported by the major fragments ($F^-$) of the precursor ions of $P_{6.0\ min}$ generated in MS/MS

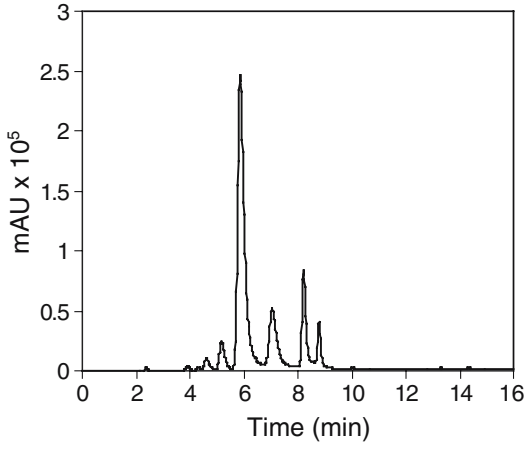


**Fig. 2** HPLC chromatogram of a culture supernatant of *Pseudomonas* sp. strain Chol1 grown anaerobically with cholate using the gradient separation program described in Materials and methods. The degradation products of cholate were detected at 244 nm wavelength. The most prominent product showed a retention time of 6.0 min and was identified as DHADD

analysis. One major fragment with $m/z = 121$ had been identified in an earlier study as containing the 1,4-diene-3-one part of 12-hydroxy-1,4-androstadiene-3,17-dione (Owen and Bilton 1983). Two major fragments with $m/z = 173$ and 157 were generated that would fit to the A- and B-rings of DHADD with two or one O-atoms, respectively. A further fragment with $m/z = 165$ would fit to the C- and D-rings, from which a further fragment of $m/z = 147$ was generated by loss of water. As axial $\alpha$-hydroxyl groups are lost as water more easily than equatorial $\beta$-hydroxyl groups (Tenneson et al. 1979; Zietz and Spiteller 1974), both hydroxyl groups of DHADD are likely to be in the $\alpha$-configuration. Based on these findings, we detected 1.1–1.2 mM DHADD in culture supernatants after complete consumption of cholate.

## Aerobic degradation

During aerobic growth with cholate, strain Chol1 had a doubling time of ca. 2.1 h. Cholate was not detectable any more in the middle of the exponential growth phase (Fig. 4). The molar growth yield was 258.7 g dry mass $(mol\ cholate)^{-1}$. Concomitant with degradation of cholate, four degradation intermediates accumulated transiently in the culture supernatant. These intermediates were degraded further during growth and were not detectable any more when the maximal $OD_{600}$ was reached. The main intermediate of aerobic degradation eluted from the HPLC column after 9.2 min ($P_{9.2\ min}$) and reached its highest concentration just when cholate had disappeared. The three minor intermediates had retention times and UV spectra identical to the products of anaerobic degradation, including DHADD. $P_{9.2\ min}$ exhibited maximal UV absorption at 279 nm, which resembled the spectra of estradiol and other steroids with a phenolic A-ring. This intermediate was purified from the culture supernatant for further characterization. Upon alkalization, the $P_{9.2\ min}$-containing supernatant turned brownish, and HPLC analysis showed that $P_{9.2\ min}$ was decomposed while several new products appeared as a result of this alkaline treatment, supporting the presence of a phenolic A-ring. In contrast, DHADD was stable under alkaline conditions. LC–MS analysis of $P_{9.2\ min}$ showed a molecular ion at $m/z = 331$ ($M^-$), corresponding to a mass of 332 Da for the uncharged molecule with the formula $C_{19}H_{24}O_5$. Two additional ions of $P_{9.2\ min}$ with $m/z = 313$ and 295 indicated the loss of two hydroxyl groups as water. Further ions of $P_{9.2\ min}$ at $m/z = 367$, 391, and 429 could be explained by adduct formation with $Cl^-$, acetate, and $H_2PO_4^-$, respectively. Compared to DHADD, $P_{9.2\ min}$ contained an additional oxygen atom. IR analysis revealed signals for keto groups at wave numbers 1,700 and 1,732 $cm^{-1}$. The latter signal was caused by the 17-keto group, which was also found in DHADD. The signal at 1,700 $cm^{-1}$ was likely to be a keto group attached to a saturated six-membered ring (Hesse et al.

