# Anaerobic and aerobic cleavage of the steroid core ring structure by *Steroidobacter denitrificans*[S]

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**Abstract** The aerobic degradation of steroids by bacteria has been studied in some detail. In contrast, only little is known about the anaerobic steroid catabolism. *Steroidobacter denitrificans* can utilize testosterone under both oxic and anoxic conditions. By conducting metabolomic investigations, we demonstrated that *S. denitrificans* adopts the 9,10-seco-pathway to degrade testosterone under oxic conditions. This pathway depends on the use of oxygenases for oxygenolytic ring fission. Conversely, the detected degradation intermediates under anoxic conditions suggest a novel, oxygenase-independent testosterone catabolic pathway, the 2,3-seco-pathway, which differs significantly from the aerobic route. In this anaerobic pathway, testosterone is first transformed to 1-dehydrotestosterone, which is then reduced to produce 1-testosterone followed by water addition to the C-1/C-2 double bond of 1-testosterone. Subsequently, the C-1 hydroxyl group is oxidized to produce 17-hydroxy-androstan-1,3-dione. The A-ring of this compound is cleaved by hydrolysis as evidenced by $H_2^{18}O$-incorporation experiments. Regardless of the growth conditions, testosterone is initially transformed to 1-dehydrotestosterone. This intermediate is a divergence point at which the downstream degradation pathway is governed by oxygen availability.[S] Our results shed light into the previously unknown cleavage of the sterane ring structure without oxygen. We show that, under anoxic conditions, the microbial cleavage of steroidal core ring system begins at the A-ring.—Wang, P-H., Y-L. Leu, W. Ismail, S-L. Tang, C-Y. Tsai, H-J. Chen, A-T. Kao, and Y-R. Chiang. **Anaerobic and aerobic cleavage of the steroid core ring structure by *Steroidobacter denitrificans*. *J. Lipid Res.* 2013.** 54: 1493–1504.

**Supplementary key words** steroid biodegradation • anaerobic metabolism • denitrifying bacteria • metabolomics • testosterone

Steroids are ubiquitous and abundant in nature. Considering their divergent functions, the structural similarity of steroids is remarkable. Mammals are unable to degrade steroids. After a modification (e.g., glucuronide and sulfate conjugations) to enhance the solubility, steroid hormones are excreted into the environment through the urinary tract of mammals (1). In addition, large amounts of steroid drugs are released from the pharmaceutical industry as environmental pollutants (2). Members of androgens and estrogens have been detected in a number of effluents of wastewater treatment plants and rivers at concentrations in the ng $l^{-1}$ range (3–6). Because of the negative environmental effects of steroid hormones, the removal of these compounds from the environment has attracted considerable interest (7–9).

The biotransformation of steroids by microorganisms is a crucial example of the successful application of microbial technology in industrial processes (10). Several species of bacteria, such as *Comamonas testosteroni*, can degrade testosterone under oxic conditions. In 1968, Coulter and Talalay (11) established the oxygenase-dependent pathway (9,10-seco-pathway; **Fig. 1**) for the degradation of testosterone by aerobes. The aerobic testosterone degradation by *C. testosteroni* starts with the oxidation of the C-17 hydroxyl group and the introduction of a double bond at C-1/C-2. Subsequently, hydroxylation at C-9 occurs. The resulting compound, 9α-hydroxy-androsta-1,4-diene-3,17-dione, is unstable and undergoes spontaneous aromatization of the A-ring and nonenzymatic cleavage of the B-ring. The aromatized A-ring is subsequently split through the *meta*-cleavage. Investigations of the details of
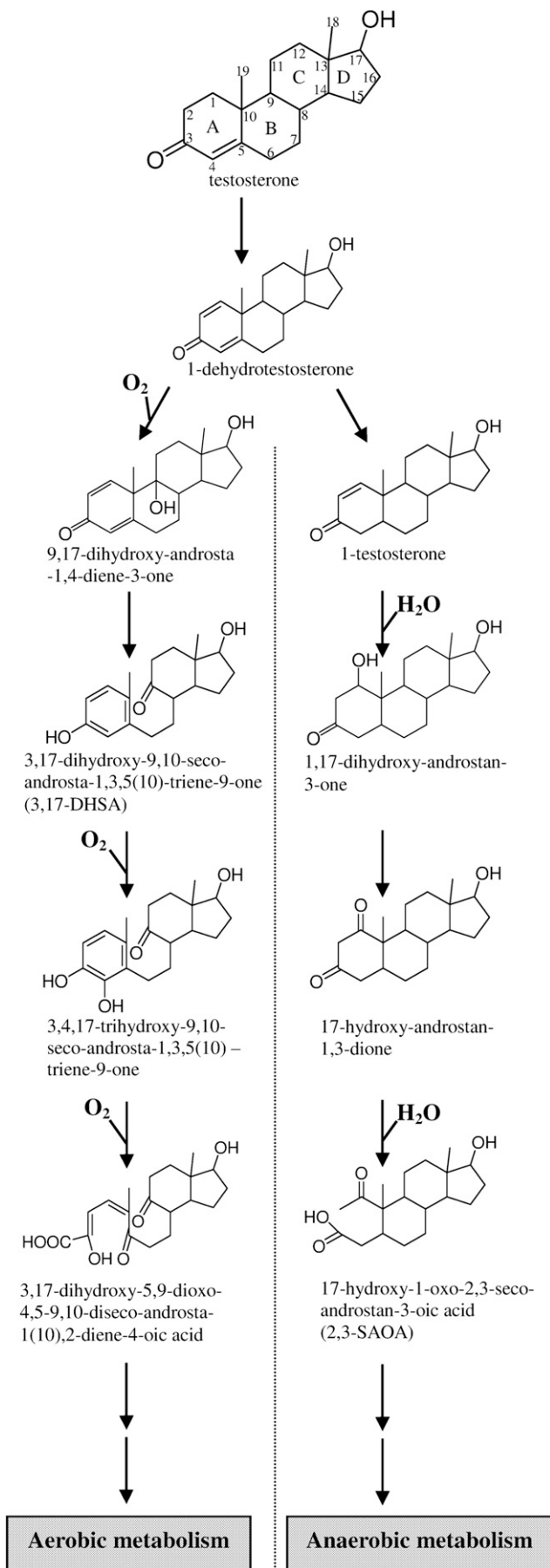
Abbreviations: APCI, atmosphere pressure chemical ionization; 2D, two-dimensional; 3,17-DHSA, 3,17-dihydroxy-9,10-seco-androsta-1,3,5(10)-triene-9-one; HMBC, heteronuclear multiple bond coherence; 2,3-SAOA, 17-hydroxy-1-oxo-2,3-seco-androstan-3-oic acid; HRMS, high-resolution mass spectrometry; UPLC, ultra-performance liquid chromatography

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one figure and one table.

Fig. 1. The proposed aerobic and anaerobic catabolic pathways of testosterone demonstrated in *S. denitrificans* DSMZ18526. All these testosterone-derived intermediates were also observed as their 17-keto structures. The ring identification (A–D) and carbon numbering systems (1–19) are shown in testosterone.

the aerobic steroid catabolic pathway are in progress. Overall, during the aerobic degradation of testosterone, three reactions are catalyzed by oxygenases. In addition to testosterone, cholesterol and phytosterols are also degraded through the common 9,10-seco-pathway, with C19 androgens as the intermediates (12).

By contrast, information on the biochemical and molecular details of anaerobic steroid degradation is very limited. Steroids, especially sterols, may remain in anoxic sediments over hundreds of millions of years (13, 14), indicating that steroids are not degraded facilely by anaerobes. Obviously, anaerobes must use a novel, oxygenase-independent catabolic strategy to degrade steroids in the absence of oxygen. Denitrifying bacteria are facultative aerobes that can use various aromatic compounds and terpenoids as sole sources of carbon and energy; thus, they play a crucial role in carbon cycling in the environment. In the last decade, a few denitrifying bacteria that can anaerobically mineralize steroids were isolated and characterized (15–18). Among them, *S. denitrificans* DSMZ18526 has an unusual ability to degrade testosterone under both oxic and anoxic conditions. The Blast results showed that *S. denitrificans* strains are widely distributed in diverse oxic and anoxic ecosystems, e.g., agriculture soil, bioremediated soil, anoxic sediment, activated sludge, and anoxic sludge (supplementary Fig. I).

