Substrate Uptake and Subcellular Compartmentation of Anoxic Cholesterol Catabolism in Sterolibacterium denitrificans

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Background: Cholesterol is ubiquitous on earth. Little is known about anoxic cholesterol catabolism.

Results: we proposed a model for cholesterol uptake and subcellular compartmentation during cholesterol catabolism by a Gram-negative bacterium.

Conclusion: The enzymes located in the periplasm are critical to cholesterol catabolism, especially during the steps of substrate activation.

Significance: This study may have potential applications in the biotechnological production of steroid drugs.

ABSTRACT

Cholesterol catabolism by actinobacteria has been extensively studied. In contrast, the uptake and catabolism of cholesterol by Gram-negative species is poorly understood. Here we investigated microbial cholesterol catabolism at the subcellular level. 13C-metabolomic analysis revealed that anaerobically grown Sterolibacterium denitrificans, a betaproteobacterium, adopts an oxygenase-independent pathway to degrade cholesterol. S. denitrificans cells did not produce biosurfactants upon growth on cholesterol and exhibited high cell surface hydrophobicity. Moreover, S. denitrificans did not produce extracellular catabolic enzymes to transform cholesterol. Accordingly, S. denitrificans accessed cholesterol through direction adhesion. Cholesterol is imported through the outer membrane via a putative FadL-like transport system, which is induced by neutral sterols. The OM steroid transporter is able to selectively import various C27 sterols into the periplasm. S. denitrificans spheroplasts exhibited a significantly higher efficiency in cholest-4-en-3-one-26-oic acid uptake than in cholesterol uptake. We separated S. denitrificans proteins into four fractions, namely the outer membrane, periplasm, inner membrane, and cytoplasm, and observed the individual catabolic reactions within them. Our data indicated that, in the periplasm, various periplasmic and peripheral membrane enzymes transform cholesterol into cholest-4-en-3-one-26-oic acid. The C27 acidic steroid is then transported into cytoplasm, in which side-chain degradation and the subsequent sterane cleavage occur. This study sheds light into microbial cholesterol metabolism under anoxic conditions.
Cholesterol is an essential structural component of eukaryotic membranes that maintains membrane permeability and fluidity. In addition to free membrane-embedded molecules, cholesterol is converted by animals to a storage form through esterification with long-chain fatty acids (1). Because of the ubiquity of cholesterol in eukaryotes, various pathogenic bacteria have evolved to produce and excrete cholesterol-dependent cytolysins to avoid phagocytosis by immune cells during infection (2) or to evade eukaryotic predation (3). In addition, an increasing number of reports have indicated that microbial steroid catabolism can modulate or interfere with eukaryotic signalling (4). Cholesterol is a feasible carbon and energy source for many aerobic bacteria. Cholesterol and related sterols (e.g., phytosterols and ergosterol) are released in large quantities through eukaryotic excretion and decomposition, and their complete degradation is relevant to the global carbon cycle (5). Cholesterol-degrading microorganisms have been used for biotechnological purposes such as the industrial production of steroid drugs from inexpensive cholesterol (6, 7). On-going research is focused on discovering new pharmaceutically useful steroids.

Bacteria have developed numerous mechanisms for importing, transforming, and degrading cholesterol (8, 9, 10). Most current knowledge on cholesterol catabolism is based on studies of Gram-positive actinobacteria (8, 11). Identification of a large regulon of cholesterol catabolic genes suggested that the utilization of host sterols is crucial for infection and persistence of Mycobacterium tuberculosis in macrophages (12). Recent progress in the investigation of the cholesterol catabolic reactions of M. tuberculosis indicated that cholesterol catabolism can be used as a therapeutic target in this human pathogen (13). Researchers have explored the cholesterol uptake of M. tuberculosis within the host cell extensively and identified an ATP-binding cassette-like cholesterol transport system, mce4, in M. tuberculosis (11, 14, 15). Moreover, analyses of genome sequences have indicated that diverse actinobacteria, including pathogens and free-living species, harbor mce loci (15, 16).

By contrast, steroid uptake systems adopted by Gram-negative bacteria (mainly proteobacteria) remain poorly understood. The additional presence of an outer membrane (OM) and periplasmic space complicates steroid uptake and catabolism in proteobacteria. The lipopolysaccharide leaflet on the outer surface of OM impedes steroids from passively diffusing through the OM bilayer (17). In addition, the void of ATP in the periplasmic space (18) excludes the possibility that a mce4-similar transporter functions in the proteobacteria OM. Over the previous two decades, microbiologists have gradually gained a greater understanding of how hydrophobic molecules pass through the OM of Gram-negative bacteria (19-23). In addition to general porins, two families of substrate-specific OM transporters, TonB-dependent outer membrane receptors (TBDRs) and long-chain fatty acid transporters (FadL family), have been examined in detail (23-25).

The subcellular localization of individual steroid catabolic enzymes in Gram-negative bacteria is poorly understood. The periplasm constitutes up to 40% of the total cell volume in Gram-negative species (26). However, with the exception of a few studies (27, 28), past research has overlooked the periplasmic proteins involved in hydrocarbon metabolism. Some cholesterol-transforming enzymes (e.g., FAD-containing cholesterol oxidase) are extracellular (29), implying that cholesterol can be transformed into other steroids before being imported by bacterial cells.

Sterolibacterium denitrificans DSMZ 13999 can degrade cholesterol under both oxic and anoxic conditions (30, 31). Recently, we examined the cholesterol catabolic pathway in aerobically grown S. denitrificans (10). In the current study, we evidenced that an oxygenase-independent pathway functions in the denitrifying S. denitrificans cells. In addition, we investigated biosurfactant production and cell surface hydrophobicity to understand the substrate uptake mechanism. The import of cholesterol and its catabolic intermediates across the outer and cytoplasmic membranes of S. denitrificans was addressed. We then studied the subcellular localization of cholesterol catabolic enzymes. Based on these data, for the first time, we delineated the subcellular compartmentation of anoxic cholesterol catabolism in a Gram-negative bacterium. Considering that this anoxic cholesterol catabolic pathway involves novel steroid intermediates and uncharacterized enzymes, our study may have potential applications in the biotechnological production of steroid drugs.
EXPERIMENTAL PROCEDURES

Chemicals and bacterial strains - 25-Hydroxycholest-4-en-3-one, 1-testosterone, androst-1-en-3,17-dione, 17-hydroxy-1-oxo-2,3-seco-androstan-3-oxic acid (2,3-SAOA), and 1,17-dioxo-2,3-seco-androstan-3-oxic acid were produced according to previously determined methods (10, 32, 33). 26-Hydroxycholest-4-en-3-one (also named 27-hydroxy-4-cholesten-3-one, 99%) and cholest-4-en-3-one-26-oxic acid (also named 3-cholestenic acid-3-one) and pregn-4-en-3-one-20-carboxylic acid (also named 4-pregnen-3-one-20β-carboxylic acid) were purchased from Steraloids. Cholesterol, [4C-13]cholesterol (99 atom % 13C), cholest-4-en-3-one, cholesta-1,4-diene-3-one, androst-4-en-3,17-dione (AD), and androsta-1,4-diene-3,17-dione (ADD) were obtained from Avanti Polar Lipids, and 3-oxo-4-cholestenoic acid (also named 3-oxo-4-cholestenoic acid, 99%) and cholest-4-en-3-one-26-oic acid were obtained from Sigma-Aldrich. Sterol[bacterium] denitrificans Chol-1S\textsuperscript{T} (DSMZ 13999) was purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). Agrobacterium tumefaciens ATCC 33970, Escherichia coli ATCC 23815, and Pseudomonas aeruginosa ATCC 27853 were obtained from the American Type Culture Collection (Manassas, VA, USA).