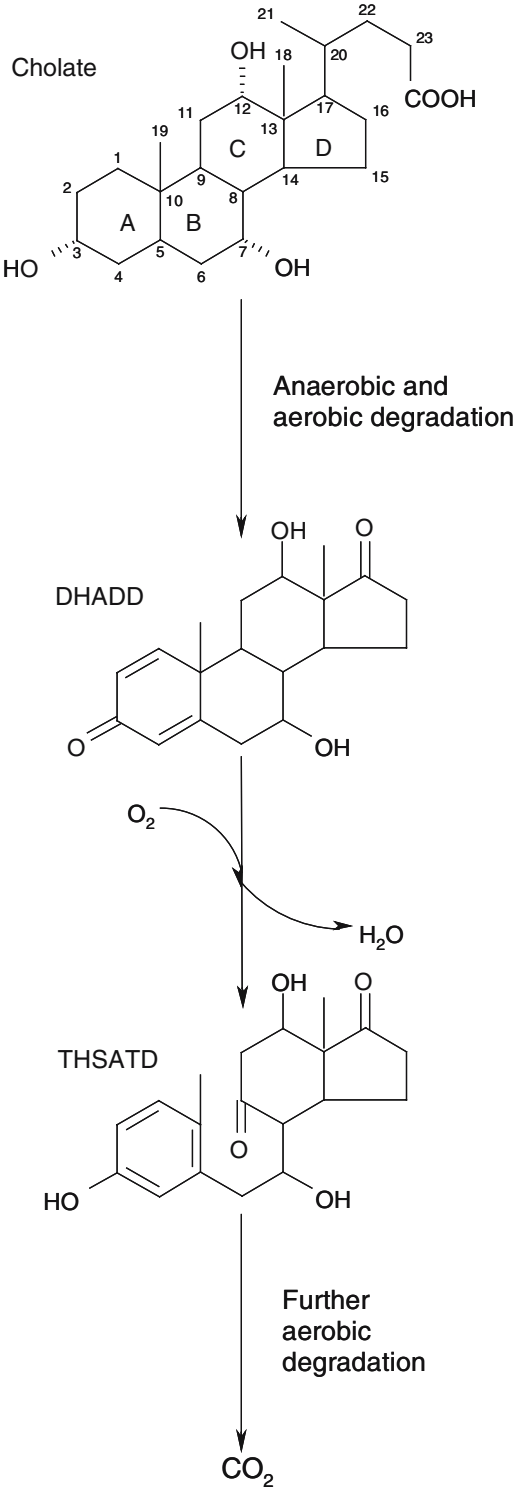


**Fig. 3** Conversion of 7,12-dihydroxy-1,4-androstadiene-3,17-dione (DHADD) to 3,7,12-trihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (THSATD). This oxygenase-dependent reaction links anaerobic with aerobic cholate degradation by *Pseudomonas* sp. strain Chol1

1995). In analogy with data from the literature, this keto group is likely to be at C9 (Sih et al. 1966). Fragments ($F^-$) generated in LC–MS/MS analysis resembled those found for phenolic 9,10-secosteroids (Park 1984). A major fragment with $m/z = 121$ was shown to contain the aromatic A-ring of different 9,10-secosteroids (Park 1984). Two fragments at $m/z = 181$ and 163 corresponded to the C- and D-rings with three or two oxygen atoms, respectively. The fragment with $m/z = 163$ was 2 Da lighter than a corresponding fragment of DHADD ($m/z = 165$), indicating a dehydrogenation of the C- or D-ring. All spectroscopic data are consistent with the hydroxylation of DHADD at the C9 position and subsequent dehydrogenation of this hydroxyl group, resulting in the cleavage of C9–C10 carbon bond concomitant with the formation of an aromatic A-ring. We therefore propose that $P_{9.2\ min}$ is 3,7,12-trihydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (THSATD; Fig. 3). As mentioned above, the easy loss of the 7,12-hydroxyl groups as water during mass spectroscopy suggests that they are in the α-configuration.

