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Study of Anoxic and Oxic Cholesterol Metabolism by *Sterolibacterium denitrificans*▽

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The initial enzymes and genes involved in the anoxic metabolism of cholesterol were studied in the denitrifying bacterium *Sterolibacterium denitrificans* Chol-1S^T. The second enzyme of the proposed pathway, cholest-4-en-3-one-Δ^1-dehydrogenase (AcmB), was partially purified. Based on amino acid sequence analysis, a gene probe was derived to screen a cosmid library of chromosomal DNA for the *acmB* gene. A positive clone comprising a 43-kbp DNA insert was sequenced. In addition to the *acmB* gene, the DNA fragment harbored the *acmA* gene, which encodes the first enzyme of the pathway, cholesterol dehydrogenase/isomerase. The *acmA* gene was overexpressed, and the recombinant dehydrogenase/isomerase was purified. This enzyme catalyzes the predicted transformation of cholesterol to cholest-4-en-3-one. *S. denitrificans* cells grown aerobically with cholesterol exhibited the same pattern of soluble proteins and cell extracts formed the same ^{14}C-labeled products from [^{14}C]cholesterol as cells that were grown under anoxic, denitrifying conditions. This is especially remarkable for the late products that are formed by anaerobic hydroxylation of the cholesterol side chain with water as the oxygen donor. Hence, this facultative anaerobic bacterium may use the anoxic pathway lacking any oxygenase-dependent reaction also under oxic conditions. This confers metabolic flexibility to such facultative anaerobic bacteria.

Steroids are a diverse group of molecules that are abundant in the environment. Large amounts of steroids and their derivatives are synthesized by eukaryotes, and these molecules play very important and diverse roles and include hormones, detergents that facilitate the absorption of lipids by the intestine, and membrane constituents (27). Some of them, cholesterol and related compounds, are ubiquitous as membrane constituent of eukaryotes (for instance, up to 10% of the dry mass of yeast is ergosterol) and as the precursors of all steroid hormones, vitamin D, and the bile acids (27). Interestingly, with exception of methane-oxidizing bacteria that contain relatively large amounts of sterols, sterols are absent from most prokaryotes. However, many bacterial membranes contain hopanoids, which are similar pentacyclic sterol-like molecules (26, 30).

The ubiquity of cholesterol and related sterols in the environment has made them a common carbon source for microorganisms belonging to various physiological groups. However, the microbial degradation of cholesterol, in particular, is challenging because of its complex chemical structure, low solubility in water, and low number of functional groups and the presence of four alicyclic rings and two quaternary carbon atoms (12). Several bacterial genera, such as *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, and *Rhodococcus*, reportedly mineralize cholesterol in the presence of molecular oxygen (19). The oxic pathway for cholesterol metabolism includes at least four transformations that require molecular oxygen as a cosubstrate (19). Despite the importance of the cholesterol-metabolizing enzymes in pharmaceutical and clinical applications, only a few of the enzymes have been studied in more detail. The first enzyme in the oxic pathway for cholesterol catabolism (cholesterol oxidase or dehydrogenase) has been purified from several bacterial strains and characterized (5, 10, 20, 24, 25, 40). Cholesterol oxidase (dehydrogenase), which catalyzes the oxidation of cholesterol to cholest-4-en-3-one, is a flavin adenine dinucleotide- or NAD(P)-dependent enzyme that requires molecular oxygen as an electron acceptor (14, 25, 31). Genes involved in oxic cholesterol metabolism have been found in *Rhodococcus* sp. (39).

Compared to the oxic metabolism of cholesterol, the metabolism of this substrate in the absence of oxygen is very poorly understood (for a review of the biotransformation of monoterpenes, bile acids, and other isoprenoids, including cholesterol, in anoxic ecosystems, see reference 15). The best-studied anoxic reactions involve mainly incomplete transformation of cholesterol. The well-known transformations include double bond reduction, which transforms cholesterol to coprostanol, by intestinal fermentative bacteria (9).

In the last few years, two bacterial strains were reported to mineralize cholesterol to carbon dioxide under anoxic conditions. Both of these strains, 72Chol and *Sterolibacterium denitrificans* Chol-1S^T (= DSMZ 13999^T), are strictly respiratory with nitrate or oxygen as the electron acceptor and are closely related to the genera *Thauera* and *Azoarcus* (β-subclass of the *Proteobacteria*) (12, 38). *S. denitrificans* can grow with cholesterol both under oxic conditions and under strictly anoxic conditions (when nitrate is supplied as an electron acceptor).

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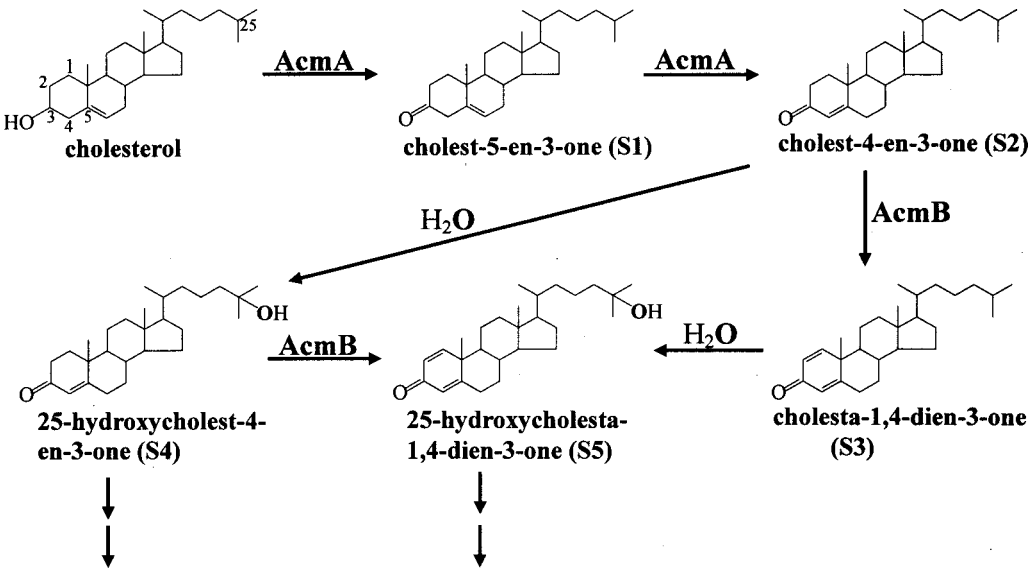

FIG. 1. Proposed initial steps in the anoxic cholesterol metabolic pathway in *S. denitrificans*.

We previously reported on the initial steps of anoxic cholesterol metabolism and identified five products formed from [^{14}C]cholesterol by cell extracts of *S. denitrificans* after growth under anoxic conditions (Fig. 1) (6). The anoxic pathway starts with the oxidation of cholesterol to cholest-5-en-3-one (Fig. 1, compound S1), which is isomerized to cholest-4-en-3-one (S2). The latter compound is then oxidized to 25-hydroxycholest-4-en-3-one (S4); alternatively, these intermediates can originate as the corresponding 1,2-dehydro structures, cholesta-1,4-dien-3-one (S3) and 25-hydroxycholesta-1,4-dien-3-one (S5). Obviously, the formation of the 25-hydroxy derivatives in an oxygen-independent reaction represents a novel enzyme reaction, since the tertiary carbon of the side chain is hydroxylated using water as the oxygen donor. This is in contrast to the oxygenase-catalyzed hydroxylation of the terminal methyl carbon in the known oxic pathway (Fig. 1). We expect that there are further unprecedented reactions and enzymes in the anoxic pathway that must replace oxygen-dependent hydroxylation and ring cleavage steps.