Denitrifying growth of S. denitrificans with [4C-13]cholesterol - A denitrifying S. denitrificans culture (500 mL) was grown with 2 mM unlabeled cholesterol in a procedure described in a previous report (33). The residual cholesterol in the culture was quantified using the o-phthalaldehyde method (34). After the consumption of cholesterol, 20 mL of the log phase culture was transferred to a sterile 25-mL glass bottle sealed with a rubber stopper. The S. denitrificans cells were subsequently fed with 1 mM [4C-13]cholesterol and anaerobically incubated at 28 °C with stirring (120 rpm). Samples (0.5 mL) were withdrawn every two hours (0–24 h). The culture samples were acidified to pH < 2, and extracted three times using the same volume of ethyl acetate to recover 13C-labeled intermediates. The ethyl acetate fractions were combined, the solvent was evaporated, and the residue was re-dissolved in 100 μL of 2-propanol. The catabolic intermediates were identified using ultra-performance liquid chromatography - high-resolution mass spectrometry (UPLC-HRMS).

Measurement of surface activity - The denitrifying S. denitrificans cells were grown on cholesterol. Culture samples were retrieved during exponential growth to detect biosurfactant production in the culture broth and the cell-free culture supernatant. The cell-free supernatant was obtained by performing centrifugation twice (room temperature, 10,000 × g for 10 min). Culture samples were tested using the oil displacement and emulsification index (with kerosene) assays described in previous research (35, 36). Surface tension was measured using a Kruss K100MK3 tensiometer (Kruss, Germany) equipped with a platinum plate by applying the Wilhelmy plate method. The instrument was calibrated by adjusting the measurement so that the surface tension of water was 72 mN/m at room temperature. The experiment was repeated three times and the results are presented as means ± standard error (SE).

Bacterial adhesion to hydrocarbons assay - The surface hydrophobicity of the S. denitrificans and P. aeruginosa cells was tested using the bacterial adhesion to hydrocarbons assay (BATH) described in a previous report (37). S. denitrificans (100 mL) was anaerobically grown with 2.5 mM cholesterol or 4 mM palmitate, while P. aeruginosa was aerobically grown in rich medium (100 mL) containing 15 g/L of tryptone, 5 g/L of soytone, and 5 g/L of NaCl (pH 7.3). Exponentially growing cells were harvested through centrifugation and washed twice with 0.1 M K\textsuperscript{2}PO\textsubscript{4} buffer (pH 6.8) containing 5 mM MgCl\textsubscript{2}. The washed cell pellets were resuspended in the same buffer and the cell suspensions were adjusted to an optical density at 600 nm (OD\textsubscript{600}) of 1.0 (optical path 1 cm). Aliquots (2 mL) of the cell suspensions were mixed with 0.5 mL of n-hexadecane. The mixtures were vortexed for 2 min and set aside to rest for 20 min to allow for phase separation. The surface hydrophobicity of the bacterial cells was calculated as the percentage of the cells that adhered to the hexadecane phase (37).

Detection of cholesterol dehydrogenase activity in the crude cell extract and cell-free medium - In a fed-batch culture (1 L), S. denitrificans was anaerobically grown with 2.5 mM cholesterol. After the bacterial cells consumed 2.2 mM cholesterol, they were harvested through centrifugation. The remaining cells were removed from the medium using
filtration (0.22 μm nitrocellulose membrane, 47 mm diameter, Millipore). Cell pellets (2.4 g) were resuspended in 20 mL of 50 mM 3-morpholinopropanesulfonic acid (MOPS)-K⁺ buffer (pH 7.5), and a French pressure cell (Thermo Fisher Scientific) was used to break the bacterial cells. Cell debris was removed through centrifugation (4 °C, 20,000 × g for 30 min). Eventually, 21.2 mL of crude cell extract was obtained. The cell-free supernatant (996 mL) was concentrated to 20 mL through ultrafiltration (10-kDa-cutoff membrane), and 1 mL of MOPS-K⁺ buffer (1 M, pH 7.5) was added to the extracellular protein fraction. The assay mixtures (1 mL) contained extracellular or cellular protein fraction (0.95 mL), 2.5 mM NAD⁺, and 0.25 mM cholesterol (from 10 mM stock dissolved in 2-propanol). After anaerobic incubation at 30 °C for 2 h, the assay mixtures were extracted using ethyl acetate. The extracts were separated on a TLC plate [Silica gel 60 F254; thickness, 0.2 mm; 20 cm (Merck)]. An n-hexane-ethyl acetate (65:35, v/v) solvent system was used. The steroid compounds were visualized by spraying the TLC plates with 30% (v/v) H₂SO₄.

Detection of the extracellular steroids in the denitrifying S. denitrificans culture - After ultrafiltration (10-kDa-cutoff membrane), the filtered cell-free medium (976 mL) was extracted with ethyl acetate. The steroids in the cell-free medium were identified using HPLC.

Steroid uptake assay using bacterial cells - S. denitrificans (1 L) was anaerobically grown with 2.5 mM cholesterol. After S. denitrificans cells completely consumed the substrate, these cells were harvested through centrifugation at room temperature. A. tumefaciens, E. coli, and P. aeruginosa cells were aerobically grown in rich medium (1 L) containing tryptone, soytone, and NaCl, and incubated at 37 °C with shaking (180 rpm) overnight. The bacterial cells were harvested through centrifugation. The cell pellets were washed twice with wash buffer [100 mL of MOPS-K⁺ buffer (25 mM, pH 7) containing 5% (v/v) 2-propanol and 5 mM MgCl₂] to remove substrates remaining on the cell surface. After centrifugation, the washed cell pellets were re-suspended in uptake buffer containing 25 mM MOPS-K⁺ buffer (pH 7) and 5 mM MgCl₂, and the cell density was adjusted to an OD₂₆₀ of 1.0. After that, 25 μM cholesterol (stock concentration = 10 mM in 2-propanol) and 25 μM 17β-estradiol (stock concentration = 10 mM in dimethyl sulfoxide) were added to the cell suspensions (1 mL). The assay mixtures were incubated at 30 °C and stirred at 120 rpm. After 10 min of incubation, the bacterial cells were recovered by filtering the assay mixtures through a 0.22 μm Durapore® membrane filter (PVDF, hydrophilic, 47 mm diameter; Millipore), and the membranes were subsequently washed with 10 mL of 25 mM MOPS-K⁺ buffer (pH 7) containing 1% (v/v) Tween-20 and 50 mL of MOPS-K⁺ buffer containing 5% (v/v) 2-propanol. After vacuum filtration, the membrane filters were extracted using ethyl acetate (10 mL) three times, and the recovered cholesterol and 17β-estradiol were quantified.

In another experiment, S. denitrificans was anaerobically grown with 2.5 mM cholesterol, cholest-4-en-3-one, AD, or 4 mM palmitate. After the complete consumption of the substrate, the resting cell suspensions (OD₂₆₀ = 1.0) were tested for their cholesterol uptake ability. 25 μM cholesterol and 25 μM 17β-estradiol (for a comparison) were added to the cell suspensions (1 mL).

To investigate the substrate preference of the cholesterol-induced OM transporter, cholesterol-grown S. denitrificans cells were resuspended in the uptake buffer, and the resulting cell suspensions were incubated with 25 μM of individual catabolic intermediates (from 10 mM stocks dissolved in 2-propanol) at 30 °C for 10 min. Ten catabolic steroids including C₂₇ sterols, acidic steroids, and C₁₉ androgens were tested for their uptake efficiency. Where indicated, 60 mM sodium azide [inhibitor of the respiratory chain (38)], 10 mM KCN [inhibitor of proton motive force (39)], 10 mM sodium orthovanadate [ATPase inhibitor (38)], or 10 mM EDTA [outer membrane permeabilizer (17)] was added to cell suspensions 10 min prior to the addition of cholesterol.