## Aerobic growth with products of anaerobic degradation

As we observed the formation of DHADD also during aerobic degradation of cholate, we wanted to investigate whether strain Chol1 could grow aerobically with the end products of anaerobic cholate degradation. For this, fully grown anaerobic cultures were aerated by transferring them from closed serum bottles into shaking flasks and incubating them like aerobic cultures. After a lag of ca. 20 h, cultures resumed growth at a rate comparable to aerobic growth with cholate (Fig. 5a). During the lag phase, the culture started to transform the products of anaerobic cholate degradation with DHADD as the main component to THSATD, the main intermediate of aerobic cholate degradation (Fig. 5b). Degradation of THSATD was concomitant with growth. To determine the growth yield, we inoculated filter-sterilized culture supernatants obtained from anaerobic cholate degradation with pre-cultures of

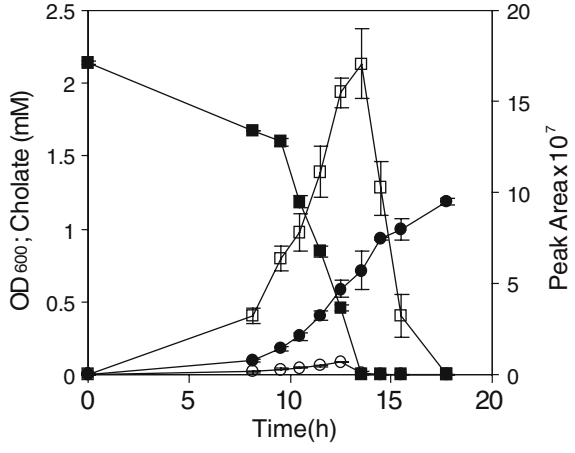


**Fig. 4** Aerobic growth of *Pseudomonas* sp. strain Chol1 with cholate: $OD_{600}$ (*filled circle*), cholate (*filled square*), DHADD (*open circle*), and THSATD (*open square*)

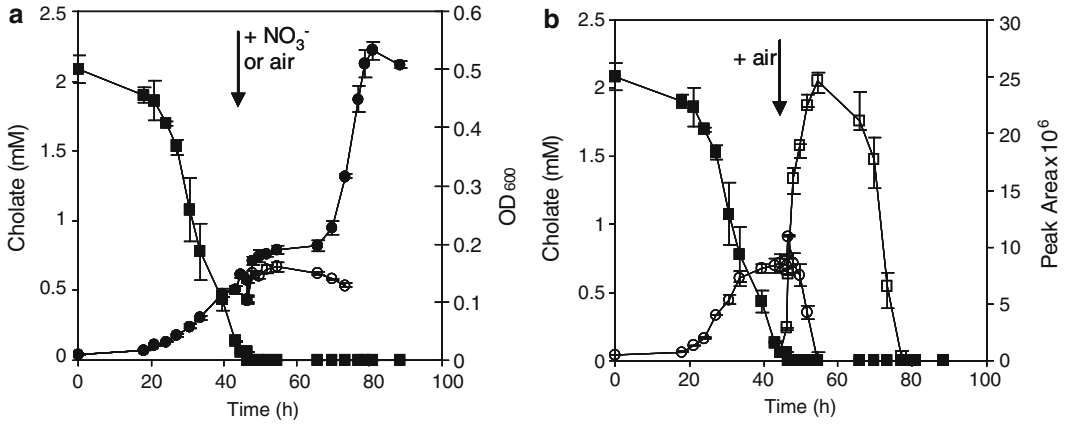


**Fig. 5** Effects of aeration or nitrate repletion on cultures of *Pseudomonas* sp. strain Chol1 grown anaerobically with cholate. The *arrows* indicate when anaerobic cultures were aerated or supplied with nitrate. **a** $OD_{600}$ of cells before (*filled circle*) and after (*open circle*) addition of nitrate (8 mM) or after aeration (*filled circle*); degradation of cholate (*filled square*). **b** Degradation of cholate (*filled square*, same values as in *a*), formation and degradation of DHADD (*open circle*), and THSATD (*open square*)

aerobically cholate-grown cells. Assuming a stoichiometric 1:1 conversion of cholate to its anaerobic degradation products, a molar growth yield of 188.3 g dry mass mol $DHADD^{-1}$ was obtained.