Here, we set out to study the initial enzymes and genes involved in anoxic cholesterol metabolism. We identified the genes encoding the two initial enzymes of the pathway on a 43-kbp DNA fragment of chromosomal DNA. The gene encoding the first enzyme was cloned and successfully expressed in *Escherichia coli*. Cells grown aerobically with cholesterol exhibited the same pattern of soluble proteins and cell extracts formed the same labeled products from [^{14}C]cholesterol as cells that were grown under anoxic, denitrifying conditions. This is especially remarkable for the late products mentioned above that are formed by anaerobic hydroxylation of the cholesterol side chain. Hence, even under oxic conditions this facultative anaerobic bacterium appears to use the anoxic pathway lacking any oxygenase-dependent reaction.

MATERIALS AND METHODS

Materials and bacterial strains. The chemicals used were analytical grade and were obtained from Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), and Roth (Karlsruhe, Germany). Biochemicals were obtained from Fermentas (St. Leon-Rot, Germany), Roche Diagnostics (Mannheim, Germany), and Amersham Biosciences (Freiburg, Germany). Proofreading DNA polymerase was obtained from Genaxxon Bioscience GmbH (Biberach, Germany). A QIAquick gel extraction kit was obtained from Qiagen (Hilden, Germany). The bacterial strains and plasmids used in this study are shown in Table 1.

Bacterial cultures. *S. denitrificans* was grown anaerobically at 30°C under a nitrogen atmosphere. Large-scale cultivation was performed in a 200-liter fermentor. The medium used for anaerobic cultivation of *S. denitrificans* has been described previously (6). Cells were harvested in the exponential growth phase at an optical density at 578 nm (OD_{578}) of 1.5 (optical path length, 1 cm). The culture was precooled to 8°C, and cells were harvested by continuous-flow cen-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference
Supercos1	*amp neo*; cloning vector for construction of the cosmid gene library	Stratagene
pMal-c2x	*amp*; overexpression vector	New England Biolabs
pWI1-5	pMal-c2x carrying *acmA*	This study
pYRC1	Supercos1 carrying the 42.7-kbp insert	This study
E. coli XL1-Blue MR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
E. coli XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 hsdR17 recA1 gyrA46 thi relA lac* F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^r)]	Stratagene
S. denitrificans Chol-1S^T (= DSMZ 13999^T)	Wild type	38

trifugation at 4°C and then stored at −70°C immediately. For aerobic growth on cholesterol, $NaNO_3$ was omitted and $NaHCO_3$ was replaced by 0.4 g liter^{-1} KH_2PO_4 and 1.2 g liter^{-1} K_2HPO_4 as the buffer. For mRNA isolation experiments, *S. denitrificans* cells were grown anaerobically or aerobically on 2.5 mM cholesterol in a 200-ml fed-batch culture. Cells were harvested by centrifugation at 4°C in the exponential growth phase at an OD_{578} of 0.6 to 0.8 (optical path length, 1 cm). The pellet was used immediately for mRNA isolation.

Preparation of cell extracts. All steps used for preparation of cell extracts were performed at 4°C. Frozen cells were suspended in 2 volumes of 20 mM 3-morpholinopropanesulfonic acid (MOPS)-K^+ buffer (pH 7.9) containing 0.1 mg of DNase I ml^{-1}. Cells were broken by passing the cell suspension through a French pressure cell (American Instruments, Silver Spring, MD) twice at 137 MPa. The cell lysate was fractionated by two centrifugation steps. The first step involved centrifugation for 30 min at 20,000 × *g* to get rid of the cell debris, unbroken cells, and residual cholesterol. The supernatant (crude cell extract) was then centrifuged at 100,000 × *g* for 1.5 h to separate soluble proteins from membrane proteins.

Partial purification of cholest-4-en-3-one-Δ^1-dehydrogenase from *S. denitrificans*. Cholest-4-en-3-one-Δ^1-dehydrogenase (AcmB) was purified from 45 g of *S. denitrificans* cells grown anaerobically on cholesterol as described previously (7).

Purification of the recombinant cholesterol dehydrogenase/isomerase from *E. coli*. Cloning of the *acmA* gene in pMal-c2x resulted in production of a recombinant protein with an N-terminal maltose binding protein tag (Mal). This recombinant cholesterol dehydrogenase/isomerase ($AcmA_{mal}$) was purified in two steps from cell extract of *E. coli* XL1-Blue MRF′ containing the overproduced recombinant enzyme.

(i) Affinity chromatography. A 25-ml amylose resin column (New England Biolabs, Frankfurt, Germany) was equilibrated with 60 ml of 20 mM Tris-HCl (pH 7.9) containing 200 mM NaCl. The column was then loaded with the cell extract (20 ml) and washed with 150 ml of the equilibration buffer. Elution was performed with 50 ml of the equilibration buffer containing 10 mM maltose. The $AcmA_{mal}$ pool was concentrated 10-fold (30-kDa-cutoff membrane; Amicon) and then stored at −20°C or used immediately for the next fractionation step. The flow rate was 1 ml min^{-1}, and the eluate was collected in 2-ml fractions.

(ii) Gel filtration chromatography. Final purification of $AcmA_{mal}$ was carried out using a 120-ml Superdex 200 HiLoad 16/60 gel filtration column (Amersham Biosciences Europa GmbH). A protein sample (2 ml) from the previous purification step was applied to the column, which was equilibrated with 3 bed volumes of the equilibration buffer (20 mM Tris-HCl, pH 7.9) containing 100 mM KCl. The flow rate was 0.5 ml min^{-1}, and 3-ml fractions were collected. The active protein pool was concentrated 10-fold and frozen at −20°C.