Recently, the initial reactions involved in the anaerobic metabolism of cholesterol and testosterone were reported (19–21), albeit the ring cleavage details of the anaerobic pathways are yet to be unraveled. In this study, we adopted a $^{13}$C metabolomic approach to investigate the anaerobic degradation of testosterone using *S. denitrificans* as a model organism. The aerobic testosterone degradation by the same model organism was also studied for comparison. The results obtained shed light into the previously unknown cleavage of the sterane ring structure without oxygen. To our knowledge, this is the first study showing that under anoxic conditions, the microbial cleavage of steroidal core ring system begins at the A-ring.

## MATERIALS AND METHODS

### Chemicals and bacterial strain

The [2,3,4C-$^{13}$C]testosterone was purchased from Isosciences. The chemicals were analytical grade and were purchased from Mallinckrodt Baker, Merck, or Sigma-Aldrich. *Steroidobacter denitrificans* DSMZ18526 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany).

TABLE 1.   UPLC-HRMS analysis of the intermediates involved in aerobic testosterone catabolism by *S. denitrificans*

| Compound ID | UPLC Behavior (RT, min) | Molecular Formula (Predicted Molecular Mass)[a] | Dominant Ion Peaks | Identification of Product Ions | Mode Observed |
|---|---|---|---|---|---|
| Testosterone | 6.15 | $C_{19}H_{28}O_2$ 288.2082 | 271.2059 289.2161 311.1983 | $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI |
| Androst-4-en-3,17-dione | 5.91 | $C_{19}H_{26}O_2$ 286.1926 | 269.1909 287.2023 309.1831 | $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI |
| 1-Dehydrotestosterone | 5.77 | $C_{19}H_{26}O_2$ 286.1926 | 269.1903 287.2009 309.1823 | $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI |
| Androsta-1,4-diene-3,17-dione | 5.45 | $C_{19}H_{24}O_2$ 284.1770 | 267.1742 285.1855 307.1666 | $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI |
| 3,17-Dihydroxy-9,10-seco-androsta-1,3,5 (10)-triene-9-one(3,17-DHSA) | 5.53 | $C_{19}H_{26}O_3$ 302.1875 | 267.1740 285.1844 303.1946 325.1766 | $[M-2H_2O+H]^+$ $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI and APCI ESI |
| 3-Hydroxy-9,10-seco-androsta-1,3,5 (10)-triene-9,17-dione | 5.35 | $C_{19}H_{24}O_3$ 300.1719 | 283.1667 301.1801 323.1625 | $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI |
| 3,4,17-Trihydroxy-9,10-seco-androsta-1,3,5 (10)-triene-9-one | 4.99 | $C_{19}H_{26}O_4$ 318.1824 | 301.1762 319.1958 341.1791 | $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI |
| 3,4-Dihydroxy-9,10-seco-androsta-1,3,5 (10)-triene-9,17-dione | 4.50 | $C_{19}H_{24}O_4$ 316.1668 | 299. 1608 317.1831 339. 1592 | $[M-H_2O+H]^+$ $[M+H]^+$ $[M+Na]^+$ | ESI and APCI ESI and APCI ESI |

RT, retention time.
[a]The predicated molecular mass was calculated using the atom mass of $^{12}C$ (12.0000), $^{16}O$ (15.9949), and $^1H$ (1.0078).

## Anaerobic in vivo transformation of [2,3,4C-$^{13}$C] testosterone

*S. denitrificans* was grown with 2 mM of unlabeled testosterone in a 250 ml glass bottle. After the unlabeled testosterone was completely consumed, 10 ml of the anoxic culture was transferred into a 12 ml glass bottle sealed with a rubber stopper. The *S. denitrificans* cells were subsequently fed with 2 mM testosterone (unlabeled testosterone and [2,3,4C-$^{13}$C]testosterone were mixed in a 1:1 molar ratio) under denitrifying conditions. The samples (1 ml) were withdrawn after 10 min, 6 h, and 12 h of incubation at 28°C. After the second sampling (6 h), 0.5 mM of mercaptopropionic acid [an inhibitor of acyl-CoA dehydrogenase (22)] was immediately added to the anoxic culture. The culture samples were immediately extracted three times with the same volume of ethyl acetate to recover testosterone-derived intermediates. The ethyl acetate fractions were combined, the solvent was evaporated, and the residue was redissolved in 100 μl of methanol. The testosterone-derived intermediates were identified using UPLC-HRMS.

## Anaerobic growth of *S. denitrificans* with unlabeled testosterone

The *S. denitrificans* was grown with 4 mM of unlabeled testosterone at 28°C in anoxic fed-batch cultures (2 l) according to published procedures (20). The amounts of residual testosterone in the anoxic culture were monitored using HPLC. After the consumption of 2 mM testosterone, 0.5 mM mercaptopropionic acid was added to the cultures, and incubation was continued for an additional 12 h. The cultures were subsequently extracted three times with the same volume of ethyl acetate to recover residual testosterone and its derivatives from the aqueous phase. Separation of ethyl acetate extracts was performed using silica gel chromatography, TLC, and HPLC. The structures of HPLC-purified intermediates were determined using NMR spectroscopy.

## Aerobic growth of *S. denitrificans* with testosterone

The *S. denitrificans* was grown in phosphate-buffered shake-flask cultures (500 ml in 2 l Erlenmeyer flasks) containing 4 mM testosterone. The cultures were incubated at 28°C in an orbital

TABLE 2.   $^1$H-NMR chemical shifts ($\delta_H$, ppm) of ring cleavage intermediates involved in aerobic or anaerobic testosterone catabolic pathways in *S. denitrificans* cells

| $^1$H | 3,17-DHSA | 2,3-SAOA |
|---|---|---|
| 1 | 6.99 (1H, d, $J$ = 8.0 Hz) | – |
| 2 | 6.59 (1H, dd, $J$ = 8.0, 2.8 Hz) | 2.18 (3H, s) |
| 3 | – | – |
| 4 | 6.66 (1H, d, $J$ = 2.8 Hz) | 1.88 (1H, m) 1.73 (1H, m) |
| 5 | – | 2.31 (1H, m) |
| 6 | 2.66 (1H, ddd, $J$ = 13.2, 4.8, 4.8 Hz) 2.43 (1H, m) | 1.78 (1H, m) 1.23 (1H, m) |
| 7 | 1.79 (1H, m) 1.58 (1H, m) | 1.98 (1H, m) 1.73 (1H, m) |
| 8 | 2.41 (1H, m) | 1.36 (1H, m) |
| 9 | – | 1.47 (1H, m) |
| 10 | – | – |
| 11 | 2.52 (1H, m) 2.37 (1H, m) | 1.62 (1H, m)[a] 1.25 (1H, m) |
| 12 | 2.02 (1H, ddd, $J$ = 6.8, 6.0, 2.0 Hz) 1.53 (1H, m) | 1.74 (1H, m) 1.08 (1H, td, $J$ = 13.2, 4.0 Hz) |
| 13 | – | – |
| 14 | 1.61 (1H, m) | 1.03 (1H, m) |
| 15 | 1.71 (1H, m) 1.51 (1H, m) | 1.38 (1H, m)[a] 0.90 (1H, m) |
| 16 | 2.19 (1H, m) 1.65 (1H, m) | 1.75 (1H, m) 1.03 (1H, m) |
| 17 | 3.76 (1H, t, $J$ = 8.4 Hz) | 3.57 (1H, t, $J$ = 8.7 Hz) |
| 18 | 1.10 (3H, s) | 0.70 (3H, s) |
| 19 | 2.25 (3H, s) | 0.92 (3H, s) |

[a]The signals of H-11 and H-15 of 2,3-SAOA are undistinguishable.