Preparation of spheroplasts of different proteobacteria - S. denitrificans (1 L) was anaerobically grown with cholesterol (2.5 mM), and A. tumefaciens, E. coli, and P. aeruginosa were aerobically grown in rich medium. Bacterial spheroplasts were prepared according to the procedure described in a previous report (27) with slight modifications. In brief, freshly harvested bacterial cells (0.8 g, wet mass) were re-suspended in 64 mL of lysis buffer containing 30 mM Tris-HCl (pH 8), 30% sucrose, 9 mM ethylenediaminetetraacetic acid (EDTA), and lysozyme (2.6 x 10⁶ units). The cell suspensions were incubated on ice for 120 min, and the
resulting spheroplasts were harvested through centrifugation (16,000 × g, 4 °C, for 20 min).

**Steroid uptake assay using bacterial spheroplasts** - The spheroplasts were gently washed with wash buffer containing MOPS-K+ buffer (25 mM, pH 7), 5% (v/v) 2-propanol, 5 mM MgCl2, and 2.5% sucrose (w/v). After centrifugation, the pellets were re-suspended in uptake buffer containing 25 mM MOPS-K+ buffer (pH 7), 5 mM MgCl2, and 30% sucrose, and the spheroplast density was adjusted to OD600 of 1. A 10 mM cholesterol solution (2.5 μL; in 2-propanol) and 2.5 μL of the second steroid (cholest-4-en-3-one, 25-hydroxycholest-4-en-3-one, 26-hydroxycholest-4-en-3-one, and cholest-4-en-3-one-26-oic acid, AD, or ADD; from 10 mM stock dissolved in 2-propanol) were added to the spheroplast suspensions (1 mL). The steroid uptake assays were incubated at 30 °C and stirred gently (80 rpm) for 10 min. The uptake assay mixtures containing 30% sucrose were unable to pass through a 0.22-μm Durapore® membrane filter within 30 min. Therefore, the spheroplasts were recovered through centrifugation. The assays were quenched by adding 9 mL of ice-cold wash buffer containing 30% sucrose, and then centrifuged at 16,000 × g and 4 °C for 20 min. The spheroplast pellets were gently washed with a wash buffer (10 mL) again. After centrifugation, the pellets were extracted with ethyl acetate to recover the steroids.

In the competitive experiment, S. denitrificans spheroplasts were incubated with cholest-4-en-3-one-26-oic acid (25 μM) and palmitate (0~100 μM) at 30 °C for 10 min. After centrifugation, the acidic steroid imported into spheroplasts was quantified using HPLC.

**Separation of subcellular compartments and the steroid transformation assays using fractionated S. denitrificans proteins** - Subcellular fractions were prepared at 4 °C under anoxic conditions. Exponentially growing S. denitrificans cells (2 L) were harvested through centrifugation, yielding a total of 4.1 g of bacterial cells (wet weight). Cell pellets (2 g; wet weight) were used to prepare the OM and the cytoplasmic membrane. The remaining 2 g of cell pellets was used to prepare periplasmic and cytoplasmic fractions.

(i) Sucrose gradient separation of S. denitrificans membranes. S. denitrificans membrane proteins were separated from soluble proteins as described previously (33). To separate the cytoplasmic membrane from the OM, a sucrose gradient centrifugation was performed according a previously described procedure (40) with some modifications. The membrane pellets were re-suspended in 0.5 mL of basal buffer containing 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.2 mM dithiothreitol. The resulting membrane suspension was layered on a sucrose bed consisting of 20% (w/v; 2 mL), 40% (3 mL), and 50% (1.5 mL) sucrose in the basal buffer. The membrane sample was centrifuged at 4 °C, 120,000 × g for 16 h. The membrane fractions (0.5 mL, per fraction) were carefully collected from the top of the gradient. The fractions containing the OM and cytoplasmic membrane were identified based on the distributions of NADH oxidase activity (specific marker of cytoplasmic membrane) and the OM-specific lipopolysaccharide 2-keto-3-deoxyoctonate (KDO). Both the cytoplasmic and OM fractions were diluted to 10 mL by using 10 mM Tris-HCl (pH 7.5).

(ii) The isolation of periplasmic and cytoplasmic proteins from S. denitrificans cells. The S. denitrificans spheroplasts were prepared using the EDTA/lysozyme treatment described above. The spheroplasts were harvested through centrifugation (16,000 × g, 4 °C for 20 min), and the supernatant was further centrifuged at 4 °C and 120,000 × g for 1.5 h to remove insoluble membrane debris. The resulting periplasmic protein fraction was concentrated to 10 mL by using an Amicon Ultra Centrifugal Filter Unit (Ultracel-10K, Millipore). The periplasmic proteins were passed through a PD-10 desalting column (GE Healthcare) to remove the sucrose, and 10 mM Tris-HCl (pH 7.5) was used to elute the proteins. The spheroplast pellet was re-suspended in 10 mL of Tris-HCl (10 mM, pH 7.5) and broken using a French pressure cell. The cell lysate was centrifuged at 120,000 × g for 1.5 h to separate cytoplasmic proteins from insoluble membranes.

(iii) Assays for subcellular markers. NADH oxidase activity was measured as described in a previous report (40), and malate dehydrogenase (a specific cytoplasmic marker) was assayed as described in another report (41). KDO was quantified by performed the thiobarbituric acid assay after membrane fractions were precipitated using 13% trichloroacetic acid (42). Cytochrome c content (periplasmic marker) in subcellular fractions was analyzed and calculated as described (43).

(iv) In vitro steroid biotransformation assays. The assay mixtures contained
fractionated *S. denitrificans* proteins (0.25 mL), 0.65 mL of Tris-HCl buffer (10 mM, pH 7.5), 0.2 mM steroid substrate (from 10 mM stock dissolved in 2-propanol), 1 mM electron acceptor or electron donor (from 100 mM stock dissolved in water), and 100 mg of (2-hydroxypropyl)-β-cyclodextrin. The reactions were initiated by the addition of the steroid substrate, and the mixtures were shaken at 200 rpm. After 2 h of anaerobic incubation at 30 °C, the steroids were extracted with ethyl acetate. The solvent was evaporated, and the residue was dissolved in 100 μL of acetonitrile for HPLC analysis. The cholesterol dehydrogenase (AcmA) activity was assayed using cholesterol as the substrate and NAD⁺ as the electron acceptor. Cholest-4-en-3-one-Δ⁴-dehydrogenase (AcmB) activity was assayed using 25-hydroxycholest-4-en-3-one and K₃[Fe(CN)₆] as the substrate and electron acceptor, respectively. Steroid C25 dehydrogenase (S25DH) contained cholesta-1,4-diene-3-one and K₃[Fe(CN)₆]. The reduction activity of 17β-hydroxysteroid dehydrogenase was assayed by using estrone as the substrate and NADH as the electron donor. The reduction activity of steroid Δ⁴-dehydrogenase was assayed using 1-dehydrotestosterone as the substrate and NADH as the electron donor, as well as 2 mM KCN to inhibit the subsequent catabolic enzyme, 1-testosterone hydratase. The 1-testosterone hydratase activity assay contained 1-testosterone and 2 mM KCN to inhibit the subsequent catabolic enzyme, 1-testosterone hydratase. The 1-testosterone hydratase activity assay contained 1-testosterone and 2 mM KCN to inhibit the subsequent catabolic enzyme, 1-testosterone hydratase. The reactions were incubated with 0.2% (w/v) Tween 20. After 10 min of anaerobic incubation at room temperature for 2 h, the steroids in the assays were separated using TLC and were observed under UV light at 254 nm.