We followed the conversion of purified DHADD to THSATD also in cell-free extracts. The activity was dependent on molecular oxygen and NAD(P)H as electron donor, both indicative of a monooxygenase, and was localized in the cytoplasmic fraction. DHADD was degraded at a very low rate of 0.4–0.8 mU mg $protein^{-1}$, and this activity was unstable. THSATD accumulated only occasionally and was often converted further to a yellow compound with an absorption maximum at 430 nm. This compound could be the product of meta-cleavage of the aromatic A-ring of THSATD (Tai and Sih 1970), but its concentration was too low for exact identification.

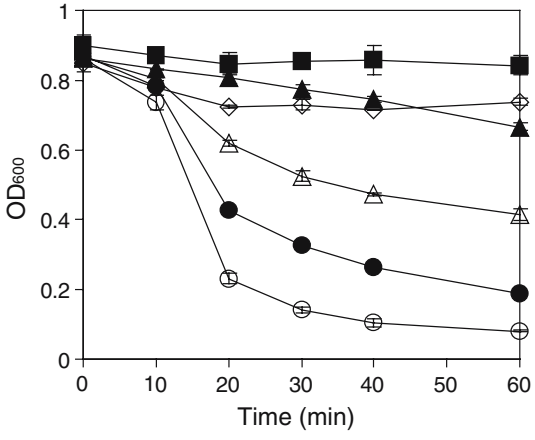


**Fig. 6** Effect of cholate at various concentrations on the optical density of cells suspensions of *Pseudomonas* sp. strain Chol1. Cholate was applied at the following concentrations (mM): 0 (*open diamond*), 2 (*filled square*), 5 (*filled triangle*), 10 (*open triangle*), 15 (*filled circle*), and 25 (*open circle*)

## Sensitivity of cell suspensions towards cholate and its degradation intermediates

We wanted to investigate whether the toxicity of cholate would influence growth with this compound as carbon and energy source. First, we had to test whether strain Chol1 was sensitive to cholate. These experiments were confined to suspensions of cells grown aerobically with cholate.

Addition of cholate at concentrations of 10 mM and above caused a decrease of the optical density to these cell suspensions (Fig. 6). In the first 10 min of incubation, the optical density remained relatively stable while it dropped rapidly in the following 10 min if cells were exposed to 10, 15, or 25 mM cholate. In all further experiments, we compared CFU counts of cell suspensions after 20 min exposure to 0 and 10 mM cholate which is below the critical micellar concentration of 13–15 mM (Helenius and Simons 1975). Exposure to 10 mM cholate caused a significant reduction (90.6%) of CFUs compared to 0 mM ($n = 63$; $P < 10^{-7}$ in independent $t$ test). Addition of the uncoupling agent CCCP (0.5 and 1 mM) increased the sensitivity to cholate and caused a reduction of CFUs between 2 and 5 orders of magnitude (not shown). Addition of EDTA (2 mM) increased the sensitivity of cholate-grown cells to cholate tremendously. Decrease of $OD_{600}$ was instantaneous upon the addition of cholate, and no CFU could be retrieved from the undiluted cell suspension (not shown). In the absence of cholate, EDTA or CCCP had no effect on $OD_{600}$ or on the number of CFU.

Further, we tested for the sensitivity of cholate-grown cells to the degradation intermediates of cholate. In the presence of EDTA (2 mM), reduction of CFU was observed by incubation of cells in culture supernatants containing DHADD or THSATD (Fig. 7). The maximum concentration that the degradation intermediates could reach in these assays was 1.7 mM. Incubation of

cells at an equivalent cholate concentration did not lead to a reduction of CFU in the presence of EDTA, indicating that the effect was caused by DHADD and THSATD rather than by residual cholate that might have been present in these supernatants. Without the addition of EDTA, CFU counts were not affected by DHADD- and THSATD-containing culture supernatants. Addition of CCCP did not cause a reduction of CFU in the presence of degradation intermediates.

