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A patchwork pathway for oxygenase-independent degradation of side chain containing steroids

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Running title: Anaerobic degradation of steroids

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Originality-Significance Statement

Steroids constitute a ubiquitous class of highly hydrophobic molecules that are components of biological membranes or compounds with endocrine activities. Their complete biodegradation is important for the global carbon cycle and for the elimination of bioactive micropollutants. While oxygenase-dependent degradation has been studied since decades, much less is known about steroid degradation at anoxic sites. We provide the complete genome of the Betaproteobacterium *Sterolibacterium denitrificans* that fully degrades numerous zoo-, phyto- and mycosterols under denitrifying conditions. Intensive proteomic and metabolomics analyses together with the characterization of a typical catabolic enzyme revealed a patchwork pathway for the complete degradation of side-chain containing steroids. It involves reaction sequences known from aerobic actinobacterial and proteobacterial steroid degradation and unique enzymatic reactions to circumvent oxygen-dependent steps. The results obtained now allow a first complete view on a 40-step anaerobic degradation pathway for side-chain containing steroids.

Abstract

The denitrifying Betaproteobacterium *Sterolibacterium denitrificans* serves as model organism for studying the oxygen-independent degradation of cholesterol. Here, we demonstrate its capability of degrading various globally abundant side chain containing zoo-, phyto- and mycosterols. We provide the complete genome which empowered an integrated genomics/proteomics/metabolomics approach, accompanied by the characterization of a characteristic enzyme of steroid side chain degradation. The results indicate that individual molybdopterin-containing steroid dehydrogenases are involved in C25-hydroxylations of steroids with different isoprenoid side chains, followed by the unusual conversion to C26-oic acids. Side chain degradation to androsta-1,4-dien-3,17-dione (ADD) via aldolytic C–C-bond cleavages involves acyl-CoA synthetases/dehydrogenases specific for the respective 26-, 24-, 22-oic acids/-oyl-CoAs, and promiscuous MaoC-like enoyl-CoA hydratases, aldolases

and aldehyde dehydrogenases. Degradation of ring A and B depends on gene products uniquely found in anaerobic steroid degraders, which after hydrolytic cleavage of ring A, again involves CoA-ester intermediates. The degradation of the remaining CD rings via hydrolytic cleavage appears to be highly similar in aerobic and anaerobic bacteria. Anaerobic cholesterol degradation employs a composite repertoire of more than 40 genes partially known from aerobic degradation in Gammaproteobacteria/Actinobacteria, supplemented by unique genes that are required to circumvent oxygenase-dependent reactions.

Introduction

The ubiquitous steroids constitute a widely abundant class of natural compounds with a common four-ringed steran ring system. Steroids without side chains are known for their endocrine function whereas those containing isoprenoid side chains mainly serve as components of biological membranes that influence fluidity and other functions. The latter are abundant in animals (e.g. cholesterol), plants (β-sitosterol and stigmasterol), fungi (ergosterol) but are also present in many microorganisms (hopanoids and cholesterol in a few cases). Bile acids contain a carboxyalkyl side chain that greatly enhances their solubility, a structural prerequisite to function as emulsifiers of dietary lipids (Dufourc, 2008; Nes, 2011; Wollam and Antebi, 2011).

Many steroids exhibit endocrine disrupting activity and are considered as micropollutants. Due to their increasing discharge to the environment by sewage plant or industrial effluents, livestock manures or agricultural applications some are classified as contaminants of emerging concern (Barbosa et al., 2016; Ting and Praveena, 2017). The degradation of steroids to CO_2 by microorganisms represents the major means of steroid elimination from the environment and is important for the global carbon cycle (Hylemon and Harder, 1998). But in particular, the isoprenoid side chain containing steroids are highly persistent due to their extremely low water solubility and the high number of tertiary and quaternary carbon atoms. Research on microbial steroid degradation is also medically relevant, because *Mycobacterium tuberculosis* is known to use cholesterol from macrophages during intracellular survival (Van der Geize et al., 2007; Wipperman et al., 2014). Finally, studying biological steroid degradation is motivated by the search of biocatalysts for the production of bioactive steroid variants from low-cost natural precursors (Donova and Egorova, 2012; Garcia et al., 2012).