Freshly harvested *S. denitrificans* cells (0.8 g) were re-suspended in 64 mL of lysis buffer and the cell suspensions were anaerobically incubated on ice for 2 h. 16 mL of 1% (w/v) Tween 20 was then slowly added to the resulting spheroplast suspension, and the Tween 20-treated spheroplasts were incubated at room temperature for 10 min. The periplasmic and dissociated membrane proteins (formerly located on the outer surface of the cytoplasmic membrane) were separated from the spheroplasts through centrifugation. The resulting supernatant was centrifuged at 4 °C and 120,000 × g for 1.5 h to remove insoluble membrane debris and then concentrated to 10 mL through ultrafiltration. The proteins were passed through a PD-10 desalting column to remove the sucrose, and 10 mM Tris-HCl (pH 7.5) was used to elute the proteins. The spheroplast pellet was re-suspended in 10 mL of Tris-HCl (pH 7.5). After passing through a French pressure cell, the cell lysate was treated with 0.2% (w/v) Tween 20. After 10 min of anaerobic incubation at room temperature, the cytoplasmic and dissociated membrane proteins (formerly located on the inner surface of the cytoplasmic membrane) were separated from the membranes through centrifugation. The AcmB activity of the resulting “peripheral-periplasmic” and “peripheral-cytoplasmic” fractions containing dissociated membranes proteins was tested, and the enzyme activity was analyzed by performing HPLC quantification of the steroid product, 25-hydroxycholesta-1,4-diene-3-one.

**Quantification of proteins and steroids** - The protein content was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to manufacturer’s instructions, and bovine serum albumin was used as the standard. The steroids (excluding cholesterol) were quantified using HPLC as described in a previous report (10). Cholesterol did not exhibit apparent UV absorption (with a HPLC-UV detection limit above 5 μg). Thus, the cholesterol was quantified using the o-phthalaldehyde method (34), which features a

proteins were then separated from membrane proteins through centrifugation. The enzyme assays (1 mL) contained 10 mM Tris-HCl (pH 7.5), various *S. denitrificans* proteins fractions (5 mg), 25-hydroxycholesterol-4-en-3-one (0.2 mM), and 1 mM K₃[Fe(CN)₆]. After anaerobic incubation for 2 h, the steroids in the assays were separated using TLC and were observed under UV light at 254 nm.
detection limit of 500 ng.

**UPLC-HRMS** - The ethyl acetate-extractable samples were analyzed using UPLC-atmospheric pressure chemical ionization (APCI)-HRMS as described previously (10).

**Statistical analysis** - One-way analysis of variance (Tukey test with a 95% confidence interval) was performed using the JMP statistical software (Version 10.0.2, SAS Corporation, Chicago, Illinois, USA).

RESULTS

Identification of the cholesterol catabolic intermediates in the denitrifying *S. denitrificans* culture - To investigate the intermediates involved in anoxic cholesterol catabolism, denitrifying *S. denitrificans* cells were grown with [4C-13C]cholesterol. We used UPLC-APCI-HRMS to identify the 13C-labeled intermediates (Table 1); a total of eleven were identified, including the characteristic 2,3-SAOA. With the exception of two intermediates, cholest-4-en-26-al-3-one and androstan-1,3,17-trione, the UPLC and HRMS behaviors of the detected intermediates were identical to those of authentic standards. Our results exclude the possibility of detecting structural isomers, which may be observed in chemical analyses relying only on mass spectrometry. In this study, we clearly revealed that 26-hydroxycholest-4-en-3-one and cholest-4-en-3-one-26-oic acid are crucial intermediates involved in the anoxic cholesterol catabolism of *S. denitrificans*.

Biosurfactant production by *S. denitrificans* - Several assays were performed to test biosurfactant production by denitrifying *S. denitrificans*. Cultures grown on cholesterol under anoxic conditions did not foam. In addition, no significant differences (*P* > 0.5) in the oil displacement activity of cell-free culture supernatants and uninoculated medium existed. Moreover, no significant surface tension reduction (*P* > 0.5) was detected in the cell suspension or cell-free culture supernatant compared with the uninoculated medium (Fig. 1A). Washed cell suspension emulsified kerosene with an emulsification index (E24) of 48% (*P* < 0.0001). No emulsions were produced when kerosene was incubated with cell-free culture supernatant or uninoculated medium (Fig. 1A).

Hydrophobicity of the *S. denitrificans* cell surface - The surface hydrophobicity of *S. denitrificans* and *P. aeruginosa* cells was tested using the BATH assay, in which *n*-hexadecane served as the organic phase. *P. aeruginosa* ATCC 27853 was widely used in surface hydrophobicity studies (44, 45) and showed kerosene adherence of 11.5 ± 0.2% (Fig. 1B). Regardless of growth substrate, the cell surface of *S. denitrificans* exhibited hydrophobic characteristics. The fraction of cholesterol-grown cells adhering to the hydrocarbon phase (37.9 ± 0.6%) was higher than that of palmitate-grown *S. denitrificans* cells (29.7 ± 0.3%) (Fig. 1B).

Cholesterol catabolic enzymes were not detected in the extracellular medium - The crude cell extract of *S. denitrificans* transformed most of the cholesterol to cholest-4-en-1-one and the ensuing intermediates (Fig. 2A4). Note that only residual cholesterol, but not cholest-4-en-3-one, was detected in the concentrated extracellular protein fraction (Fig. 2A5). Cholesterol remained after 2 h of incubation with extracellular proteins (Fig. 2A6), indicating that these proteins did not exhibit cholesterol dehydrogenase activity. In addition, the extracellular steroid profile of the denitrifying *S. denitrificans* culture indicated that cholesterol was the only steroid present in the extracellular medium (Fig. 2B).

Steroid uptake by whole bacterial cells - The steroid uptake abilities of cholesterol-grown *S. denitrificans* (β-proteobacterium), *P. aeruginosa* (γ-proteobacterium), *A. tumefaciens* (α-proteobacterium), and *E. coli* (γ-proteobacterium) were tested. Cholesterol and 17β-estradiol (a comparison) were added to cell suspensions in a 1:1 molar ratio. It is worth mentioning that 17β-estradiol is unable to serve as the carbon and energy source for all tested bacterial strains, and the non-*Sterolibacterium* species cannot utilize cholesterol. As shown in Fig. 3A, small amounts of 17β-estradiol were detected in the cells of all tested bacterial strains. Three non-*Sterolibacterium* proteobacteria imported comparable amounts of cholesterol and 17β-estradiol, and exhibited steroid uptake rates of less than 0.5 nmol/min/mg protein. *S. denitrificans* exhibited significantly greater cholesterol uptake ability (*P* < 0.0001) than the non-cholesterol degrading proteobacteria (Fig. 3A). After residual cholesterol remaining in bacterial cells was excluded, the cholesterol-grown *S. denitrificans* cells imported 20.3 ± 3.2 nmol of cholesterol/mg protein after 10 min of incubation. The 17β-estradiol uptake rate was
lower than that of cholesterol in the S. denitrificans cell suspension (P < 0.0001). Moreover, no significant differences in the 17β-estradiol uptake rate existed among the tested cell suspensions (P > 0.05).