TABLE 3. $^{13}$C-NMR chemical shifts ($\delta_C$, ppm) of ring cleavage intermediates involved in aerobic or anaerobic testosterone catabolic pathways in *S. denitrificans* cells

| $^{13}$C | 3,17-DHSA | 2,3-SAOA |
|---|---|---|
| 1 | 131.4 | 216.8 |
| 2 | 113.0 | 25.5 |
| 3 | 154.1 | 176.6 |
| 4 | 116.1 | 40.9 |
| 5 | 142.7 | 42.5 |
| 6 | 31.5 | 28.0 |
| 7 | 27.6 | 30.4 |
| 8 | 50.9 | 36.1 |
| 9 | 212.7 | 50.5 |
| 10 | 128.4 | 57.1 |
| 11 | 38.4 | 24.2[a] |
| 12 | 35.9 | 37.6 |
| 13 | 43.7 | 44.1 |
| 14 | 50.1 | 52.0 |
| 15 | 24.5 | 24.2[a] |
| 16 | 31.5 | 32.1 |
| 17 | 80.8 | 82.4 |
| 18 | 11.1 | 11.6 |
| 19 | 18.7 | 9.9 |

[a]The signals of C-11 and C-15 of 2,3-SAOA are undistinguishable.

shaker (180 rpm). After the consumption of 2 mM testosterone, 1 mM of 3-chlorocatechol [a *meta*-cleavage inhibitor (23)] was added to the cultures, and incubation continued for an additional 12 h. The cultures were extracted using ethyl acetate, and testosterone-derived intermediates present in the extract were analyzed using UPLC-HRMS. Separation of ethyl acetate extracts was performed using silica gel chromatography, TLC, and HPLC.

### *S. denitrificans* grown under various concentrations of oxygen

A *S. denitrificans* culture (500 ml) was first anaerobically grown on 2 mM testosterone. After testosterone and its derivatives were completely consumed, 50 ml of the preculture was mixed with 450 ml of fresh phosphate-buffered medium (pH 7.0) containing 2.2 mM testosterone, 10 mM NH$_4$Cl (the nitrogen source), and 10 mM NaNO$_3$ (the potential electron acceptor). The resulting cultures (100 ml) were transferred to five 1 l glass bottles sealed with rubber stoppers and were incubated under various concentrations of oxygen [headspace (900 ml); 0, 2.5, 5, 10, and 20% (v/v)]. The culture containing 20% oxygen in headspace was prepared in air. The remaining four cultures were prepared in an anaerobic chamber containing 95% nitrogen and 5% hydrogen gas. Oxygen gas was injected into the headspace after passing through a 0.22 μm membrane filter (Millipore). The fed-batch cultures were incubated at 28°C with shaking (180 rpm). Samples (3 ml) were retrieved every 4 h to measure the growth of bacterial cells (measured as total proteins), the residual amount of nitrate and testosterone, and the production of ring cleavage intermediates (3,17-DHSA and 2,3-SAOA). NaNO$_3$ was added continuously to 10 mM when the nitrate added initially was consumed. After the consumption of 1 mM testosterone, 0.5 mM mercaptopropionic acid and 1 mM 3-chlorocatechol were added to the cultures, and incubation was continued for an additional 12 h.

### Measurement of protein content and nitrate

The protein content in the culture samples and in cell extracts was determined using a BCA protein assay according to manufacturer's instructions, with BSA as the standard. Nitrate was determined by using the 2,6-dimethylphenol photometric method as described elsewhere (21).

### Silica gel chromatography

A 385 ml silica gel column (55 × 3 cm; SiliaFlash P60; Silicycle) was equilibrated with 2 bed volumes of dichloromethane/ethyl acetate/ethanol (14:4:1, v/v). The ethyl acetate extract (approximately 350 mg dissolved in 3 ml ethyl acetate) was loaded to the column and eluted with the same solvent system at a flow rate of 2 ml min$^{-1}$. The eluate was collected in 5 ml fractions, and a 0.5 ml sample was withdrawn from each fraction. The solvent was evaporated until dry, and the residue was redissolved in 10 μl of methanol. The samples after silica gel chromatography (SGC) were analyzed using TLC. The fractions that contained the same compounds were pooled and evaporated to dryness, and 200 μl of methanol was used to redissolve the residue. Further purification of testosterone-derived intermediates was performed using TLC.

### Thin layer chromatography

The steroid standards and products were separated on silica gel aluminum TLC plates (Silica gel 60 F$_{254}$, thickness, 0.2 mm, 20 × 20 cm; Merck). The following developing solvent system was used: dichloromethane/ethyl acetate/methanol (14:4:1, v/v). The steroid compounds were visualized under UV light at 254 nm or by spraying the TLC plates with 30% (v/v) H$_2$SO$_4$.

### High-performance liquid chromatography

A reversed-phase Hitachi high-performance liquid chromatography (HPLC) system was used for the final separation. The separation was achieved on an analytical RP-C$_{18}$ column [Luna 18 (2), 5 μm, 150 × 4.6 mm; Phenomenex] with a flow rate of 0.5 ml min$^{-1}$. The separation was performed isocratically at room temperature with 50% (v/v) methanol as an eluent. The steroid products were detected in the range of 200–300 nm using a photodiode array detector. In addition, HPLC was used for the quantification of steroids present in the *S. denitrificans* cultures. The quantity of steroids (testosterone, 3,17-DHSA, and 2,3-SAOA) was calculated from their respective peak areas using a standard curve of individual standards. The $R^2$ values for the standard curves were greater than 0.98. Data are averages of three measurements.

### $^{18}$O-Incorporation experiments

The denitrifying growth of *S. denitrificans* with testosterone and the preparation of cell extracts were performed as previously described (21). To determine the origins of the oxygen atoms at C-1 and/or C-3 of 17-hydroxy-androstan-1,3-dione and 17-hydroxy-1-oxo-2,3-seco-androstan-3-oic acid (2,3-SAOA), two in vitro assays were performed. The two reaction mixtures (3 ml for each assay) were prepared anaerobically and were incubated at 30°C for 16 h with shaking. The steroid products were extracted from the assays using ethyl acetate, and the extracts were analyzed using UPLC-APCI-mass spectrometry.

*Control assay.* The 3 ml reaction mixture contained 50 mM Tris-HCl buffer (pH 7), soluble proteins (15 mg) of *S. denitrificans*, 0.5 mM mercaptopropionic acid, and 200 μl of 67.5 mM 1-testosterone solution (in 2-propanol). The final concentration of the steroid substrate in the reaction mixture was 4.5 mM. The final 2-propanol content was 6.67%.
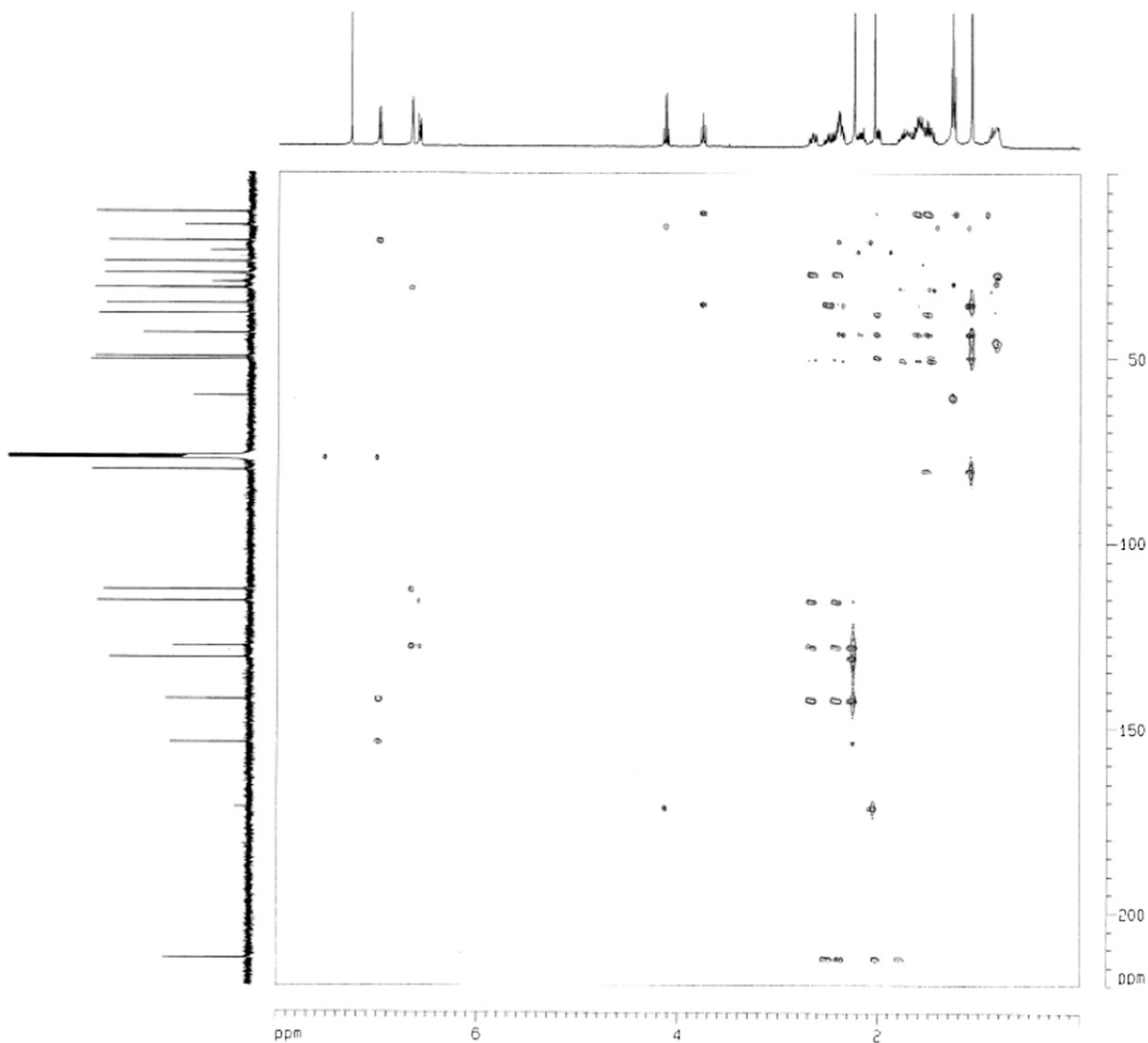
**Fig. 2.** HMBC spectrum of the characteristic intermediate (3,17-DHSA) involved in aerobic testosterone catabolism by *S. denitrificans*.