### Growth with cholate at various concentrations

We tested whether strain Chol1 could grow with toxic cholate concentrations. Aerobic growth of strain Chol1 with up to 50 mM cholate was observed (Fig. 8). At 10 mM and above, lag phases were greatly prolonged and growth rates decreased. Microscopic cell aggregates and large flocs were observed in these cultures. HPLC analysis of cultures growing with 10 mM cholate showed a similar pattern of cholate consumption and product formation as during growth with 2 mM cholate (Fig. 4). Again, cholate was consumed in the middle of exponential growth, THSATD was formed, but in contrast to growth with 2 mM cholate, degradation intermediates with the characteristic absorption maximum at 245 nm accumulated to a larger extent.

## Discussion

The goals of this study were to investigate whether growth with bile acids is possible under anoxic conditions and whether the toxicity of bile acids affects growth with these compounds.

We have shown that the new bacterial isolate *Pseudomonas* sp. strain Chol1 could grow by utilizing cholate and other bile acids under anoxic conditions with nitrate as electron acceptor. Cholate was not degraded completely but transformed into the steroid compound DHADD. The identification of DHADD (as well as of THSATD) was based on UV, MS, and IR spectroscopic data, which were partly identical to those obtained in earlier studies on bacterial bile acid degradation in which DHADD (Tenneson et al. 1979; Owen and Bilton 1983) and THSATD (Park 1984) had been identified completely. Thus, although we could not perform NMR analysis due to limited quantity of material, we state that our spectroscopic data justify proposing these chemical structures to the two degradation products of cholate. The transformation of cholate to DHADD should proceed by oxidation of the A-ring and $\beta$-oxidation of the acyl side chain. As the steroid skeleton could obviously not be broken down in the absence of oxygen (see below), only the acyl side chain of cholate was available for dissimilation and assimilation under anoxic conditions. During anaerobic degradation of cholate, we detected eight products in culture supernatants that had an oxidized A-ring as indicated by the characteristic absorption maximum around 245 nm. Differences in the retention times of these products suggest that they reflected different intermediate steps in side chain removal. DHADD was by far the most abundant product, reaching concentrations of up to 1.2 mM formed in cultivation with 2 mM cholate. As this concentration is in the range of the maximal solubility of DHADD in aqueous solution, more DHADD might have been formed but did not dissolve in the medium. Assuming that all cholate is eventually oxidized to DHADD ($C_{19}H_{24}O_4$), dissimilation of cholate ($C_{24}H_{40}O_5$) proceeds according to the equation:

$$C_{24}H_{40}O_5 + 6.8NO_3^- + 6.8H^+ \rightarrow C_{19}H_{24}O_4 + 5CO_2 + 3.4N_2 + 11.4H_2O.$$

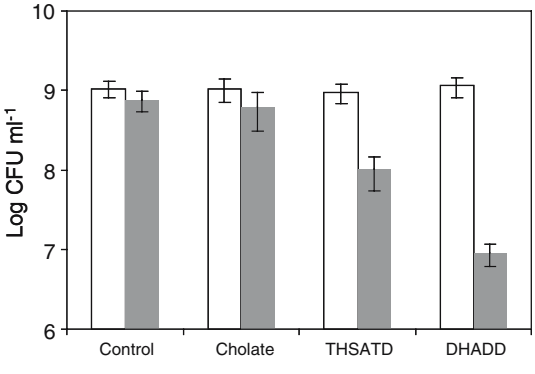


**Fig. 7** Viable counts determined by CFU after 20 min exposure of *Pseudomonas* sp. strain Chol1 cell suspensions to cholate (1.7 mM, in medium MMChol) and to culture supernatants containing THSATD or DHADD in the absence (*open bars*) and presence (*grey bars*) of EDTA (2 mM). As control, MMChol without cholate was used; ± indicates standard deviation ($n = 6$)