Our model organism can only utilize a few hydrophobic compounds as the carbon source (30). Here, S. denitrificans cells grown on different substrates were tested for their cholesterol uptake ability (Fig. 3B). Regardless of the carbon source, the S. denitrificans cells imported more cholesterol than 17β-estradiol (presumably imported via passive diffusion). However, sterol-grown S. denitrificans cells exhibited greater (twofold) cholesterol uptake rate (P < 0.0001) than that of the cells grown on AD or palmitate. The result indicates that C37 sterols can induce the production of the cholesterol OM transporter, although this transporter seems to be produced at a basal level with other substrates.

We then tested the substrate preference of the OM cholesterol transporter system. As shown in Fig. 4A, its uptake efficiencies for cholesterol and cholest-4-en-3-one were significantly higher than those of C19 androgens (P < 0.0001). This was in accordance with the observation that AD-grown S. denitrificans cells cannot efficiently import cholesterol (Fig. 3B). The OM steroid transporter prefers 4-en-3-one structures over 1,4-diene-3-one compounds. It appears that cholesterol, cholest-4-en-3-one, and cholest-4-en-3-one-26-oic acid are the most favorable substrates for this OM steroid transporter (Fig. 4A).

Cholesterol uptake activity was not abolished by pre-incubation of cells with the respiratory inhibitor (60 mM sodium azide) or the inhibitor of proton motive force (10 mM KCN) (Fig. 4B), suggesting that a TonB-dependent transporter system (24, 25, 39) is not involved in cholesterol uptake of S. denitrificans. In addition, the ATPase inhibitor (10 mM vanadate) did not reduce the cholesterol uptake activity (Fig. 4B), indicating that the steroid uptake is ATP-independent. Our data thus exclude the possibility that a Mce-type steroid transporter works in cholesterol uptake of S. denitrificans (15, 16). It is known that the cation chelator EDTA is able to improve the membrane permeability of Gram-negative bacteria by chelating the divalent cations that cross-bridge the lipopolysaccharide covering the cell surface (17). EDTA (10 mM) did not enhance the cholesterol uptake of S. denitrificans cells (Fig. 4B), indicating that this hydrophobic substrate is not mainly absorbed via passive diffusion. The EDTA-induced decrease in cholesterol uptake (39%) may have resulted from the damage or disintegration of the OM and the OM-located cholesterol transporter system.

Steroid uptake by the bacterial spheroplasts - Spheroplasts were first prepared from anaerobically cholesterol-grown S. denitrificans cells by using lysozyme/EDTA treatment. The steroid uptake efficiency of the S. denitrificans spheroplasts was tested. Fig. 5A shows the individual steroid uptake rates (nmol/min/mg protein) divided by that of the cholesterol. The uptake efficiency for cholest-4-en-3-one-26-oic acid and 26-hydroxycholest-4-en-3-one was significantly higher than that of the other tested steroids (P < 0.0001). It appears that cholest-4-en-3-one-26-oic acid is the most favorable substrate for S. denitrificans spheroplasts. Interestingly, the uptake efficiency of the two C37 hydroxysteroid isomers was apparently different (P < 0.0001). S. denitrificans spheroplasts seemed to prefer the 26-hydroxysteroid to the 25-hydroxy isomer. The C19 androgens, AD and ADD, were poorly imported by S. denitrificans spheroplasts (Fig. 5A).

In another experiment, the steroid uptake abilities of various proteobacteria spheroplasts were tested (Fig. 6A). Regardless of the bacterial species, > 80% of the malate dehydrogenase activity remained in spheroplasts (Fig. 6B), indicating that the bacterial spheroplasts were properly prepared. Significant differences were observed between the uptake efficiencies of cholesterol and cholest-4-en-3-one-26-oic acid (P < 0.0001), and all spheroplasts preferentially took up cholest-4-en-3-one-26-oic acid (Fig. 6A). Surprisingly, spheroplasts in all tested strains, including three non-cholesterol degraders, imported comparable amounts of cholest-4-en-3-one-26-oic acid after 10 min of incubation.

Because (i) the cytoplasmic transporter responsible for cholest-4-en-3-one-26-oic acid seems to be ubiquitous in proteobacteria, and (ii) S. denitrificans can also utilize fatty acids, one can envisage that S. denitrificans may use cytoplasmic membrane-located fatty acid transporter to import fatty acids and the acidic steroid intermediate. We incubated S. denitrificans spheroplasts with cholest-4-en-3-one-26-oic acid (25 μM) and palmitate (0–100 μM), and tested the effects of palmitate on the
uptake efficiency of this acidic steroid. Regardless of the concentration of palmitate, *S. denitrificans* spheroplasts showed no significant difference in the cholest-4-en-3-one-26-oic acid uptake rate (*P* > 0.05) (Fig. 5B). Our result suggests that *S. denitrificans* does not adopt the same cytoplasmic transporter to import palmitate and cholest-4-en-3-one-26-oic acid.

Subcellular localization of cholesterol catabolic enzymes in *S. denitrificans* - To identify the subcellular locations where individual cholesterol catabolic reactions occurred, we separated *S. denitrificans* cells into four subcellular fractions by using lysozyme/EDTA treatment of whole cells to isolate the periplasmic and cytoplasmic fractions and density gradient centrifugation to separate the OM and cytoplasmic membrane. The periplasmic marker cytochrome *c* was largely released to the supernatant (87%) after the lysozyme/EDTA treatment of bacterial cells, whereas 79% of the cytoplasmic marker malate dehydrogenase was retained in the spheroplasts (Fig. 7A). The protein distribution pattern of the sucrose gradient fractions indicated two major peaks (Fig. 7B). The distribution of the OM marker (KDO) and the cytoplasmic membrane marker (NADH oxidase) suggested that the two membrane fractions were efficiently resolved.

We then tested the cholesterol catabolic activities of the four resulting protein fractions by using HPLC-based assays. No cholesterol catabolic activity was majorly distributed in the outer membrane fraction (Fig. 8), indicating that OM-bound proteins are not critical to cholesterol catabolism. Cholesterol dehydrogenase (AcmA) activity was mainly detected in the periplasmic fraction (*P* < 0.0001) (Fig. 8A). Conversely, cytoplasmic membranes exhibited the greatest cholest-4-en-3-one-Δ4-dehydrogenase (AcmB) and steroid C25 dehydrogenase (S25DH) activities (*P* < 0.0001). However, the two enzymes were also detected in the periplasmic and cytoplasmic fractions (Figs. 8B and 8C). The cytoplasmic fraction exhibited significantly greater androgen transformation activities (Figs. 8D-8G), suggesting that C1-hydroxylation and A-ring cleavage of C19 intermediates occurred in the cytoplasm of *S. denitrificans*.

The subcellular localization of AcmB was further investigated. We first showed the dissociation of AcmB from membranes (containing outer and cytoplasmic membranes) by a 0.2% (w/v) Tween 20 treatment for 10 min (Fig. 9A). We then incubated *S. denitrificans* spheroplasts with 0.2% Tween 20 for various amounts of time (0-40 min), during which most malate dehydrogenase activity remained in the cytoplasm (Fig. 9B). These results indicated that a 10-min treatment with 0.2% Tween 20 solubilized membrane-bound AcmB, but did not apparently lyse *S. denitrificans* spheroplasts. We then used 0.2% Tween 20 to solubilize the peripheral proteins located on the periplasmic and cytoplasmic sides of the cytoplasmic membrane, and tested the AcmB activity of the resulting “peripheral-periplasmic” and “peripheral-cytoplasmic” protein fractions. Most enzyme activity was detected in the peripheral-cytoplasmic protein fraction (Fig. 9C), indicating that AcmB was located on the cytoplasmic side of the inner membrane.