<sup>18</sup>*O-Labeled water-treated assay.* A total of 1.5 ml of ^{18}O-labeled water (97 atom %, Aldrich) was added to 1.5 ml of 100 mM Tris-HCl buffer (pH 7) containing soluble proteins of *S. denitrificans* (15 mg) and 1 mM mercaptopropionic acid. The final ^{18}O-water content was approximately 48.5%. The reaction was started by adding 4.5 mM of 1-testosterone to the anoxic assay. The 2-propanol content was also 6.67%.

## UPLC-APCI-HRMS

The ethyl acetate extractable samples or purified steroid intermediates were analyzed using UPLC-MS with UPLC coupled to an atmospheric pressure chemical ionization (APCI) high-resolution mass spectrometry (HRMS). Mass spectral data were obtained using a Waters HDMS-QTOF synapt mass spectrometer (Waters) equipped with a standard APCI source operating in the positive ion mode. Separation was achieved on a reversed-phase $C_{18}$ column (Acquity UPLC BEH C18, 1.7 μm, 100 × 2.1 mm; Waters) with a flow rate of 0.4 ml min$^{-1}$ at 35°C (column oven temperature). The mobile phase comprised a mixture of two solvents: Solvent A [2% (v/v) acetonitrile containing 0.1% formic acid to enable excellent ionization in the APCI] and Solvent B (methanol containing 0.1% formic acid). Separation was

achieved with a linear gradient of Solvent B from 10% to 99% in 8 min. In APCI-MS analysis, the temperature of the ion source was maintained at 100°C. Nitrogen desolvation gas was set at a flow rate of 500 l h$^{-1}$ and the probe was heated to 400°C. Nitrogen was used as the APCI nebulizer gas. The corona current was maintained at 20 μA, and the electron multiplier voltage was set to1700 eV. The parent scan was in the range of $m/z$ 50–500. The predicted elemental composition of individual intermediates was calculated using MassLynx Mass Spectrometry Software (Waters).

## UPLC-ESI-HRMS

The ethyl acetate extractable samples or TLC-purified testosterone-derived intermediates were also analyzed using UPLC-ESI-HRMS. The separation conditions for UPLC were the same as those for UPLC-APCI-HRMS. Mass spectral data were collected in +ESI mode in separate runs on a Waters HDMS-QTOF synapt mass spectrometer operated in a scan mode from $m/z$ 50 to 500. The capillary voltage was set at 3000 V; the source and desolvation temperatures were 100°C and 250°C, respectively. The cone gas flow rate was 50 l h$^{-1}$.
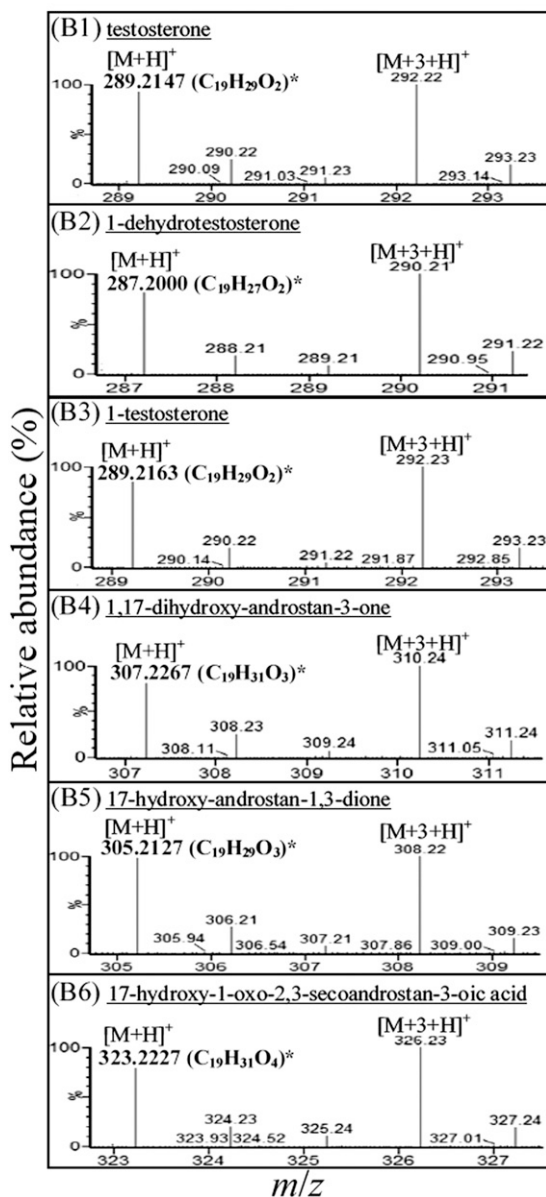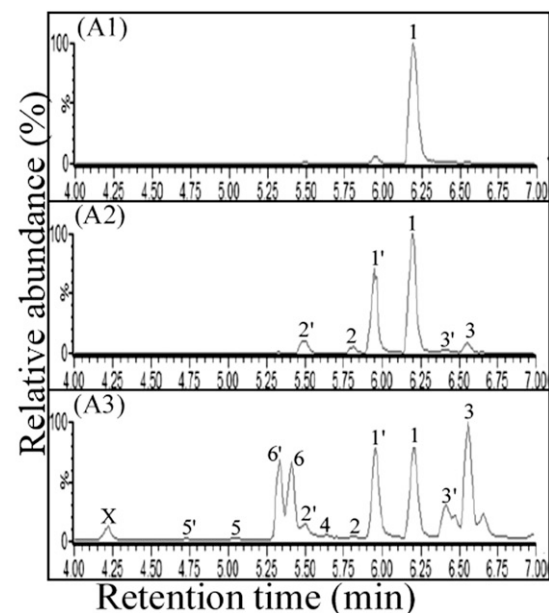
## NMR spectroscopy

The $^1$H- and $^{13}$C-NMR spectra were recorded at 27°C using a Bruker AV600_GRC 600 MHz NMR (in the case of 2,3-SAOA) or a Bruker Avance-400 FT-NMR [for 3,17-dihydroxy-9,10-seco-androsta-1,3,5(10)-triene-9-one (3,17-DHSA)] spectrometer. Chemical shifts (δ) were recorded and shown as ppm values with deuterated methanol (99.8%, $^1$H: δ = 3.31 ppm; $^{13}$C: δ = 49.0 ppm; in the case of 2,3-SAOA) or chloroform (99.5%, $^1$H: δ = 7.26 ppm; $^{13}$C: δ = 77.0 ppm; for 3,17-DHSA) as the solvents and internal references.