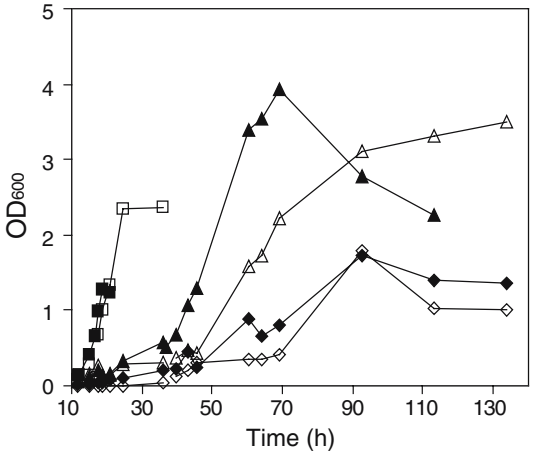


**Fig. 8** Growth of *Pseudomonas* sp. strain Chol1 with cholate at the following concentrations (mM): 2 (*filled square*), 5 (*open square*), 10 (*filled triangle*), 15 (*open triangle*), 25 (*filled diamond*), and 50 (*open diamond*)

To assimilate the acyl side chain for the production of biomass ($<C_4H_7O_3>$), cholate has to be oxidized to DHADD according to the equation:

$$C_{24}H_{40}O_5 + 3.4NO_3^- + 3.4H^+ \rightarrow C_{19}H_{24}O_4 + <C_4H_7O_3> + CO_2 + 1.7N_2 + 6.2H_2O.$$

Based on yield determinations, 51.3% of cholate is oxidized to DHADD for energy production, and the remaining 48.7% is oxidized to DHADD for assimilation. The overall anaerobic growth with cholate thus proceeds according to the equation:

$$C_{24}H_{40}O_5 + 5.144NO_3^- + 5.144H^+ \rightarrow C_{19}H_{24}O_4 + 0.487<C_4H_7O_3> + 3.052CO_2 + 2.572N_2 + 9H_2O.$$

This equation indicates a cholate to nitrate stoichiometry of 1:5. In our growth experiments, 10 mM nitrate was required to oxidize 2 mM cholate to DHADD. Thus, this assumed equation is supported by experimental data. This ratio of cholate and nitrate also agrees with the proposed metabolic pathway: oxidation of 51.3% of cholate for energy production is equivalent to a release of 17.43 mol electrons (3.07 from the oxidation of A-ring and 14.36 from the complete oxidation of the acyl side chain). For assimilation of the remaining 48.7% of cholate, 8.279 mol electrons are released because cholate is more reduced than the biomass. Altogether, 25.725 mol electrons are derived from anaerobic growth with cholate and match exactly the required amount for complete reduction of 5.144 mol nitrate to $N_2$. Assuming a complete conversion of cholate to DHADD, the electron recovery including biomass was 97.2%. To our knowledge, this is the first quantitative study demonstrating anaerobic growth of a bacterium by transformation of a bile acid.

Aerobic degradation also involved the transient release of DHADD and further compounds with an oxidized A-ring, and we conclude that the pathways for anaerobic and aerobic degradations start with identical reaction steps (Fig. 3). Anaerobic degradation was limited to the formation of DHADD because its further degradation to THSATD depended on an oxygenase-dependent reaction step which we detected in the soluble fraction of the cell-free extract. Such 9α-steroid-hydroxylases have been found in Gram-positive bacteria (Strijewski 1982; van der Geize et al. 2002). Altogether, aerobic cholate degradation by strain Chol1 appears to proceed through the route that Hayakawa (1982) proposed for Gram-negative bacteria.

Analysis of the degradation pathway was a prerequisite to approach our second goal, namely the question whether the bacteria were affected by the toxic effects of cholate while using it as carbon and energy source. Clearly, cholate was toxic to strain Chol1 because it caused cell lysis at concentrations of 10 mM and above. However, growth with cholate at such toxic concentrations was possible, and, consequently, strain Chol1 must possess resistance mechanisms to overcome these toxic effects. Our sensitivity tests suggested that the outer membrane and the proton motive force were essential for resistance against cholate. Complete lysis of cholate-grown cells occurred in such experiments when EDTA was added, which is known to destabilize the outer membrane of Gram-negative bacteria (Hancock 1984). Strong lysis occurred when the proton motive force was abolished by the uncoupling agent CCCP, and this detrimental effect hints at the importance of proton-dependent efflux pumps. These findings show that strain Chol1 requires very similar protection mechanisms against bile acids as those bacteria that live in the intestinal tract (Gunn 2000) and support our hypothesis that growth with bile acids requires an additional energetic investment.