Enzyme assays. In vitro assays were routinely performed at 30°C for 2 h. Assay mixtures were incubated under anoxic conditions when anaerobically grown cells were tested and under oxic conditions when aerobically grown cells were tested. The assay mixtures (0.6 ml) contained protein (50 μg to 10 mg), 20 mM MOPS-K^+ buffer (pH 7.9), 5 mM artificial electron acceptor (NAD^+ for cholesterol dehydrogenase/isomerase and 2,6-dichlorophenolindophenol for cholest-4-en-3-one-Δ^1-dehydrogenase), and a steroid substrate (0.2 mM [4C-^{14}C]cholesterol [120,000 dpm] for cholesterol dehydrogenase/isomerase and 0.5 mM cholest-4-en-3-one for cholest-4-en-3-one-Δ^1-dehydrogenase). For production of S1 to S3 and AE1 to AE3, the assay mixtures (0.6 ml) contained soluble proteins (10 mg) extracted from *S. denitrificans* grown anaerobically or aerobically on cholesterol, 20 mM MOPS-K^+ buffer (pH 7.9), 5 mM NAD^+, and 0.2 mM [4C-^{14}C]cholesterol (120,000 dpm). For production of S4, S5, AE4, and AE5, soluble proteins extracted from *S. denitrificans* were precipitated at 25% ammonium sulfate saturation at 4°C. After centrifugation (20,000 × *g*, 4°C, 20 min) the protein pellet was redissolved in 20 mM MOPS-K^+ buffer (pH 7.9). The assay mixtures (0.6 ml) contained ammonium sulfate-precipitated protein (1 mg), 20 mM MOPS-K^+ buffer (pH 7.9), 5 mM $K_3[Fe(CN)_6]$, and 0.5 mM cholest-4-en-3-one. The assay mixtures were first extracted twice with an equal volume of ethyl acetate, and the ethyl acetate fraction was concentrated under vacuum. The standards and extracted products were separated on silica gel aluminum thin-layer chromatography (TLC) plates (Silica Gel 60 F_{254}; thickness, 0.2 mm; 20 by 20 cm; Merck, Darmstadt, Germany). The following solvent system was used: *n*-hexane–ethyl acetate (65:35, vol/vol). The products were localized by autoradiography with a phosphorimaging plate (Fuji Photo Film Co., Ltd., Kanagawa, Japan) or were visualized under UV light at 254 nm. To produce large amounts of these intermediates for high-performance liquid chromatography (HPLC) analysis, the reaction mixtures were enlarged to 6 ml.

HPLC. The following systems were used for separation and identification of products formed from [4C-^{14}C]cholesterol and cholest-4-en-3-one.

(i) System 1. For separation and identification of products S1 to S3 and AE1 to AE3, an analytical RP-C_{18} column (LiChrospher 100; end capped; 5 μm; 120 by 4 mm; Merck, Darmstadt, Germany) was used with a flow rate of 0.8 ml min^{-1} at room temperature. The mobile phase comprised a mixture of two solvents, solvent A (30% [vol/vol] acetonitrile) and solvent B (80% [vol/vol] 2-propanol). Separation was performed with a linear 80 to 90% solvent B gradient in 30 min. ^{14}C-labeled products were detected by using two detectors (a UV detector at 210 or 240 nm and a flowthrough radioactivity detector with a solid scintillation cell connected to the HPLC system) in series.

(ii) System 2. An analytical RP-C_{18} column [Luna 18(2); 5 μm; 150 by 4.6 mm; Phenomenex, Aschaffenburg, Germany] was used for separation and identification of products S4, S5, AE4, and AE5. Solvents A and B in the mobile phase and the detectors were the same as those described above for system 1. Separation was performed with a linear 40 to 70% solvent B gradient in 30 min and a flow rate of 0.8 ml min^{-1} at room temperature.

Determination of total protein concentration. The total protein concentration was determined by using the Bradford method (3) with bovine serum albumin as the standard.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) was performed with a discontinuous buffer system (23). Protein bands were visualized by Coomassie blue staining. The following protein standards were used: phosphorylase B (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), lactate dehydrogenase (34 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa).

2D gel electrophoresis. For two-dimensional (2D) gel electrophoresis, separation in the first dimension was performed by isoelectric focusing and separation in the second dimension was performed by SDS-PAGE. For the first dimension, carrier ampholyte (pH 5 to 8) gels were prepared in glass tubes (diameter, 2.3 mm; length, 16 cm) (1). Separation in the second dimension was performed by SDS-PAGE (10% polyacrylamide) using a gel that was 20 by 20 by 0.1 cm. Soluble proteins (300 μg) extracted from *S. denitrificans* cells grown aerobically and anaerobically on cholesterol were used as the protein samples for comparison. Proteins on the SDS-polyacrylamide gels were visualized by colloidal Coomassie blue staining.

In-gel digestion of protein samples. Each SDS-PAGE gel spot was dried under vacuum pressure. In-gel digestion was performed with an automated protein digestion system (MassPREP station; Micromass, Manchester, United Kingdom). The gel slices were washed three times in a mixture containing 25 mM NH_4HCO_3 and acetonitrile (1:1, vol/vol). The cysteine residues were reduced with 50 μl of 10 mM dithiothreitol at 57°C and were alklyated with 50 μl of 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were cleaved in the gel with 40 μl of a 12.5-ng μl^{-1} solution of modified porcine trypsin (Promega, Madison, WI) in 25 mM NH_4HCO_3 at room temperature for 14 h. The resulting tryptic peptides were extracted with 60% acetonitrile in 5% formic acid and then with 100% (vol/vol) acetonitrile. The peptide extracts were used for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS and data interpretation. Nano-scale capillary LC-MS/MS analysis of the resulting tryptic peptides was performed using a CapLC capillary LC system (Micromass) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF II; Micromass). Chromatographic separation was performed with a Pepmap C_{18} reverse-phase capillary column (inside diameter, 75 μm; length, 315 mm; LC Packings, Sunnyvale, CA) and a flow rate of 200 nl min^{-1}, using a precolumn split. External calibration was performed using a 2-pmol μl^{-1} [Glu1]-fibrinopeptide B solution. Mass data acquisition was piloted by MassLynx 4 software (Micromass) using automatic switching between MS and MS/MS modes. Classical protein database searches were performed using a local Mascot (Matrix Science, London, United Kingdom) server. To be accepted for identification, an error of less than 100 ppm for the parent ion mass was tolerated, and the sequences of the peptides were manually checked. One missed cleavage per peptide was allowed, and some modifications were taken into account (carbamidomethylation for Cys and oxidation for Met). In addition, the searches were performed without constraining protein M_r and pI and without any taxonomic specifications. These searches did not always lead to positive identification since the *S. denitrificans* genome has not been sequenced yet. In such cases, the use of a de novo sequencing approach was necessary for successful identification. For this purpose, the MS/MS spectra were interpreted with the PepSeq tool from the MassLynx 4 (Micromass) software, as well as the PEAKS Studio software (version 3; Bioinformatics Solutions, Waterloo, Canada). The resulting peptide sequences were submitted to the BLAST program provided at the EMBL site (http://dove.embl-heidelberg.de/Blast2/msblast.html) in order to identify them by homology with proteins present in the databases. We used the MS-BLAST specifically modified PAM30MS scoring matrix, no filter was set, and the nrdb95 database was used for the searches, as