**DISCUSSION**

We previously proposed an oxygenase-independent cholesterol catabolic pathway for the aerobically grown *S. denitrificans* cells (10). In this study, we applied a 13C-metabolomic approach to demonstrate that the denitrifying *S. denitrificans* adopts a highly similar strategy to degrade cholesterol. The highly similar protein patterns of the anaerobically and aerobically grown cells (31) suggest that *S. denitrificans* may use the same catabolic enzymes to degrade cholesterol regardless of oxygen availability. It is worth mentioning that the denitrifying *S. denitrificans* cells produce mainly 17-hydroxyl catabolic intermediates (Fig. 10), while aerobically grown cells accumulate 17-ketosteroids (10).

Our current results provided clues to the potential cholesterol uptake mechanism adopted by *S. denitrificans*. The microbial degradation of hydrophobic substrates, like cholesterol, is usually hampered by their low solubility in water (poor bioavailability). Some hydrocarbon degraders overcome this problem by producing solubilizing/emulsifying agents such as biosurfactants, which enhance the aqueous solubility, and therefore, the bioavailability of the hydrophobic substrates (46). *S. denitrificans* does not seem to belong to this group of microbes. The lack of foaming, surface tension reduction, and emulsification activity in the cell-free culture medium excludes the possibility that *S. denitrificans* produces and excretes biosurfactants into the extracellular medium. Accordingly, *S. denitrificans* cells possibly
interact directly with insoluble cholesterol through adhesion to facilitate the uptake process. This is another known hydrocarbon uptake mechanism by which bacteria circumvent the poor bioavailability of the hydrocarbon substrates (47). These bacteria are characterized by a hydrophobic cell surface enabling efficient adhesion to hydrocarbons (48). The emulsification of kerosene with washed \textit{S. denitrificans} cells indicates the ability of the cells to adhere to hydrocarbons via a hydrophobic surface. This was corroborated by the results of the cell surface hydrophobicity assay, which clearly showed that \textit{S. denitrificans} cells possess a hydrophobic surface. We discuss further evidence for the proposed adhesin-mediated cholesterol uptake later in the context of the uptake experiments.

Accumulating evidence indicates that the OM of Gram-negative bacteria serves as a selective permeability barrier, because its asymmetric structure with lipopolysaccharides results in an unusually slow influx of hydrophobic compounds such as steroids (49). Previous investigations have revealed that the diffusion of steroids across the OM of non-steroid degrading Gram-negative bacteria was approximately two orders of magnitude slower than diffusion through a cytoplasmic membrane containing phospholipid bilayers (17, 49). Although steroids could passively diffuse through the OM bilayer of \textit{S. denitrificans}, our current data disproved the crucial role of passive diffusion in the cholesterol uptake of \textit{S. denitrificans} because (i) the cholesterol uptake efficiency of cholesterol-grown \textit{S. denitrificans} cells was significantly higher than that of palmitate-grown \textit{S. denitrificans} cells and other tested proteobacterial species, (ii) the steroid uptake pattern of cholesterol-grown \textit{S. denitrificans} cells exhibited high substrate-selectivity, and (iii) the cholesterol uptake of \textit{S. denitrificans} is not enhanced by pre-incubation with EDTA. It is well documented that the OM of Gram-negative bacteria contains proteins that form channels for the facilitated diffusion of small molecules (20). Three types of OM channel have been observed: the porins, the FadL family, and the TBDRs (19-25). Our current data implied that the OM channel of \textit{S. denitrificans} is critical in cholesterol uptake, and the inducible transporter system appears to selectively import cholesterol across the OM. Unfortunately, the cholesterol transport system of \textit{S. denitrificans} is not currently studied at molecular levels. Nevertheless, it appears that the induced cholesterol transporter is neither a porin nor a TBDR-similar protein because (i) the porins are not substrate-specific and show no apparent differences in the uptake of different steroids; (ii) the OM cholesterol transporter is not sensitive to the respiratory inhibitor (azide) or the inhibitor of proton motive force (cyanide); and (iii) TBDRs, the active transporters, mediate the uptake of large (> 600 Da) hydrophilic molecules such as vitamin B \textsubscript{12} and iron siderophores (24, 25). Moreover, in the absence of carrier molecules [e.g., (2-hydroxypropyl)-\textbeta-cyclodextrin], \textit{S. denitrificans} cannot import cholesterol at concentrations below 1 \textmu M. Relevant literature demonstrated that, with the aid of active transporter system of TBDRs, bacteria are able to salvage vitamin B \textsubscript{12} from the environment, even below 50 nM (50). A study on the OM channels responsible for the uptake of monoaromatic hydrocarbons indicated that the OM proteins (TbuX and TodX) are highly substrate-specific (20). Both TbuX and TodX (belong to the FadL family) cannot transport long-chain fatty acids, although their primary structures are similar to that of the FadL in \textit{E. coli}. The OM steroid transporter of \textit{S. denitrificans} also exhibited high substrate-specificity which efficiently imports various C\textsubscript{27} sterols but not C\textsubscript{19} androgens. In addition, these hydrophobic channels contain covalent-bound detergent molecules located on the extracellular surface, corresponding to the observation of high surface hydrophobicity and the lack of biosurfactants in the cell-free medium of cholesterol-grown \textit{S. denitrificans} cultures.

As mentioned previously, bacteria cannot easily access cholesterol because of its extremely low aqueous solubility. One may envisage that bacteria have to activate and solubilize this substrate by secreting extracellular enzymes. Our data clearly showed that \textit{S. denitrificans} does not produce any extracellular cholesterol catabolic enzymes. Furthermore, no catabolic intermediates were detected in the cell-free media of the cholesterol-grown \textit{S. denitrificans} cultures. These results contradict those of previous studies which showed that some microorganisms produce extracellular enzymes (e. g., cholesterol oxidase) to transform cholesterol (29). In addition, during oxic degradation of bile acids by the Gram-negative \textit{Pseudomonas} sp. strain Chol1, catabolic intermediates are exported to the media (4, 51).
Similar extracellular accumulation of metabolites has been observed in aerobically cholate-grown *Rhodococcus jostii* RHA1 (52). It is appealing to postulate that, in case of *S. denitrificans*, cholesterol uptake precedes catabolic reactions.

In a previous investigation, AcmA was identified as an NAD+-dependent enzyme belonging to the short-chain dehydrogenase/reductase family (31). Here we showed that AcmA is a periplasmic protein. The existence of free NAD(P)⁺ in the periplasm seems unlikely for various reasons (53), although there is no direct experimental evidence regarding this matter. The primary structure of AcmA is highly similar to that of 3β-hydroxysteroid dehydrogenase isolated from various bacteria including *Comamonas testosteroni* (31). The 3β-hydroxysteroid dehydrogenase of *C. testosteroni* [formerly named *Pseudomonas testosteroni* (54)] was also reported to be a periplasmic protein (55). In the past decades, various NAD(P)⁺-dependent enzymes, including glucose-fructose oxidoreductase from *Zymomonas mobilis* (53) and glyceraldehydes-3-phosphate dehydrogenase from *Aeromonas hydrophila* (56), were also identified as periplasmic proteins. These periplasmic enzymes contain tightly associated NAD(P)⁺ which is not released during the catalytic cycle.