## Phylogenetic analysis of *S. denitrificans* strains

Detection of the phylogenetic relationship of *S. denitrificans* strains was conducted using Clustal W and MEGA 5.0 (24). Sixty-three 16S rRNA gene sequences of *S. denitrificans* were retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI).

## RESULTS

### Aerobic testosterone catabolism by *S. denitrificans*

*S. denitrificans* is able to grow with testosterone under aerobic conditions. It was unclear whether the bacterium utilizes the well-studied 9,10-seco-pathway to degrade testosterone in the presence of oxygen. Metabolites of the aerobic testosterone degradation by *S. denitrificans* were extracted using ethyl acetate from shake-flask cultures. The addition of the *meta*-cleavage inhibitor 3-chlorocatechol resulted in the accumulation of at least seven intermediates, which were identified using UPLC-HRMS (**Table 1**). The initial intermediates (1-dehydrotestoterone, androst-4-en-3,17-dione, and androsta-1,4-diene-3,17-dione) were identified by comparison with authentic standards by UPLC-HRMS. The 3,17-dihydroxy-9,10-seco-androsta-1,3,5(10)-triene-9-one (3,17-DHSA), 3,4,17-trihydroxy-9,10-seco-androsta-1,3,5(10)-triene-9-one, and their 17-keto derivatives exhibited maximal UV absorption of approximately 280 nm, indicating the presence of the phenolic A-ring structure in these compounds. The chemical structure of 3,17-DHSA was further confirmed

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**Fig. 3.** UPLC-HRMS analysis of ethyl-acetate extracts of *S. denitrificans* cells grown anaerobically on testosterone (2 mM). In the in vivo assay, the steroid substrate was composed of [2,3,4C-$^{13}$C] testosterone and unlabeled testosterone (mixed in 1:1 molar ratio). (A) UPLC chromatograms of ethyl-acetate extracts: (A1) 10 min after the anaerobic incubation; (A2) 6 h after the anaerobic incubation; and (A3) an additional 6 h anaerobic incubation with 0.5 mM mercaptopropionic acid. Abbreviations of testosterone-derived intermediates present in the chromatograms: 1: testosterone; 2: 1-dehydrotestosterone; 3: 1-testosterone; 4: 1,17-dihydroxy-androstan-3-one; 5: 17-hydroxy-androstan-1,3-dione; 6: 17-hydroxy-1-oxo-2,3-seco-androstan-3-oic acid. Numbers with an apostrophe represent their 17-keto derivatives (for their mass spectra, see Fig. 4). (B) The high-resolution mass spectra of 17-hydroxyl intermediates derived from testosterone. *The predicted elemental composition of individual intermediates was calculated using Mass-Lynx Mass Spectrometry Software (Waters).
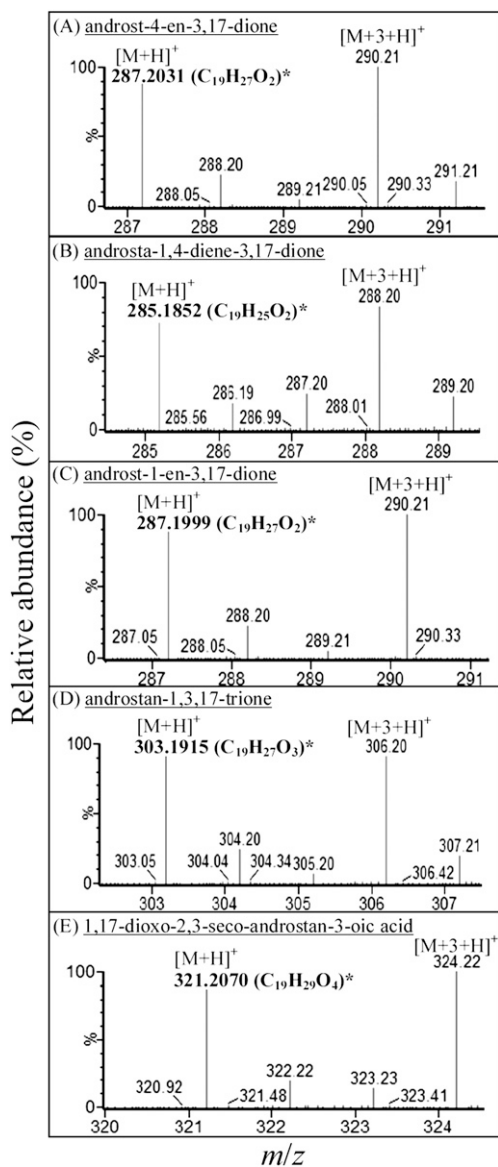
**Fig. 4.** APCI-HRMS spectra data of 17-keto intermediates involved in anaerobic testosterone degradation by *S. denitrificans*. (A–E) In the in vivo assay, the steroid substrate was composed of [2,3,4C-$^{13}$C]testosterone and unlabeled testosterone (mixed in 1:1 molar ratio). *The predicted elemental composition of individual molecular adduct ions was calculated using MassLynx Mass Spectrometry Software (Waters).

using NMR (**Tables 2** and **3** for the $^1$H- and $^{13}$C-NMR spectral data, respectively). In the $^1$H-NMR spectrum of 3,17-DHSA, a set of mutually coupled aromatic protons was observed at $\delta_H$ 6.99 (1H, d, $J$ = 8.0 Hz; H-1); 6.66 (1H, d, $J$ = 2.8 Hz; H-4); and 6.59 (1H, dd, $J$ = 8.0, 2.8 Hz; H-2). Furthermore, two methyl groups were present at 2.25 (3H, s; H-19) and 1.10 (3H, s; H-18). In the $^{13}$C-NMR spectrum of 3,17-DHSA, a carbonyl signal was observed at $\delta_C$ 212.7 (C-9). An additional oxygenated methine proton and a corresponding oxygenated carbon were present at $\delta_H$ 3.76 (1H, t, $J$ = 8.4 Hz, H-17) and $\delta_C$ 80.8 (C-17), respectively. Final structural elucidation of this compound was performed using two-dimensional (2D) NMR. In the

heteronuclear multiple bond coherence (HMBC) spectrum of 3,17-DHSA, the $^2J$ long-range coupling signals were observed between $\delta_H$ 2.37, 2.52 (H-11)/$\delta_C$ 212.7 (C-9). In addition, the $^3J$-correlation signals between $\delta_H$ 1.79 (H-7) and $\delta_C$ 212.7 (C-9) and H-12 ($\delta_H$ 2.02)/$\delta_C$ 212.7 (C-9) were identified (**Fig. 2**). These results suggest that *S. denitrificans* adopts the 9,10-seco-pathway to degrade testosterone in the presence of oxygen, with 3,17-DHSA as the key intermediate (Fig. 1).

### Anaerobic testosterone catabolism by *S. denitrificans*

The metabolites of the anaerobic testosterone catabolism were extracted using ethyl acetate from in vivo transformation assays. These testosterone-derived intermediates were identified using UPLC-HRMS. The steroid substrate was composed of [2,3,4C-$^{13}$C]testosterone and unlabeled testosterone (mixed in 1:1 molar ratio). Therefore, pairs of molecular adduct ions (with the $m/z$ difference of 3) were observed in the mass spectra of testosterone-derived intermediates (**Fig. 3**). The mass spectra of their 17-keto structures are shown in **Fig. 4**. At the beginning of the assay, only testosterone was detected (Fig. 3A1). After 6 h of anaerobic incubation, 1-dehydrotestoterone, 1-testosterone, and their 17-keto derivatives appeared (Fig. 3A2). These steroid compounds were identified by comparison with authentic steroid standards by UPLC-HRMS. After a further incubation (6 h) with 0.5 mM mercaptopropionic acid (an acyl-CoA dehydrogenase inhibitor), a few new intermediates were present (Fig. 3A3). This phenomenon suggests that β-oxidation may play a role in the degradation of downstream intermediates. The testosterone-derived intermediates detected in the anaerobic *S. denitrificans* cultures are summarized in supplementary Table I.