TABLE 2. Primers used in this study

Primer	Sequence[a]	Use
KSDH_for	GTSGTSCTSGGCGCSGGCGGCTTCGA	PCR, for a 857-bp DNA probe
KSDH_rev	AAGGCGAASGTCATSGCSGGGCC	PCR, for a 857-bp DNA probe
MOE3_for	ACGAGGTCGTCACCGGCTTC	RT-PCR, ORF1 (fragment 1, 298 bp)[b]
MOE3_for	CGCTTGCCGTTCCGTTCGAT	RT-PCR, ORF1
CDE1_for	TCCCTGGCTGTTCGGATTCCAT	RT-PCR, *acmA* (fragment 2, 413 bp)[b]
CDE1_rev	AATGCGCGAGCAGATAACGTCG	RT-PCR, *acmA*
TRANS_for	GCTTCGAACATGGCGACGTT	RT-PCR, ORF2 (fragment 3, 451 bp)[b]
TRANS_rev	GAGAGGCGCTGCGTTACAT	RT-PCR, ORF2
FBP_for	GAATTCGCGCCGGTAATGAG	RT-PCR, ORF3 (fragment 4, 565 bp)[b]
FBP_rev	AGCAGTGAAGCAGAGAACGT	RT-PCR, ORF3
TREG_for	AAATCAGGCATCGATCCGCT	RT-PCR, ORF4 (fragment 5, 523 bp)[b]
TREG_rev	AGTCGAAATCGCAGACGAGGT	RT-PCR, ORF4
KSDHE2_for	CTGGAAAGCGCGCCGCAGAT	RT-PCR, *acmB* (fragment 6, 501 bp)[b]
KSDHE2_rev	CGTCTGGCCGTTGCGCTTGA	RT-PCR, *acmB*
INT1_for	ACCTTCTCGTCGAATCCCGT	RT-PCR, INT1 (fragment 7, 430 bp)[b]
INT1_rev	ATGGCACCGCAAGCGCCGGT	RT-PCR, INT1
INT2_for	ACGTTATCTGCTCGCGCATT	RT-PCR, INT2 (fragment 8, 897 bp)[b]
INT2_rev	AACGTCGCCATGGTCGAAGC	RT-PCR, INT2
INT3_for	CGGTGTTGATCCAGTTGTCTA	RT-PCR, INT3 (fragment 9, 1,648 bp)[b]
INT3_rev	CGGCTCGGAACTCCATCGCT	RT-PCR, INT3
INT4_for	ACGTTCTCTGCTTCACTGCT	RT-PCR, INT4 (fragment 10, 862 bp)[b]
INT4_rev	AGCGGATCGATGCCTGATTT	RT-PCR, INT4
NC0_for[c]	AGAGTCAGCAATTGGTTCTT	RT-PCR, negative control (520 bp)
NC0_rev[c]	ATTGAGATTGAAGTTGAGAT	RT-PCR, negative control
AcmA-EcoRI_for	CCGGAATTCAAGACGGTGCTCGTCACC	PCR, cloning *acmA*
AcmA-HindIII_rev	CCCAAGCTTTCAGGAAAGATAGAGGCGGCG	PCR, cloning *acmA*

[a] S indicates a mixture of G and C.
[b] See Fig. 4.
[c] NC0_for and NC0_rev were used to amplify the DNA fragment at positions 32,300 to 32,820 of the 43-kbp DNA insert.

described by Castro et al. (4). Statistical evaluation of the results and validation of the matches were performed as described by Shevchenko et al. (36).

Gas chromatography-electron impact MS. MS analyses were performed using an Agilent Technologies 6890N gas chromatograph equipped with a split/splitless programmed temperature injector and an HP-5MS fused silica column (30 m by 0.25 mm; film thickness, 0.25 μm) connected to an Agilent 5975 inert MSD quadrupole spectrometer. The mass spectrometer was operated in electron impact mode at 70 eV, and spectra were recovered over a mass range from m/z 50 to 500 with a cycle time of 1.6 scans s^{-1}. The oven temperature was programmed to increase from 180 to 300°C at a rate of 6°C min^{-1}, and then the oven was kept isothermal for 10 min. The other conditions were as follows: helium split, 1:10; constant flow rate, 1.5 ml/min; transfer line temperature, 280°C; and MS source temperature, 230°C. Samples were injected using the split mode and a ratio of 1:10 with an autoinjector; the temperature of the injector was 280°C.

DNA manipulations. Standard protocols were used for DNA cloning, transformation, amplification, and isolation (2, 33).

PCR. A PCR mixture containing a *Taq* polymerase, genomic DNA isolated from *S. denitrificans*, and degenerate primers (Table 2) deduced from tryptic peptides of cholest-4-en-3-one-Δ^1-dehydrogenase was used to amplify a 857-bp DNA probe from the *acmB* gene. The following PCR program was used: 5 min at 95°C, followed by 40 cycles of 95°C for 45 s, 70°C for 30 s, and 72°C for 3 min and then incubation for 5 min at 72°C. The *acmA* gene encoding cholesterol dehydrogenase was amplified from genomic DNA of *S. denitrificans* using primers AcmA-EcoRI_for and AcmA-HindIII_rev (Table 2). The following conditions were used: 5 min at 95°C, followed by 35 cycles of 95°C for 45 s, 65°C for 30 s, and 72°C for 2 min. After the 35 cycles the PCR mixtures containing proofreading DNA polymerase (Pfunds DNA polymerase) were incubated for 5 min at 72°C.

RT-PCR. Total RNA was isolated from *S. denitrificans* cells grown anaerobically or aerobically on cholesterol by using an RNeasy mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Contaminating DNA was removed from the isolated total RNA by treating it with fast protein liquid chromatography-purified RNase-free DNase I (2 U per μg of total RNA; Amersham Pharmacia Europa GmbH, Freiburg, Germany) at 37°C for 30 min; 2.5 μg of purified total RNA was used to prepare cDNA. Reverse transcription-PCR (RT-PCR) was performed at 45°C for 1 h by using a Moloney murine leukemia virus reverse transcriptase (RevertAidM-MuLV RT) and a mixture of completely random hexanucleotides (RevertAid first strand cDNA synthesis kit; Fermentas, St. Leon-Rot, Germany) for random priming. Gene expression was studied by amplification of regions in the open reading frames (ORFs) and intergenic regions between ORFs. The sequences of the primers used are shown in Table 2. Complete removal of residual DNA from the total RNA preparation was verified by amplifying the intergenic region between two ORFs (the ORF coding for putative peptidoglycan-binding LysM and the ORF coding for putative *N*-formylmethionyl-tRNA deformylase) coding in different directions, with cDNA as the template. A mixture of random hexanucleotides was used for random priming.

Construction and screening of a cosmid gene library of *S. denitrificans*. A cosmid gene library of *S. denitrificans* was constructed as described by Redenbach et al. (32) by using a SuperCos1 cosmid vector kit and a Gigapack III Gold packaging extract kit (Stratagene, Amsterdam, The Netherlands). The 857-bp DNA probe used to screen the cosmid gene library of *S. denitrificans* was labeled with digoxigenin-11-dUTP. DNA-DNA hybridization was performed at 70°C overnight. The probe signals were detected by using a digoxigenin nucleic acid detection kit (Roche Diagnostics, Mannheim, Germany) containing anti-digoxigenin-alkaline phosphatase antibody conjugate and the color substrates 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate. The cosmid DNA with a positive signal was isolated with a NucleoBond PC 20 (Macherey-Nagel, Düren, Germany) plasmid DNA purification kit.

DNA sequencing. Sequencing of the PCR products was performed by G. Igloi (Freiburg, Germany), whereas shotgun sequencing of the cosmid DNA was performed by GATC Biotech (Konstanz, Germany). DNA sequences and the deduced amino acid sequences were analyzed by using the BLAST programs provided by the National Center for Biotechnology Information. Multiple alignments were generated by using the CLUSTALW program in the DNAman software package (Lynnon, Montreal, Canada).