In a recent study, the S25DH of *S. denitrificans* was isolated and characterized (28), and suggested to be associated with the periplasmic side of the cytoplasmic membrane. In the oxic cholesterol catabolism of *Mycobacterium tuberculosis*, cholest-4-en-3-one is oxidized to cholest-4-en-3-one-27-oic acid by the bifunctional steroid C27 monooxygenase (CYP125A1), with 27-hydroxycholest-4-en-3-one serving as the intermediate (57). This oxygenase cannot function in anoxic cholesterol catabolism, and *S. denitrificans* adopts an alternative strategy to activate the acyl side-chain of cholesterol. The *S. denitrificans* enzymes catalyzing the oxidation of 25-hydroxycholest-4-en-3-one to cholest-4-en-3-one-26-oic acid have yet to be studied. The pyrroloquinoline quinone-dependent and FAD-dependent alcohol dehydrogenases and aldehyde dehydrogenases located on the periplasmic side of the Gram-negative bacterium cytoplasmic membranes have been thoroughly examined (58). However, these enzymes have not been reported to play a role in steroid metabolism.

Aerobic actinobacteria (7) and anaerobic *S. denitrificans* (this study) have been proposed to degrade side-chain of cholesterol through β-oxidation-like reactions. The current data suggested that side-chain degradation occurs in the *S. denitrificans* cytoplasm: (i) the C₁₉ androgens did not efficiently pass through the cytoplasmic membrane of *S. denitrificans*, and we detected no C₁₉ intermediates in the periplasm of *S. denitrificans*; and (ii) the fatty acid degradation through β-oxidation is a cytoplasmic process due to the ATP void in the periplasm (59). Although C₂₇-C₂₂ acidic intermediates were detected in the aerobically (10) and anaerobically (this study) cholesterol-grown *S. denitrificans* cultures, the proposed CoA-thioesters of the acidic steroid intermediates have never been observed. Therefore, the side-chain degradation mechanism awaits further investigations.

A previous study (60) suggested cholest-4-en-3-one to be the native substrate of AcmB; however, the enzymatic assays indicated that this FAD-dependent enzyme also catalyzes the oxidation of various steroid intermediates, including progesterone (C₂₁) and AD (C₁₉). Interestingly, AcmB appeared to prefer the C₂₁ and C₁₉ steroids over cholest-4-en-3-one (60). Our current results indicated that AcmB exists on the cytoplasmic side of the inner membrane of *S. denitrificans*. In the cholesterol catabolism of *S. denitrificans*, cholest-4-en-3-one is not the native substrate for AcmB, because its product, cholesta-1,4-diene-3-one, was never detected in the cholesterol-grown *S. denitrificans* cultures. By contrast, ADD, the 1,4-diene structure of AD, has been detected in aerobically (10) and anaerobically grown *S. denitrificans* cells. According to our results from subcellular localization and the substrate preference of AcmB, as well as the metabolomic investigations of cholesterol-grown *S. denitrificans* cultures, we suggest that AD is the physiological substrate of AcmB.

**SUMMARY**

Here we studied anoxic cholesterol catabolism at the subcellular level. Based on the presented results, we propose a model for cholesterol uptake and subcellular compartmentation during cholesterol catabolism by a Gram-negative bacterium (Fig. 10). The cells of *S. denitrificans* access cholesterol via direct adhesion followed by outer membrane transport. The facilitated diffusion might be
mediated by a member of the FadL family. In the periplasm, initial catabolic reactions, including oxidation of the 3-hydroxy group, double bond isomerization, and a series of side-chain activation reactions, occur. These reactions result in the transformation of cholesterol to cholest-4-en-3-one-26-oic acid, which then crosses the cytoplasmic membrane. In the cytoplasm, the side-chain degradation occurs through β-oxidation-similar reactions, followed by sterane cleavage and further catabolism. The bacterial cytoplasm was considered the major compartment in which hydrocarbon catabolism occurs. However, in this study, we showed that the enzymes located in the periplasmic space are critical to cholesterol catabolism, especially during the steps of substrate activation.
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FOOTNOTES

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The abbreviations used are: AcmA, cholesterol dehydrogenase; AcmB, cholest-4-en-3-one-Δ1-dehydrogenase; AD, androst-4-en-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; APCI, atmosphere pressure chemical ionization; HRMS, high-resolution mass spectrometry; MOPS, 3-morpholinopropanesulfonic acid; 2,3-SAOA, 17-hydroxy-1-oxo-2,3-seco-androstan-3-oic acid; S25DH, steroid C25 dehydrogenase; UPLC, ultra-performance liquid chromatography.

FIGURE LEGENDS

FIGURE 1. (A) Emulsification index (E24) and surface tension (ST) measurements in the S. denitrificans culture anaerobically grown on cholesterol. (B) Fraction of bacterial cells partitioned to the hydrocarbon phase measured using the BATH assay. The data shown are the means ± SE of three experimental measurements.

FIGURE 2. (A) Thin-layer chromatograms showing that cholesterol dehydrogenase activity was not detected in the cell-free medium. Lanes: 1, authentic steroid standards; 2, negative control (steroid substrate only, without the addition of S. denitrificans proteins); 3, negative control (crude cell extract only, without the
addition of a steroid substrate); 4, crude cell extract incubated with cholesterol; 5, negative control
(extracellular proteins only, without the addition of a steroid substrate); 6, extracellular proteins incubated with
cholesterol. (B) The extracellular steroid profile extracted from the denitrifying cholesterol-grown S.
denitrificans culture. Three steroid standards (10-100 μg) are shown in the upper HPLC chromatogram.

**FIGURE 3.** (A) Steroid uptake by whole cells of various proteobacteria. Cholesterol (25 μM) and 17β-estradiol
(25 μM) were added to the whole cell assays, which were incubated for 10 min. (B) Cell suspensions of S.
denitrificans anaerobically grown with different substrates (cholesterol, cholest-4-en-3-one, AD, or palmitate)
were tested for their cholesterol uptake ability. The data shown are the means ± SE of three experimental
measurements.

**FIGURE 4.** (A) Specific uptake rates of different steroids by cholesterol-grown S. denitrificans cells. (B)
Effects of the respiratory inhibitor (60 mM sodium azide), proton motive force inhibitor (10 mM KCN),
ATPase inhibitor (10 mM sodium orthovanadate), and OM permeabilizer (10 mM EDTA) on the cholesterol
uptake of S. denitrificans. The data shown are the means ± SE of three experimental measurements.

**FIGURE 5.** Steroid uptake by the spheroplasts of S. denitrificans anaerobically grown with cholesterol. (A)
Relative steroid uptake efficiency of S. denitrificans spheroplasts. The uptake efficiency of cholesterol (the
internal control) in each assay was set at one and the uptake rate of the second steroid substrate is shown
relative to that of cholesterol. (B) Effect of palmitate (0–100 μM) on the cholest-4-en-3-one-26-oic acid uptake
by S. denitrificans spheroplasts. The data shown are the means ± SE of three experimental measurements.

**FIGURE 6.** (A) Steroid uptake by the spheroplasts of various proteobacteria. S. denitrificans was anaerobically
grown on cholesterol, and the other proteobacteria were aerobically grown in rich medium. (B) Subcellular
distribution of malate dehydrogenase (a cytoplasmic marker protein) in the periplasm and cytoplasm of four
tested proteobacteria.

**FIGURE 7.** The subcellular fractionation of S. denitrificans proteins. (A) The distribution of malate
dehydrogenase and cytochrome c (periplasmic marker) in the periplasmic, cytoplasmic, and membrane
fractions. (B) The membrane fractions separated using sucrose density gradient centrifugation were collected
from the top of the gradient (left of the figure). The fractions containing the cytoplasmic membrane and OM
were identified based on NADH oxidase activity and KDO content, respectively.