To produce a sufficient amount of intermediates for NMR analysis, an enlarged anoxic culture (2 l) containing *S. denitrificans* cells, testosterone (4 mM), and mercaptopropionic acid (0.5 mM) was produced. The intermediates were sequentially purified using liquid-liquid partition, silica gel chromatography, TLC, and HPLC. The structures of 17-hydroxy-1-oxo-2,3-seco-androstan-3-oic acid (2,3-SAOA) were elucidated using NMR spectroscopy. According to the UPLC-HRMS data, the elemental composition of 2,3-SAOA was calculated as $C_{19}H_{30}O_4$ (Fig. 3B6). Its H-17, H-18, and H-19 signals were exhibited at $\delta_H$ 3.57 (1H, t, $J$ = 8.7 Hz), 0.70 (3H, s), and 0.92 (3H, s), respectively, in the $^1$H-NMR spectrum (Table 2). An additional methyl signal (H-2) was observed at $\delta_H$ 2.18 (3H, s), indicating that the single bond between C-2 and C-3 was broken. In the $^{13}$C-NMR spectrum, a ketone group and a carboxyl group appeared at $\delta_C$ 216.8 and 176.6, respectively (Table 3). The investigation of the final structure of this compound was performed using 2D NMR. The $^2J$ and $^3J$ long-range coupling signals were observed between $\delta_H$ 2.18 (H-2)/$\delta_C$ 216.8 (C-1) and $\delta_H$ 0.92 (H-19)/$\delta_C$ 216.8 (C-1), respectively, in the HMBC spectrum (**Fig. 5**). The hydroxyl group at C-17 was confirmed by the appearance of a $^3J$-correlation signal between H-18 ($\delta_H$ 0.70) and the oxygenated carbon ($\delta_C$ 82.4).
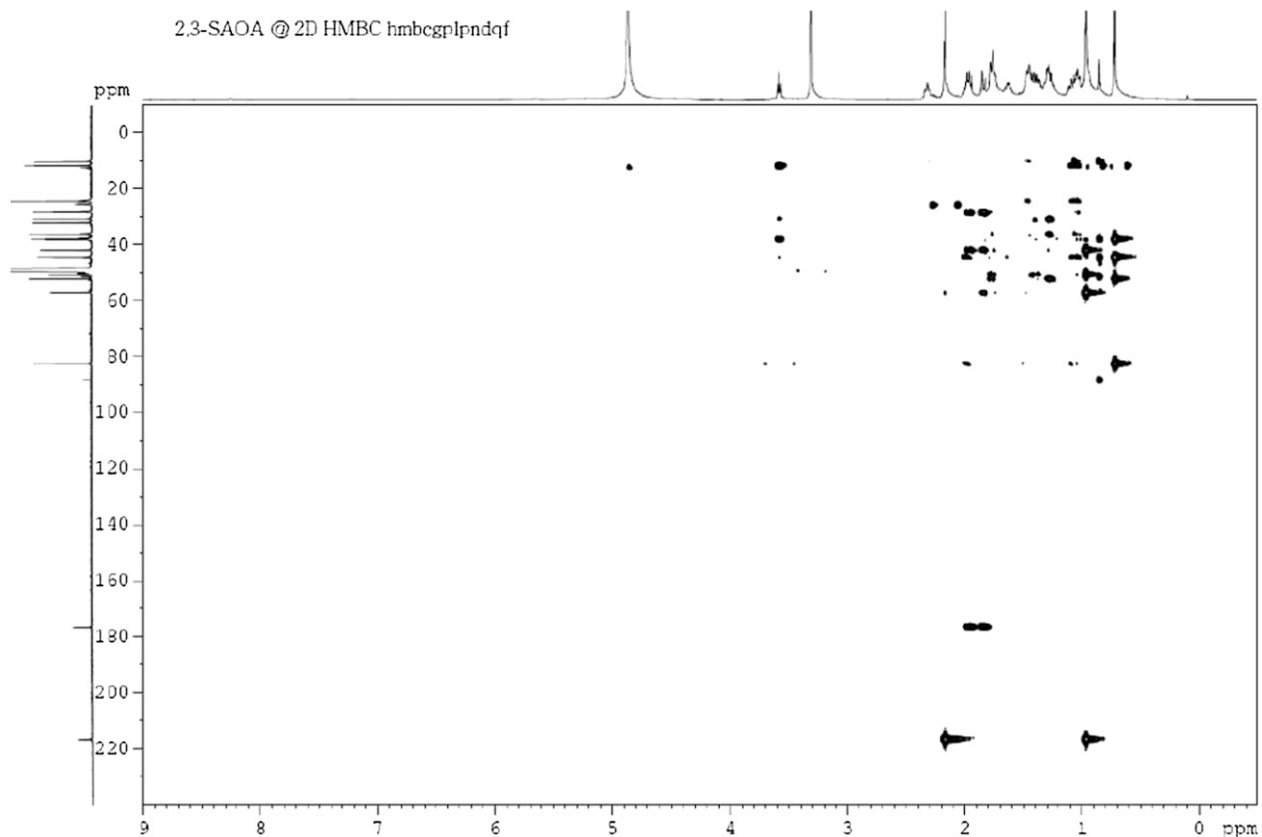
**Fig. 5.** HMBC spectrum of the characteristic intermediate (2,3-SAOA) involved in anaerobic testosterone catabolism by *S. denitrificans.*

To determine the origins of the oxygen atoms at C-1 and/or C-3 of 17-hydroxy-androstan-1,3-dione and 2,3-SAOA, we conducted two in vitro transformation assays using 1-testosterone as the substrate, as follows: *i*) an $^{18}$O-labeled water-treated assay contained approximately 48.5% $^{18}$O-labeled water (mole/mole) in the anoxic reaction mixture; and *ii*) a control assay was incubated under anoxic conditions without the addition of $^{18}$O-labeled water. Compared with the 17-hydroxy-androstan-1,3-dione purified from the control assay (**Fig. 6A**), an additional $^{18}$O-isotopic molecular ion ([M+2+H]$^+$, *m/z* 307.2162) was observed in the APCI-mass spectrum of the 1,3-dioxo product purified from the $^{18}$O-labeled water-treated assay (Fig. 6B). The APCI-mass spectrum of 2,3-SAOA purified from the $^{18}$O-labeled water-treated assay showed three dominant protonated molecular ions ([M+H]$^+$, *m/z* 323.2227, 325.2273, and 327.2320; Fig. 6D). Their elemental composition was calculated as $C_{19}H_{31}{}^{16}O_4$, $C_{19}H_{31}{}^{16}O_3{}^{18}O_1$, and $C_{19}H_{31}{}^{16}O_2{}^{18}O_2$. By contrast, the APCI mass spectrum of 2,3-SAOA purified from the control assay showed only single protonated adduct ion (*m/z* 323.2230; Fig. 6C). These data indicated that under anoxic conditions, after the activation of the A-ring through a hydration reaction, the cleavage of the steroidal core ring system begins with the A-ring by a hydrolysis reaction. Moreover, the presence of 10 mM KCN [an inhibitor generally inactivates members of the xanthine oxidase family (25, 26)] considerably

inhibited the in vitro transformation of 1-testosterone to 1,17-dihydroxy-androstan-3-one (data not shown), indicating that the hydration reaction occurring at C-1/C-2 of 1-testosterone may be catalyzed by a molybdopterin-containing enzyme.

A testosterone-derived intermediate (compound X, Fig. 3A3) with 17 carbons was identified using UPLC-HRMS. Its ESI-mass spectrum (**Fig. 7**) indicated that this compound is labeled with two $^{13}$C. We assumed that the C-1 and C-2 of intermediate X was removed because *i*) testosterone, the steroid substrate, was labeled with three $^{13}$C at C-2/C-3/C-4, and *ii*) in the case of 2,3-SAOA, the single bond between C-2 and C-3 was broken. So far, we cannot produce a sufficient amount of compound X for NMR analysis. Therefore, the exact structure of the intermediate X remains unclear.