Cloning and expression of the gene coding for cholesterol dehydrogenase/isomerase (AcmA). The *acmA* gene was amplified from chromosomal DNA of *S. denitrificans* using the primer pairs shown in Table 2. The PCR product and the cloning vector pMal-c2x (Table 1) were digested with EcoRI and HindIII, gel purified, and ligated. *E. coli* XL1-Blue MRF′ harboring the recombinant plasmid (pWI1-5 [Table 1]) was grown in 5 liters of LB medium containing ampicillin (100 μg ml^{-1}) at 37°C with shaking. When the OD_{578} reached 0.6 to 0.8, the

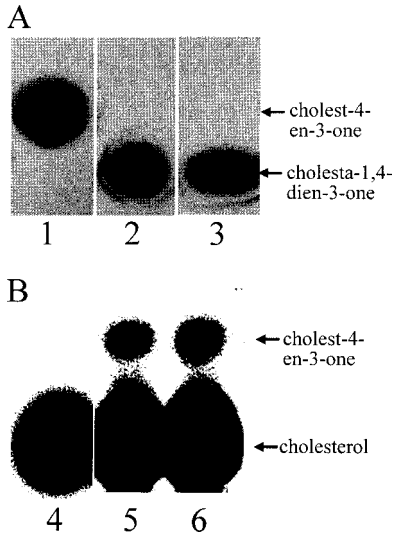

FIG. 2. Formation of products from cholesterol and cholest-4-en-3-one. (A) TLC showing the anoxic production of cholesta-1,4-dien-3-one from cholest-4-en-3-one in the presence of 2,6-dichlorophenolindophenol by partially purified cholest-4-en-3-one-Δ^1-dehydrogenase. Lane 1, negative control without protein; lane 2, mixture with partially purified cholest-4-en-3-one-Δ^1-dehydrogenase (0.5 mg); lane 3, same as lane 2 but incubated under oxic conditions. (B) TLC showing the anoxic production of ^{14}C-labeled cholest-4-en-3-one from [4C-^{14}C]cholesterol in the presence of NAD^+ by recombinant $AcmA_{mal}$ protein purified from *E. coli*. Lane 4, negative control with soluble protein fraction (10 mg) from *E. coli* carrying an empty plasmid; lane 5, mixture with purified recombinant $AcmA_{mal}$ protein (100 μg); lane 6, same as lane 5 but incubated under oxic conditions.

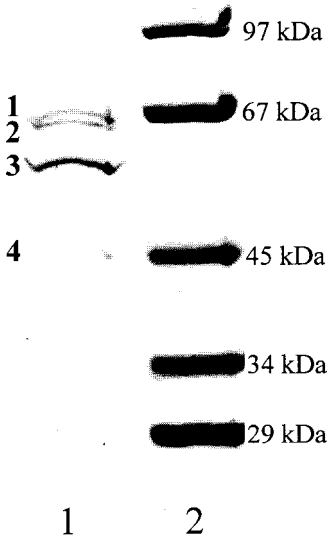

FIG. 3. SDS-PAGE analysis of partially purified cholest-4-en-3-one-Δ^1-dehydrogenase from *S. denitrificans* cells grown anaerobically on cholesterol. Lane 1, active pool after the gel filtration chromatography; lane 2, molecular mass standards.

inducer isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was then incubated at 37°C for 2 h.

Nucleotide sequence accession number. The sequence data reported in this study have been deposited in the GenBank database under accession number EU004090.

RESULTS

Partial purification of cholest-4-en-3-one-Δ^1-dehydrogenase (AcmB) from *S. denitrificans*. AcmB (*a*noxic *c*holesterol *m*etabolism) catalyzes the oxidation of cholest-4-en-3-one to cholesta-1,4-dien-3-one. From 2.3 g of soluble protein in cell extract only 3 mg protein in the active pool of the final gel filtration step was recovered. Accumulation of the expected product was observed both under anoxic and oxic conditions (Fig. 2A), indicating that AcmB activity was not affected by oxygen and did not require oxygen.

Analysis of the active gel filtration pool by SDS-PAGE revealed four proteins with the following molecular masses: protein 1, 67 kDa; protein 2, 65 kDa; protein 3, 60 kDa; and protein 4, 45 kDa (Fig. 3, bands 1 to 4, respectively). A database search revealed that the tryptic peptides originating from protein 3 are highly similar to 3-ketosteroid-Δ^1-dehydrogenase from *Comamonas testosteroni* (accession no. Q7WSH6) (24% coverage). The protein 3 band was dominant in the most active fractions. Hence, protein band 3 represents AcmB. It has a hydrophobic surface as it did not bind to ion exchangers (anion or cation) in the pH range from 6.0 and 9.0; in contrast, it bound strongly to butyl-Sepharose (hydrophobic interaction matrix) and required a detergent (Tween 20) for detachment from the matrix.

Contaminant protein 1 is similar to long-chain fatty acid-coenzyme A (CoA) ligase of *Azoarcus* sp. strain EbN1 (accession no. gi 56314165), protein 2 shows similarity to orotate phosphoribosyltransferase from *Mycobacterium avium* (gi 41409955), and protein 4 is similar to a subunit of an NADH: ubiquinone oxidoreductase of *Burkholderia fungorum* (gi 48784228).

Cloning and sequencing of genes coding for anoxic cholesterol metabolism. To search for catabolic genes coding for enzymes involved in anoxic cholesterol metabolism, degenerate primers (for DNA sequence) (Table 2) were derived from the amino acid sequences of tryptic peptides of AcmB (Fig. 3, band 3). Using these primers, an 857-bp DNA probe was amplified from the genomic DNA of *S. denitrificans*, which was used to screen a cosmid DNA library. Eleven positive clones were obtained during screening of 800 clones, and each of them had an insert whose average size was 40 kbp. Sequencing of one of the positive clones revealed a 42.7-kbp insert which harbored several ORFs, some of which were related to the anoxic metabolism of sterols. Sequence analysis also showed relatively large intergenic regions, as well as large noncoding segments.

Database similarity search and functional analysis of the ORFs. The putative functions of the sequenced genes and deduced gene products are summarized in Table 3. At the 5′ end of the sequenced DNA fragment, an incomplete ORF (ORF1) was found (Fig. 4). This ORF encodes the C-terminal region of a protein whose amino acid sequence is similar to that of a molybdenum-pterin binding protein of *Burkholderia cenocepacia* (Table 3). The *acmA* gene is 258 bp downstream of ORF1. AcmA is highly similar to cholesterol dehydrogenase from *Nocardia* sp. (Table 3), which catalyzes the first two reactions (oxidation and isomerization) during the oxic metab-

TABLE 3. Properties of genes and gene products assumed or proven to be involved in cholesterol metabolism in *S. denitrificans*

Gene	Gene properties		Protein properties[a]		Protein correspondence[b]			Similar protein in databases	Accession no.
	Position	Length (bp)	Deduced molecular mass (kDa)	Deduced pI	% Identity	% Similarity	E value		
ORF1[c]	2–832	831			30	36	1e-09	Molybdenum-pterin binding protein (*Burkholderia cenocepacia*)	ZP_00464910
acmA	1,091–2,161	1,071	38,629	9.5	34	48	2e-29	Cholesterol dehydrogenase (*Nocardia* sp.)	Q03704
ORF2	2,882–4,060	1,179	43,960	11.1	29	40	0.016	Bile acid:sodium symporter (*Paracoccus denitrificans*)	ZP_00632607
ORF3	5,546–6,694	1,149	42,436	11.9	27	34	0.009	Iron-sulfur binding protein with unknown function (*Kineococcus radiotolerans*)	ZP_00617246
ORF4	7,413–8,507	1,095	40,250	8.3	32	49	8e-36	Transcriptional regulator (*Burkholderia* sp.)	YP_373743
acmB	40,782–42,467	1,686	61,370	8.4	56	72	1e-167	3-Ketosteroid-Δ^1-dehydrogenase (*Comamonas testosteroni*)	Q7WSH6

[a] The molecular masses were derived from the 1997 IUPAC standard atomic weights, assuming pH 7.0. The isoelectric points were calculated by using the algorithm in ExPASy's compute pI/MW program.