**FIGURE 8.** Steroid transformation activities detected in various protein fractions. Proteins of anaerobically
cholesterol-grown S. denitrificans cells were separated into OM (0.4 mg/mL), periplasmic (1.5 mg/L),
cytoplasmic membrane (1M; 0.7 mg/mL), and cytoplasmic fractions (3.3 mg/L). Four protein fractions were all
adjusted to 10 mL, and 0.25 mL of the fractionated S. denitrificans proteins was added to individual steroid
transformation assay mixtures. The data shown are the means ± SE of three experimental measurements.

**FIGURE 9.** Subcellular localization of AcmB. (A) Dissociation of membrane-bound AcmB induced by a 0.2%
Tween 20 treatment. A1, total proteins (without a Tween 20 treatment); A2, membrane-bound proteins (without
a Tween 20 treatment); A3, soluble proteins (without a Tween 20 treatment); A4, membrane-bound proteins
(with a Tween 20 treatment); A5, soluble proteins (with a Tween 20 treatment). (B) Malate dehydrogenase
activity detected in peripheral-periplasmic and peripheral-cytoplasmic protein fractions of the Tween 20-treated
S. denitrificans spheroplasts. (C) AcmB activity detected in the peripheral-periplasmic and peripheral-
cytoplasmic protein fractions of the Tween 20-treated S. denitrificans spheroplasts.

**FIGURE 10.** The proposed model of substrate uptake and subcellular compartmentation of anoxic cholesterol
catabolism by S. denitrificans cells.
| Compound ID                  | UPLC behavior (RT\textsuperscript{a},min) | Molecular formula/ predicted molecular mass\textsuperscript{b} | Most dominant ion peak/ predicted elemental composition | Identification of product ion |
|-----------------------------|------------------------------------------|---------------------------------------------------------------|--------------------------------------------------------|-------------------------------|
| Cholesterol*                | 11.78                                    | C\textsubscript{27}H\textsubscript{46}O\textsubscript{3} 386.3537 384.3381 | 370.3555 12C\textsubscript{26} 13CH\textsubscript{45} | [M-H\textsubscript{2}O+1+H]\textsuperscript{+} |
| Cholest-4-en-3-one*         | 11.48                                    | C\textsubscript{27}H\textsubscript{44}O\textsubscript{2} 386.3504 12C\textsubscript{26} 13CH\textsubscript{46}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| 25-Hydroxycholest-4-en-3-one** | 8.04                                    | C\textsubscript{27}H\textsubscript{44}O\textsubscript{2} 400.3330 | 402.3453 12C\textsubscript{26} 13CH\textsubscript{46}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| 26-Hydroxycholest-4-en-3-one* | 8.37                                    | C\textsubscript{27}H\textsubscript{44}O\textsubscript{2} 400.3330 | 402.3430 12C\textsubscript{26} 13CH\textsubscript{46}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| Cholest-4-en-26-al-3-one    | 8.53                                     | C\textsubscript{27}H\textsubscript{42}O\textsubscript{2} 398.3174 | 400.3295 12C\textsubscript{26} 13CH\textsubscript{46}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| Cholest-4-en-3-one-26-oic acid* | 8.24                                    | C\textsubscript{27}H\textsubscript{42}O\textsubscript{3} 414.3123 | 416.3246 12C\textsubscript{26} 13CH\textsubscript{46}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| Cholest-4-en-3-one-24-oic acid* | 5.81                                    | C\textsubscript{24}H\textsubscript{36}O\textsubscript{3} 372.2655 | 374.2768 12C\textsubscript{23} 13CH\textsubscript{36}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| Pregn-4-en-3-one-20-carboxylic acid* | 5.10                                    | C\textsubscript{22}H\textsubscript{32}O\textsubscript{3} 346.2463 | 348.2343 12C\textsubscript{21} 13CH\textsubscript{32}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| Androst-4-en-3,17-dione (AD)* | 3.62                                    | C\textsubscript{20}H\textsubscript{26}O\textsubscript{2} 286.1926 | 288.2052 12C\textsubscript{18} 13CH\textsubscript{26}O\textsubscript{2} | [M+1+H]\textsuperscript{+} |
| Androsta-1,4-diene-3,17-dione (ADD)* | 3.21                                    | C\textsubscript{20}H\textsubscript{26}O\textsubscript{2} 284.1770 | 286.1994 12C\textsubscript{18} 13CH\textsubscript{26}O\textsubscript{2} | [M+1+H]\textsuperscript{+} |
| Androstan-1,3,17-trione     | 2.40                                     | C\textsubscript{19}H\textsubscript{26}O\textsubscript{3} 304.1994 12C\textsubscript{18} 13CH\textsubscript{26}O\textsubscript{3} | [M+1+H]\textsuperscript{+} |
| 17-hydroxy-1-oxo-2,3-seco-androstan-3-oic acid (2,3-SAOA)** | 2.76                                     | C\textsubscript{19}H\textsubscript{30}O\textsubscript{4} 322.2136 | 306.2150 12C\textsubscript{18} 13CH\textsubscript{26}O\textsubscript{3} | [M-H\textsubscript{2}O+1+H]\textsuperscript{+} |

\textsuperscript{a}RT, retention time. \textsuperscript{b}The predicted molecular mass was calculated using the atom mass of 12C (12.0000), 16O (15.9949), and 1H (1.0078). *, compounds exhibited identical UPLC-HRMS behavior with steroid standards purchased from Sigma-Aldrich, Avanti Polar Lipids, or Steraloids. **, compounds exhibited identical UPLC-HRMS behavior with NMR-confirmed steroids produced in our lab.
Fig. 1

(A) Emulsification index (%) and surface tension (mN/m) for cell suspension, cell-free supernatant, and uninoculated medium.

(B) Fraction partitioned to n-hexadecane for P. aeruginosa and S. denitrificans (palmitate-fed) and S. denitrificans (cholesterol-fed).
Fig. 2

(A) cholest-4-en-3-one

cholesterol

1 2 3 4 5 6

(B) cholesta-1,4-diene-3-one (10 µg)
cholest-4-en-3-one (10 µg)
cholesterol (100 µg)

Absorbance at 215 nm (arbitrary units)

Retention time (min)
Fig. 3

(A)

(B)
Fig. 4

(A) Steroid uptake rate (nmol/min/mg protein)

(B) Cholesterol uptake rate (nmol/min/mg protein)
Fig. 5
Fig. 6

(A) Steroid uptake rate (nmol/min/mg protein)

- S. denitrificans (β-subgroup)
- P. aeruginosa (γ-subgroup)
- A. tumefaciens (α-subgroup)
- E. coli (γ-subgroup)

(B) Malate DH activity (nmol/min/mg protein)

- Cytoplasmic fraction
- Periplasmic fraction

S. denitrificans  P. aeruginosa  A. tumefaciens  E. coli
Fig. 7

(A) 

(B) 

Protein concentration (µg/mL) 

NADH oxidase activity (µmol/mL/min) 

KDO concentration (µg/mL) 

Membrane fraction
Fig. 8

(A) Relative Aox1 activity (%)
(B) Relative Aox1 activity (%)
(C) Relative SOD1 activity (%)
(D) Relative TIP42 oxidoreductase activity (%)
(E) Relative steroid 14α-DH activity (%)
(F) Relative 1-ketoestradiol synthase activity (%)
(G) Relative 1-Methanol synthase activity (%)
Fig. 9

(A) 25-hydroxycholest-4-en-3-one
25-hydroxycholesta-1,4-diene-3-one

(B) Incubation time (min)

Relative malate dehydrogenase (cytoplasm/periplasm)

(C) Peripheral-periplasmic fraction
Peripheral-cytoplasmic fraction

AcmB activity (nmol/min/mg protein)
Fig. 10
Substrate uptake and subcellular compartmentation of anoxic cholesterol catabolism in *Sterolibacterium denitrificans*

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