**Modes of respiration and testosterone catabolism by**
***S. denitrificans* grown under various oxygen concentrations**

To test *i*) whether *S. denitrificans* adopts the oxygen-independent 2,3-seco-pathway under microaerobic conditions and *ii*) whether the coexistence of the 2,3-seco- and 9,10-seco-pathways is possible, we grew *S. denitrificans* under various oxygen concentrations (0, 2.5, 5, 10, and 20%, v/v). The volume ratio of the *S. denitrificans* cultures (100 ml) to its headspace (900 ml) was 1 to 9; thus, the oxygen concentration in the headspace was not heavily changed during
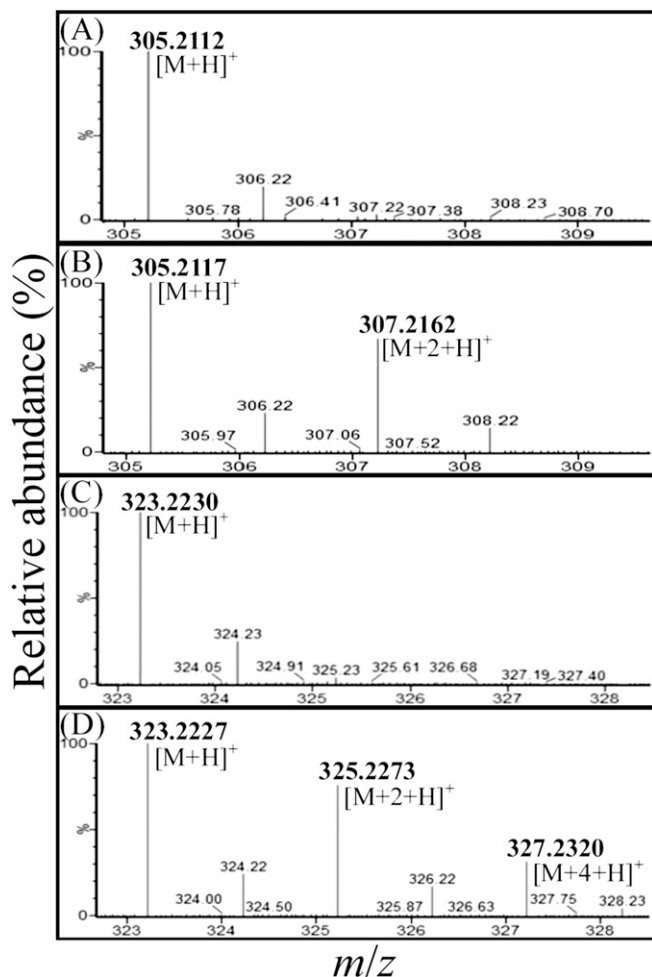
**Fig. 6.** APCI-mass spectra (positive ion mode) of 17-hydroxy-androstan-1,3-dione and 2,3-SAOA. (A) 17-hydroxy-androstan-1,3-dione purified from the anaerobic control assay. (B) 17-hydroxy-androstan-1,3-dione purified from the $^{18}O$-labeled $H_2O$-treated assay. (C) 2,3-SAOA purified from the anoxic control assay. (D) 2,3-SAOA purified from the $^{18}O$-labeled $H_2O$-treated assay.

bacterial growth. The bacterial growth was fastest in the presence 20% oxygen in the headspace, whereas the slowest growth was observed under 2.5% oxygen (**Fig. 8A**). *S. denitrificans* consumed nitrate as the terminal electron acceptor only under strictly anaerobic conditions (Fig. 8A). A tiny amount of nitrate was consumed in the other four cultures. The production of ring cleavage intermediates (3,17-DHSA and 2,3-SAOA) by *S. denitrificans* cells in five cultures was quantified using HPLC at 12 h after the addition of 3-chlorocatechol and mercaptopropionic acid. The HPLC detection limits of 2,3-SAOA and 3,17-DHSA concentrations in the bacterial cultures were 100 ng ml$^{-1}$ and 25 ng ml$^{-1}$, respectively.

The characteristic ring cleavage intermediate of the 9,10-seco-pathway, 3,17-DHSA, was produced by *S. denitrificans* cells at 5~20% oxygen in the headspace (Fig. 8B). Under 20% oxygen, the concentration of 3,17-DHSA in the culture was 24 ± 3 μg ml$^{-1}$. On the other hand, production of 2,3-SAOA, the key intermediate of the 2,3-seco-pathway, was only observed in the complete

absence of oxygen and under 2.5% oxygen (Fig. 8B). 2,3-SAOA concentration in the strictly anaerobic culture was 7 ± 1 μg ml$^{-1}$. It is worth mentioning that the two ring cleavage intermediates apparently never coexisted in any tested bacterial cultures. Our data showed that *S. denitrificans* adopts only one testosterone catabolic pathway at any time, depending on oxygen availability.

## DISCUSSION

Under oxic conditions, the degradation of testosterone by *S. denitrificans* appeared to follow the 9,10-seco-pathway reported previously (27). The microbial oxygenolytic cleavage of cycloalkane and aromatic rings under oxic conditions is widely distributed in nature, for example, aerobic catabolism of cyclohexanol and phenol by bacteria (28). The insertion of hydroxyl groups into the organic substrates by oxygenases is a common catabolic strategy that enables the microbial cells to overcome the inherent inertness of these compounds (29, 30).

The metabolomic data presented in this study show that anaerobic degradation of testosterone by *S. denitrificans* occurs through a catabolic route that differs fundamentally from the aerobic degradation pathway. The crucial differences appear in the mechanisms adopted for the cleavage of the core ring structure of testosterone (Fig. 1). In the aerobic pathway, the cleavage starts with the B-ring, whereas in the anaerobic pathway, the A-ring is opened first. Another significant difference nicely reflects the influence of oxygen availability on the actual ring cleavage mechanism. In contrast to the oxygenase-catalyzed oxygenolytic ring fission under oxic conditions, the opening of the A-ring under anoxic conditions occurs through the oxygen-independent hydrolytic mechanism. The $^{18}O$-incorporation experiments
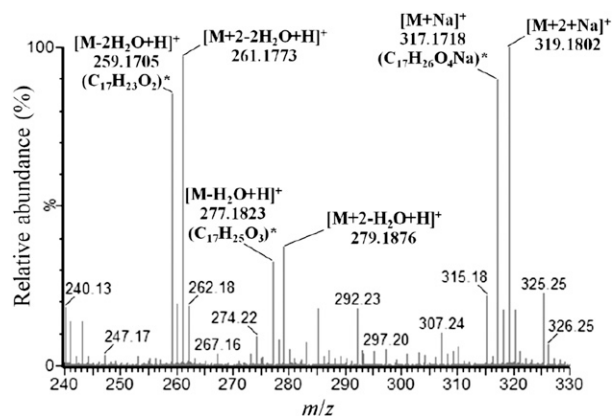


**Fig. 7.** ESI-mass spectra of a $C_{17}$ testosterone-derived intermediate involved in anaerobic testosterone degradation by *S. denitrificans*. *The predicted elemental composition of the product ions was calculated using MassLynx Mass Spectrometry Software (Waters).
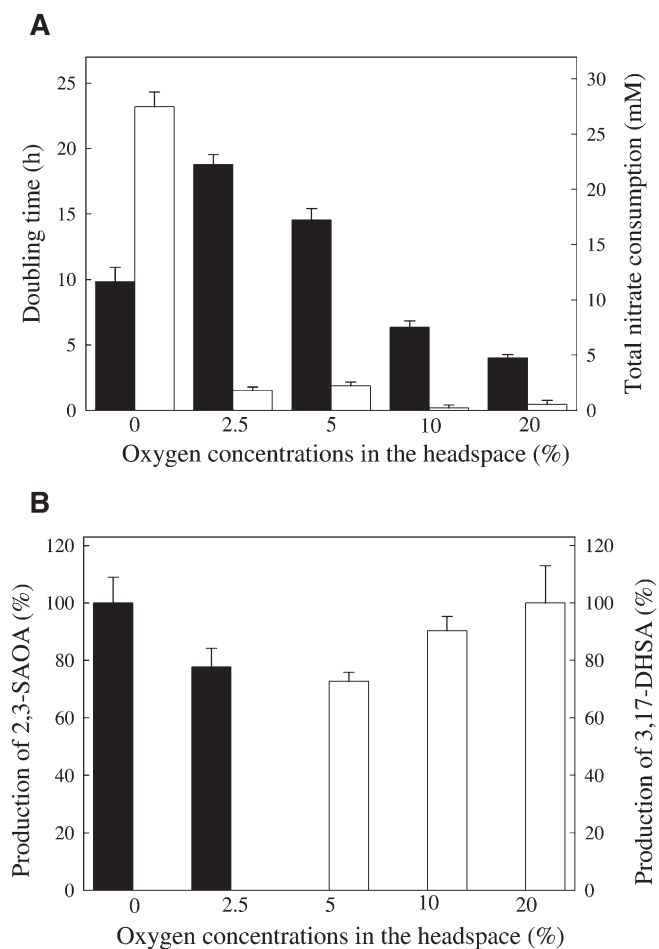
**A**