[b] Similarity searches were done with the program BLASTP (http:/www.ncbi.nlm.nih.gov/BLAST/). The percentage of identity was defined as the percentage of amino acids that were identical in the proteins. The percentage of similarity was defined as the percentage of amino acids that were identical or conserved in the proteins. The E value is an estimate of the statistical significance of the match, which specifies the number of matches with a given score that is expected in a search of a database of the size absolutely by chance.

[c] ORF1 is incomplete.

olism of cholesterol (14). Since the same oxidation and isomerization were observed as initial steps in anoxic cholesterol metabolism in *S. denitrificans* (6), *acmA* probably encodes cholesterol dehydrogenase/isomerase. ORF2 codes for a protein that is similar to a bile acid:sodium symporter of *Paracoccus denitrificans* (Table 3). Considering the structural similarity between cholesterol and bile acids, the ORF2 product may be involved in the transport of cholesterol. The protein encoded by ORF3 is similar to an iron-sulfur binding protein of *Kineococcus radiotolerans* with an unknown function. ORF4 codes for a protein that is very similar to a transcriptional regulator from *Burkholderia* sp. Thus, the ORF4 product may be a transcriptional regulator of some of the genes of cholesterol metabolism. At the 3′ end of the 42.7-kbp DNA fragment is the *acmB* gene (Fig. 4), which matched the DNA probe exactly. AcmB is highly similar to 3-ketosteroid-Δ^1-dehydrogenase from *C. testosteroni* (Table 3). This protein catalyzes dehydrogenation of the C-1—C-2 bond during the oxic metabolism of testosterone (13).

Apparently, the genes involved in anoxic cholesterol metabolism are not clustered in defined catabolic units or operons, as is the case for many other catabolic genes. Between ORF4 and *acmB* there are many other ORFs encoding proteins with functions possibly unrelated to cholesterol metabolism. Some of these ORFs are oriented in the same direction as ORF1 to ORF4, *acmA*, and *acmB*; others have the opposite orientation. Some examples of the gene products are a phage integrase, a transposase, a luciferase-like protein, and a thioesterase (data not shown).

Gene expression under anoxic and oxic conditions. The induction of ORFs during aerobic and anaerobic growth on cholesterol was studied by performing RT-PCR experiments using mRNA from *S. denitrificans* cells grown aerobically and anaerobically on cholesterol as the template (Table 4). The primers used in the following PCR were designed to amplify DNA fragments inside the ORFs and in the intergenic regions (Table 2 and Fig. 4). The data showed that ORF1 to ORF3, *acmA*, and *acmB* were induced during anaerobic growth on cholesterol (Table 4). Two of these genes, ORF1 and *acmB*, were detected even in highly (100-fold) diluted cDNA samples. The results were compared to RT-PCR products derived from mRNA from cells grown aerobically on cholesterol. In this case, ORF1, *acmA*, and *acmB* were also highly induced, whereas ORF2 and ORF3 were less induced (Table 4). These genes therefore may function in both oxic and anoxic cholesterol metabolism. Most of the intergenic regions could not be amplified; the exception was the region between ORF1 and the *acmA* gene (fragment 7) (Table 4 and Fig. 4). In contrast and as expected, all the intergenic regions tested could be amplified with genomic DNA as the template. Hence, ORF1 and the

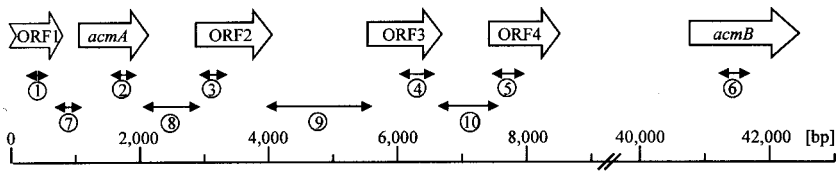

FIG. 4. Genes proposed to be involved in cholesterol metabolism in *S. denitrificans*. Bidirectional arrows indicate the regions (fragments) targeted for RT-PCR experiments.

TABLE 4. Induction of the expression of the proposed cholesterol degradation genes, as determined by using cDNA obtained from *S. denitrificans* cells grown anaerobically and aerobically on cholesterol

Amplified fragment[a]	Fragment length (bp)	Detection with the following templates:						
		Genomic DNA	Undiluted cDNA		cDNA diluted 10-fold		cDNA diluted 100-fold	
			Anaerobic conditions	Aerobic conditions	Anaerobic conditions	Aerobic conditions	Anaerobic conditions	Aerobic conditions
1	298	+[b]	+	+	+	+	+	+
2	413	+	+	+	+	+	−	+
3	451	+	+	+	+	+	−	−
4	565	+	+	+	+	−	−	−
5	523	+	−	−	−	−	−	−
6	501	+	+	+	+	+	−	−
7	430	+	+	+	+	+	−	−
8	897	+	−	−	−	−	−	−
9	1,648	+	−	−	−	−	−	−
10	862	+	−	−	−	−	−	−
11[c]	520	+	−	−	−	−	−	−

[a] See Fig. 4.
[b] +, DNA fragment was detected; −, no DNA fragment was detected.
[c] Complete removal of residual DNA from the total RNA preparation was verified by amplifying the intergenic region between two ORFs transcribed in different directions, with cDNA as the template.

acmA gene appear to be cotranscribed, whereas the other ORFs are independently transcribed.

Heterologous expression of the *acmA* gene and experimental analysis of its function. AcmA is postulated to represent the first enzyme of the cholesterol pathway, cholesterol dehydrogenase/isomerase, which catalyzes the first two reactions in anaerobic cholesterol metabolism. To validate this hypothesis, the *acmA* gene was amplified, cloned, and successfully expressed in *E. coli* as an N-terminal fusion with maltose binding protein (MalE). The recombinant protein ($AcmA_{mal}$) was purified by using amylose resin affinity chromatography followed by gel filtration chromatography. The purified active protein fraction produced a dominant protein band in SDS-PAGE at an apparent molecular mass of 80 kDa (Fig. 5). When the molecular mass of the maltose binding protein (42.7 kDa) was subtracted, the monomer size of the untagged protein was 37 kDa. This experimental value agrees well with the calculated molecular mass, 38.6 kDa (Table 3).