In the presence of EDTA, DHADD and THSATD were apparently even more toxic for strain Chol1 than cholate because equimolar amounts of cholate (1.7 mM) did not affect viable counts. It has been reported that the diffusion of uncharged steroids through the outer membrane can be enhanced by EDTA (Plesiat and Nikaido 1992), and that androstadienediones are toxic for bacteria although their exact mode of action is not known yet (Lee and Liu 1992; Perez et al. 2003). The toxicity of DHADD and THSTAD could affect growth with cholate because the enzymes for further metabolism of these degradation intermediates were obviously located in the cytoplasm meaning that sensitive parts of the cells are inevitably exposed to these compounds. Therefore, the observed kinetics of aerobic cholate degradation could be the outcome of an adaptive strategy to keep the intracellular pool of these toxic steroid metabolites low: cholate is quickly converted into DHADD and further to THSATD which is extracellularly stored before its further degradation (Fig. 4). This hypothesis is supported by the observation that if DHADD was provided as substrate, growth did not commence until all of it had been converted to THSATD which accumulated in the medium before it was degraded (Fig. 5a, b). Such extracellular accumulation of uncharged steroids has been observed in other studies on bile acid (Smith and Park 1984; Tenneson et al. 1979) or testosterone degradation (Horinouchi et al. 2003), but it remains to be investigated whether this phenomenon is based on simple diffusion or on active efflux.

In natural environments, the release of androstenediones or secosteroids during bile acid degradation could have an ecotoxicological impact because accumulation of hormonally active compounds in the environment is believed to influence mammalian fertility (Colborn 2004). It has been shown that androstenediones can act as androgens that might contribute to the masculinization of fish (Jenkins et al. 2003). In conclusion, our physiological study has now paved the way for molecular investigations to elucidate the interplay of the

degradation of and the sensitivity to cholate and its steroid degradation intermediates with its link to ecological aspects.

**Acknowledgements** The authors like to thank Antje Karst and René Schoenenberger for excellent technical assistance and Normen Szesni, Dirk Schmitt-Wagner, Melanie Hempel, and Steffi Schneider for help with some experiments. Janosch Klebensberger is acknowledged for valuable discussions. Financial support to B.P. by the Deutsche Forschungsgemeinschaft (project PH71/2-1) is acknowledged.

## References

Barnes PJ, Bilton RF, Mason AN, Fernandez F, Hill MJ (1975) The coupling of anaerobic steroid dehydrogenation to nitrate reduction in *Pseudomonas* N.C.I.B. 10590 and *Clostridium paraputrificum*. Biochem Soc Trans 3:299–301

Bortolini O, Medici A, Poli S (1997) Biotransformations on steroid nucleus of bile acids. Steroids 62:564–577

Colborn T (2004) Endocrine disruption overview: are males at risk? Adv Exp Med Biol 545:189–201

van der Geize R, Hessels GI, van Gerwen R, van der Meijden P, Dijkhuizen L (2002) Molecular and functional characterization of *kshA* and *kshB*, encoding two components of 3-ketosteroid 9alpha-hydroxylase, a class IA monooxygenase, in *Rhodococcus erythropolis* strain SQ1. Mol Microbiol 45:1007–1018

Gunn JS (2000) Mechanisms of bacterial resistance and response to bile. Microbes Infect 2:907–913

Hancock RE (1984) Alterations in outer membrane permeability. Annu Rev Microbiol 38:237–264

Hayakawa S (1982) Microbial transformation of bile acids. A unified scheme for bile acid degradation, and hydroxylation of bile acids. Z Allg Mikrobiol 22:309–326

Helenius A, Simons K (1975) Solubilization of membranes by detergents. Biochim Biophys Acta 415:29–79

Hesse M, Meier H, Zeeh B (1995) Spektroskopische Methoden in der organischen Chemie, 5th edn. Thieme, Stuttgart

Hoben HJ, Somasegaran P (1982) Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl Environ Microbiol 44:1246–1247