**B**



**Fig. 8.** The modes of respiration and testosterone catabolism adopted by *S. denitrificans* grown under various oxygen concentrations. Samples were withdrawn after 12 h incubation with 0.5 mM mercaptopropionic acid and 1 mM 3-chlorocatechol. The data shown are from five independent experiments. Data are means ± SE of three replicates in the representative experiment. (A) Bacterial growth (black column) and nitrate consumption (white column) of *S. denitrificans* cultures. (B) The production of 2,3-SAOA (black column) and 3,17-DHSA (white column) by *S. denitrificans* cultures. For white columns, the amount of 3,17-DHSA produced by *S. denitrificans* grown under 20% oxygen concentration (aerobic treatment) was set at 100%, and those of other four treatments are shown relative to that of the aerobic treatment. For black columns, the amount of 2,3-SAOA produced by *S. denitrificans* grown under strictly anaerobic conditions was set at 100%, and those of other four treatments are shown relative to that of the anaerobic treatment.

corroborate the proposed hydrolytic ring cleavage mechanism.

It is well established that the microbial cleavage of the rings of cycloalkane and aromatic compounds under anoxic conditions usually proceeds through hydrolytic mechanism (31–34). Dangel et al. (31, 32) showed that the hydrolytic ring cleavage substrate in anaerobic cyclohexanol degradation is 1,3-cyclohexanedione. Interestingly, in the anaerobic testosterone catabolism, the hydrolytic ring cleavage substrate, 17-hydroxy-androstan-1,3-dione, has a 1,3-dioxo structure in its A-ring (**Fig. 9**).

Recently, 2-cyclohexenone hydratase catalyzing the addition of water to the C = C bond of α,β-unsaturated carbonyl compounds was purified and characterized from the cyclohexanol-degrading bacterium *Alicycliphilus denitrificans* (35). This heterotrimeric enzyme (MhyADH) contains molybdopterin, FAD, and [2Fe-2S] clusters and belongs to the xanthine oxidase family. Our data showed that KCN effectively inhibited the hydration reaction of 1-testosterone, suggesting that this reaction may be catalyzed by a similar molybdopterin-binding enzyme. So far, the ring cleavage enzyme involved in anaerobic cyclohexanol catabolism, 1,3-cyclohexanedione hydrolase, was only partly characterized. 1,2-cyclohexanedione serves as a competitive inhibitor for this enzyme (32). However, the addition of 1,2-cyclohexanedione (up to 1 mM) to the in vivo or in vitro assays did not inhibit the hydrolytic A-ring cleavage of steroid substrates (data not shown).

A crucial finding of our studies is that testosterone is transformed to 1-dehydrotestosterone regardless of the growth conditions. Subsequently, the catabolism proceeds through divergent pathways depending on the availability of oxygen (Fig. 1). Therefore, 1-dehydrotestosterone can be considered a common intermediate or a divergence point for testosterone degradation. According to our current data, *S. denitrificans* adopts only one catabolic pathway (either the 2,3-seco- or 9,10-seco-pathway) to degrade testosterone, depending on oxygen tension. This mode of catabolism might have an ecological significance. It is more energetically efficient to start the degradation of a substrate through some common intermediate(s) with the same enzyme(s), regardless of the prevailing conditions. Subsequently, the last common intermediate can be channeled into the relevant pathways. This will help denitrifying bacteria to readily switch their catabolic enzyme inventory between the oxic and anoxic mode and consequently increase their metabolic competence. This hypothesis is supported by the wide distribution of 16S rRNA gene sequences of *S. denitrificans* strains in oxic and anoxic environments. Similar (but not as extreme) cases were reported in the literature for a number of facultative anaerobes, such as *Thauera aromatica* (36) and *Azoarcus evansii* (37). These bacteria can use benzoate and phenylacetate under oxic and anoxic conditions (34, 36–38). In both cases, the substrate is initially transformed to a common intermediate benzoyl-CoA or phenylacetyl-CoA by the same or isoenzymes. Then, depending on the availability of oxygen, the common substrate is driven into the relevant pathway. Under oxic conditions, the recently disclosed epoxybenzoyl-CoA or epoxyphenylacetyl-CoA pathway is used; while in the absence of oxygen, the anaerobic benzoyl-CoA is preferred.
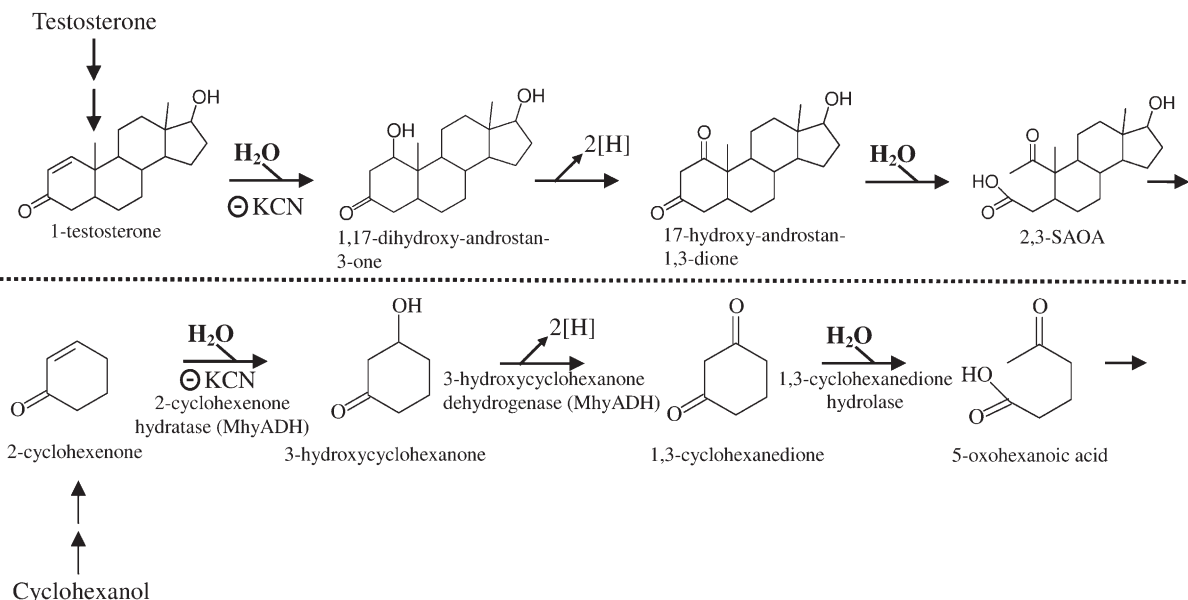
**Fig. 9.** Comparison of the intermediates involved in anaerobic testosterone catabolic pathway (demonstrated in *S. denitrificans* DSMZ 18526) with those of anaerobic cyclohexanol catabolism (by *Alicycliphilus denitrificans* DSMZ 14773). The scheme of anaerobic cyclohexanol catabolic pathway is based on Jin et al. (36). 2-cyclohexenone hydratase belongs to the xanthine oxidase family, which is inactivated by KCN. This bifunctional molybdoenzyme (MhyADH) also catalyzes the subsequent alcohol oxidation to form 1,3-cyclohexanedione. According to our data, KCN effectively inhibited the hydration reaction of 1-testosterone.

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