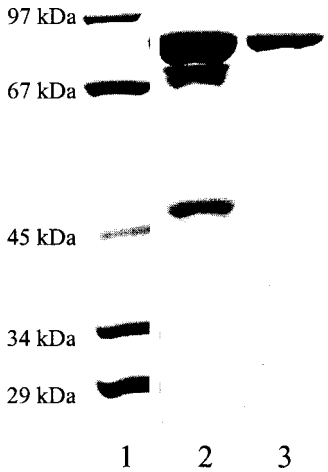

FIG. 5. SDS-PAGE analysis of purification of recombinant cholesterol dehydrogenase/isomerase ($AcmA_{mal}$) overproduced in *E. coli*. Lane 1, molecular mass standards; lane 2, active pool after amylose resin affinity chromatography; lane 3, active pool after gel filtration chromatography.

The activity of the enzyme was assayed using NAD^+ as an electron acceptor. Incubation of the purified recombinant protein with [4C-^{14}C]cholesterol and NAD^+ under anoxic and oxic conditions resulted in accumulation of one labeled product (Fig. 2B), which cochromatographed with authentic cholest-4-en-3-one. The electron impact mass spectrum (data not shown) with a dominant fragment ion peak at *m/z* 124 and a molecular ion peak at *m/z* 384 matched exactly the spectrum of cholest-4-en-3-one in a spectral database (www.aist.go.jp/RIODB/SDBS).

In vitro transformation of cholesterol under oxic conditions. *S. denitrificans* can utilize cholesterol both in the presence of oxygen and in the absence of oxygen, provided that nitrate is supplied as an electron acceptor (38). To determine whether the product pattern differs depending on the availability of oxygen, the products (AE1 to AE3) formed from [^{14}C]cholesterol with NAD^+ as an electron acceptor by extracts (soluble protein fractions) of cells grown under oxic conditions were analyzed. These products were compared to those previously identified as products formed by extracts of anaerobically grown cells (6) (Fig. 1). To find out if other intermediates of the anoxic pathway (S4 and S5) were produced, the soluble protein fraction of *S. denitrificans* cells grown aerobically was precipitated using ammonium sulfate and incubated with cholest-4-en-3-one (S2) as a substrate and $K_3[Fe(CN)_6]$ as an electron acceptor under oxic conditions. The results showed that there was an exact match between the TLC behavior and HPLC behavior, as well as between the UV absorption maxima of the five products detected (AE1 to AE5) and those of the reference compounds. The results indicate that in *S. denitrificans* oxic cholesterol metabolism and anoxic cholesterol metabolism may follow the same route, at least through the initial steps.

Comparison of soluble protein patterns of *S. denitrificans* grown aerobically and anaerobically on cholesterol. We used 2D gel electrophoresis to compare the pattern of soluble pro-

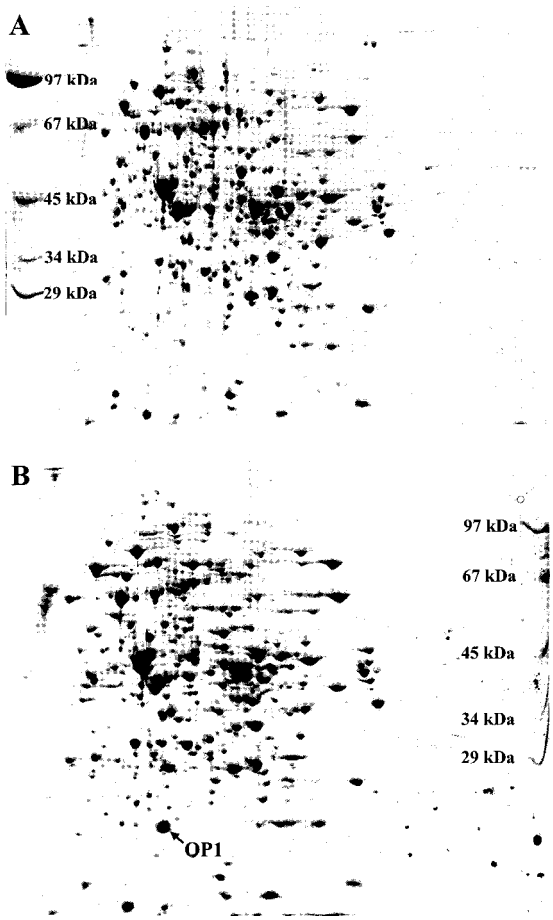

FIG. 6. Two-dimensional gel electrophoresis of soluble proteins (300 μg) extracted from *S. denitrificans* cells grown anaerobically (A) and aerobically (B) on cholesterol.

teins of *S. denitrificans* cells grown aerobically on cholesterol with the pattern obtained for anaerobically grown cells. Since most proteins were focused on a pH gradient between 5 and 8 (data not shown), isoelectric focusing electrophoresis was performed using a linear gradient from pH 5 to 8. Surprisingly, except for one protein spot (OP1), the patterns of the soluble proteins were virtually the same for the two samples (Fig. 6). OP1 appeared only when *S. denitrificans* was grown aerobically on cholesterol (Fig. 6B). In-gel digestion with trypsin, amino acid sequencing of the tryptic peptides by LC-MS/MS, and BLAST analysis revealed high similarity to alkyl hydroperoxide reductase (accession no. gi 13424540) of *Caulobacter crescentus*, which is expressed only under oxidative stress conditions (8, 16). Hence, OP1 may respond to oxygen stress. The very similar soluble protein patterns of the two samples thus corroborate the conclusion that the oxic and anoxic cholesterol metabolic pathways in *S. denitrificans* may be the same or at least highly similar. This would explain the finding that cultures did not show any remarkable lag phase during growth on cholesterol when they were shifted from anoxic growth conditions to oxic growth conditions and vice versa.

DISCUSSION

Initial enzymes of anoxic cholesterol metabolism. The first step in anoxic cholesterol metabolism (transformation of cholesterol to cholest-4-en-3-one) is catalyzed by AcmA (cholesterol dehydrogenase/isomerase). AcmA is a bifunctional enzyme which catalyzes oxidation of the 3-OH group, followed by Δ^5-to-Δ^4 isomerization (identical to the corresponding reaction under oxic conditions) (Fig. 1). The deduced amino acid sequence encoded by the *acmA* gene exhibits high similarity to NAD(P)-dependent 3β-hydroxysteroid dehydrogenase/isomerase from *Nocardia* sp. (accession no. Q03704) and *Gallus gallus* (chicken) (Q91997). AcmA belongs to the short-chain dehydrogenase/reductase (SDR) superfamily (18, 29) that comprises a growing number of NAD(P)-dependent non-metallo-oxidoreductases, which bind NAD(P)(H) with a Rossman fold motif (18). Alignment of amino acid sequences from AcmA and related members from the SDR superfamily revealed a highly conserved nucleotide binding motif at the N terminus [G-X-G-$(X)_2$-G-$(X)_{10}$-G, where X is any amino acid) (Fig. 7) (14, 35). Another fingerprint of the SDR enzymes is the highly conserved catalytic triad serine-tyrosine-lysine (11) in the middle part of AcmA at amino acids 147 to 151 (Fig. 7), although the serine residue is variable (28). The highly similar organization suggests that the members of this enzyme family have similar reaction mechanisms.