Horinouchi M, Hayashi T, Yamamoto T, Kudo T (2003) A new bacterial steroid degradation gene cluster in *Comamonas testosteroni* TA441 which consists of aromatic-compound degradation genes for seco-steroids and 3-ketosteroid dehydrogenase genes. Appl Environ Microbiol 69:4421–4430

Hylemon PB, Harder J (1998) Biotransformation of monoterpenes, bile acids, and other isoprenoids in anaerobic ecosystems. FEMS Microbiol Rev 22:475–488

Jenkins RL, Wilson EM, Angus RA, Howell WM, Kirk M (2003) Androstenedione and progesterone in the sediment of a river receiving paper mill effluent. Toxicol Sci 73:53–59

Lee CY, Liu WH (1992) Production of androsta-1,4-diene-3,17-dione from cholesterol using immobilized growing cells of *Mycobacterium* sp. NRRL B-3683 adsorbed on solid carriers. Appl Microbiol Biotechnol 36:598–603

Leppik RA, Park RJ, Smith MG (1982) Aerobic catabolism of bile acids. Appl Environ Microbiol 44:771–776

van Niel CB, Allen MB (1952) A note on *Pseudomonas stutzeri*. J Bacteriol 64:413–422

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656

Owen RW, Bilton RF (1983) The degradation of cholic acid by *Pseudomonas* sp. N.C.I.B. 10590 under anaerobic conditions. Biochem J 216:641–654

Palleroni NJ, Doudoroff M, Stanier RY, Solanes RE, Mandel M (1970) Taxonomy of the aerobic pseudomonads: the properties of the *Pseudomonas stutzeri* group. J Gen Microbiol 60:215–231

Park RJ (1984) Phenolic 9,10-secosteroids as products of the catabolism of bile acids by a *Pseudomonas* sp. Steroids 44:175–193

Park RJ, Dunn NW, Ide JA (1986) A catecholic 9,10-seco steroid as a product of aerobic catabolism of cholic acid by a *Pseudomonas* sp. Steroids 48:439–450

Perez C, Falero A, Llanes N, Hung BR, Herve ME, Palmero A, Marti E (2003) Resistance to androstanes as an approach for androstandienedione yield enhancement in industrial mycobacteria. J Ind Microbiol Biotechnol 30:623–626

Plesiat P, Nikaido H (1992) Outer membranes of gram-negative bacteria are permeable to steroid probes. Mol Microbiol 6:1323–1333

Poole K (2004) Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol Infect 10:12–26

Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A (2003) Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). Appl Environ Microbiol 69:6007–6017

Sih CJ, Lee SS, Tsong YY, Wang KC (1966) Mechanisms of steroid oxidation by microorganisms. 8. 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione, an intermediate in the microbiological degradation of ring A of androst-4-ene-3,17-dione. J Biol Chem 241:540–550

Smith MG, Park RJ (1984) Effect of restricted aeration on catabolism of cholic acid by two *Pseudomonas* species. Appl Environ Microbiol 48:108–113

Strijewski A (1982) The steroid-9 alpha-hydroxylation system from *Nocardia* species. Eur J Biochem 128:125–135

Tai HH, Sih CJ (1970) 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-dioxygenase from *Nocardia restrictus*. I. Isolation of the enzyme and study of its physical and chemical properties. J Biol Chem 245:5062–5071

Tenneson ME, Owen RW, Mason AN (1977) The anaerobic side-chain cleavage of bile acids by *Escherichia coli* isolated from human faeces. Biochem Soc Trans 5:1758–1760

Tenneson ME, Baty JD, Bilton RF, Mason AN (1979) The degradation of cholic acid by *Pseudomonas* sp. N.C.I.B. 10590. Biochem J 184:613–618

Thanassi DG, Cheng LW, Nikaido H (1997) Active efflux of bile salts by *Escherichia coli*. J Bacteriol 179:2512–2518

Widdel F, Pfennig N (1981) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. Arch Microbiol 129:395–400

Widdel F, Kohring GW, Mayer F (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. Arch Microbiol 134:286–294

Zietz E, Spiteller G (1974) Zur Lokalisierung funktioneller Gruppen mit Hilfe der Massenspektrometrie-XI. Tetrahedron 30:585–596