The second step of (an)oxic cholesterol metabolism, oxidation of cholest-4-en-3-one (S2) to cholesta-1,4-dien-3-one (S3) (Fig. 1), is catalyzed by cholest-4-en-3-one-Δ^1-dehydrogenase (AcmB) under both oxic and anoxic conditions (Fig. 2A). The enzyme is highly similar to the flavin adenine dinucleotide-dependent 3-ketosteroid-Δ^1-dehydrogenase (17). The induction of expression of *acmB* upon (an)oxic growth on choles-

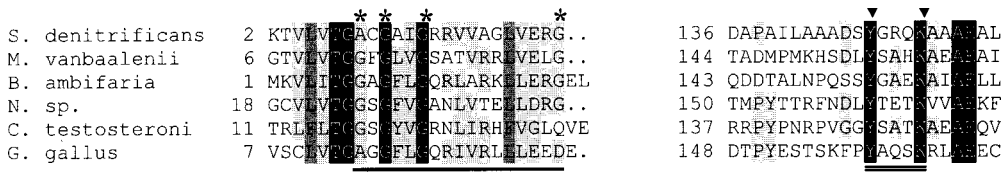

FIG. 7. Partial alignment of the deduced amino acid sequence encoded by the *acmA* gene of *S. denitrificans* with the sequences of NAD-dependent epimerase/dehydratase from *Mycobacterium vanbaalenii* (accession no. YP_955010) and *Burkholderia ambifaria* (ZP_01556894), NAD(P)-dependent cholesterol dehydrogenase from *Nocardia* sp. (N. sp.) (Q03704), and NAD(P)-dependent 3β-hydroxysteroid dehydrogenase/isomerase from *C. testosteroni* (ZP_01521768) and *G. gallus* (Q91997). Conserved amino acids are indicated by black, dark gray, and light gray backgrounds depending on their levels of similarity (from high to low). The NAD(P) binding region [G-X-G-$(X)_2$-G-$(X)_{10}$-G] is underlined, and four G residues conserved in this region are indicated by asterisks. The substrate binding region [Y-$(X)_3$-K] is double underlined, and the conserved Y and K residues are indicated by arrowheads.

terol as shown by RT-PCR (Table 4) corroborates the involvement of AcmB in (an)oxic cholesterol metabolism.

The third step in the anoxic cholesterol pathway involves the unprecedented oxygen-independent hydroxylation at the tertiary C-25 of cholest-4-en-3-one (S2) and cholesta-1,4-en-3-one (S3) (Fig. 1). The oxygen of the introduced hydroxyl group is derived from water. Enzymes catalyzing a reaction like this belong to the molybdenum-containing hydroxylase enzyme family, and they use water as a source of the oxygen atoms incorporated into their substrates (e.g., ethylbenzene dehydrogenase [21]). The 43-kbp DNA fragment did not contain a similar gene. However, ORF1 located at the 5′ end of the DNA fragment encodes the C terminus of a protein whose primary structure is similar to that of a molybdenum-pterin binding protein from *B. cenocepacia* (Table 3). ORF1 is expressed, as shown by RT-PCR.

Organization of genes involved in cholesterol metabolism by *S. denitrificans*. The DNA fragment harboring the *acmA* and *acmB* genes encoding the first two enzymes of the pathway contained four ORFs (ORF1 to ORF4) which may also play a role in this pathway. The functions of the other ORFs are unknown and may not be related to cholesterol metabolism. It appears that the cholesterol catabolic genes are not in catabolic gene clusters as usual in specialized bacteria. A similar situation was reported for oxic cholesterol metabolism by *Rhodococcus* sp. strain RHA1 (39). Many of the 572 genes related to oxic cholesterol metabolism are scattered throughout the 9.7-Mbp genome. The fact that the metabolic genes and necessary regulatory elements are scattered may be due to late acquisition of this metabolic capability, possibly by lateral gene transfer. The presence of large intergenic regions and genes encoding transposase and phage integrase in the DNA fragment supports this suggestion.

Indication of a common pathway for cholesterol metabolism under anoxic and oxic conditions. Our findings suggest that anoxic cholesterol metabolism and oxic cholesterol metabolism by *S. denitrificans* follow the same route, at least during the initial phase. (i) The same intermediates were produced when the in vitro assays were performed in the presence of oxygen and when they were performed in the absence of oxygen (6), indicating that the enzymes involved in the anoxic pathway are not sensitive to molecular oxygen. (ii) The intermediates formed by extracts of aerobically grown cells are identical to those that we reported previously for the anoxic pathway. (iii) Furthermore, with the exception of the three initial products, no other products typical of oxic cholesterol metabolism were found during in vitro assays (Fig. 1). Notably, the two later intermediates, which are formed by anoxic (the oxygen comes from water) rather oxygen-dependent hydroxylation of the side chain, have not been reported previously for oxic cholesterol metabolism. (iv) The patterns of soluble proteins were virtually identical in cells grown under anoxic conditions and cells grown under oxic conditions. One aerobically induced protein, probably an alkyl hydroperoxide reductase, seems to be involved in the oxidative stress response (8, 16). For comparison, transcription of *Rhodococcus* sp. strain RHA1 revealed upregulation of 572 genes during oxic growth on cholesterol compared to growth on pyruvate (39). (v) The RT-PCR assays revealed expression of *acmA*, *acmB*, and ORF1 in cells grown either aerobically or anaerobically on cholesterol.

A negative control is usually included in RT-PCR experiments to substantiate the results. Unfortunately, we have not been able to establish growth of *S. denitrificans* under noninducing conditions, because this bacterium seems to possess an extremely narrow substrate spectrum. We have tried to establish growth of *S. denitrificans* on palmitic acid to use it as negative control for proteomic and gene expression studies. Surprisingly, the soluble protein fraction from *S. denitrificans* cells grown anaerobically on palmitic acid also transformed cholesterol to the intermediates S1, S2, and S3, and the protein pattern did not differ significantly (data not shown). These data indicate that palmitic acid may act as a gratuitous inducer of the cholesterol metabolic enzymes. However, the possibility that the cholesterol metabolic genes studied here may be expressed constitutively cannot be ruled out. Similar phenomena have been reported for oxic cholesterol metabolism by some microorganisms. Hexanoic acid is the most effective inducer for the expression of cholesterol oxidase in *Rhodococcus* sp. strain GK1 (22), and cholesterol oxidase production by *Schizopyllum commune* depends on the presence of oleic acid in the culture broth (37).

Advantage of a common oxic and anoxic pathway. The use of a common strategy to metabolize a single substrate under oxic and anoxic growth conditions has been described previously. Denitrifying bacteria are periodically subjected to fluctuations of oxygen availability inherent to their natural environment. Consequently, these organisms have to switch their metabolic machinery between oxic and anoxic modes of metabolism. Therefore, initiating the metabolism of a substrate via the same enzyme(s) regardless of the prevailing conditions and then channeling the last common intermediate into separate pathways depending on the prevailing conditions would increase their metabolic competence. A similar situation was reported for oxic and anoxic metabolism of benzoate in denitrifying bacteria. In the presence and in the absence of oxygen benzoate is converted to benzoyl-CoA. This activation step is catalyzed by a single enzyme, benzoate-CoA ligase (34). Benzoyl-CoA then induces a separate oxic or anoxic pathway depending on the availability of oxygen.

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