The aerobic degradation of cholesterol as side chain containing model steroid has been studied in actinobacterial *Mycobacterium* and *Rhodococcus* strains (Yam et al., 2011, Wipperman et al., 2014; Lovewell et al., 2016). In contrast cholic acid degradation has predominantly been studied in the gammaproteobacterial *Pseudomonas* sp. Chol1 (Holert et al., 2013b; Holert et al., 2014) and *Comamonas testosteroni* (Horinouchi et al., 2003; Horinouchi et al., 2012), but also in *Rhodococcus jostii* (Mohn et al., 2012). Degradation of both, cholesterol and cholic acid is initiated by the isomerization of the A-ring to cholest-4-en-3-one either by a 3-ketosteroid dehydrogenase (Fernández de las Heras et al., 2012) or by an oxidase (Fig. 1) (Vrielink and Ghisla, 2009). The cholest-4-en-3-one then may undergo 1,2-desaturation in the A-ring to a di-α,β enone by a 3-ketosteroid-Δ1-dehydrogenase. The side- chain of cholesterol is hydroxylated at C26 by CYP125 monoxygenase either before or after 3-ketosteroid-Δ1-dehydrogenase reaction, followed by the oxidation to the corresponding C26-oic acid, and the activation to its CoA-ester by a specific acyl-CoA synthetase (Wippermann et al., 2014, Yam et al., 2011); likewise cholic acid is activated to a C24-oyl-CoA (Barrientos et al., 2015). The degradation of cholesterol- or cholic acid-derived acyl-CoA side chains proceeds via β-oxidation involving acyl-CoA dehydrogenases and enoyl-CoA hydratases. A major difference between both pathways is the thiolytic (cholesterol) and aldolytic (cholic acid) C–C-bond cleavage. The latter involves aldolases, aldehyde dehydrogenases and acyl-CoA synthetases instead of 3-hydroxyacyl-CoA dehydrogenases/thiolases. Both pathways are considered to yield the central intermediate androsta-1,4-diene-3-one (ADD) (Wippermann et al., 2014, Yam et al., 2011, Holert et al., 2013; Fig. 1), though modifications at the steran ring may precede complete side chain degradation in some cases. The further degradation involves three sequentially acting oxygenases that result in cleavage of ring A and B via a name-giving 9,10-*seco* intermediate pathway. The CD-rings of the steran skeleton are still present in the bicyclic 3aα-H-4α(3'-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP) intermediate (Van der Geize et al., 2007; Wipperman et al., 2014). They are cleaved by two distinct hydrolases finally yielding acetyl-CoA, propionyl-CoA and succinyl-CoA (Crowe et al., 2017), (Fig. 1).

Much less is known about the bacterial degradation of steroids in the absence of oxygen. In recent years, the denitrifying Betaproteobacterium *Sterolibacterium (Stl.) denitrificans* has been established as cholesterol-degrading model organism (Tarlera and Denner, 2003). The initial isomerization/dehydrogenation steps are identical to those in aerobic cholesterol degradation and involve the two dehydrogenases AcmA and AcmB (Fig. 1) (Chiang et al., 2008a; Chiang et al., 2008b). However, the initial activation of the side chain fundamentally differs from aerobic cholesterol catabolism and yields 25-hydroxy instead of the 26-hydroxy-cholest-3-one-4-ene (Chiang et al., 2007). The water-dependent hydroxylation of the tertiary C25 is catalyzed by a molybdopterin-containing enzyme of the DMSO reductase family (Dermer and Fuchs, 2012). The cholesterol C25 dehydrogenase (C25DH) even catalyzed the hydroxylation of vitamin D_3 ($VitD_3$) to 25-hydroxy-$VitD_3$; a reaction of potential biotechnological interest for the synthesis of the clinically relevant form of $VitD_3$ (Warnke et al., 2016). C25DH is composed of three subunits: the α-subunit harbors the active site molybdopterin-cofactor, whereas β- (FeS-clusters) and γ-subunits (heme b) are involved in electron transfer. A shotgun genome sequence revealed that *Stl. denitrificans* contains at least eight homologous genes encoding C25DH-like α-subunits; the function of each has remained unknown (Dermer and Fuchs, 2012). The steps from 25-hydroxy intermediate to 26-carboxylic acid has remained enigmatic and requires an unprecedented isomerization of the hydroxyl-group from a tertiary to a primary carbon atom. Mass spectrometry (MS)-based analyses of ethyl acetate extractable metabolites from *Stl. denitrificans* suggested that the side chain is first degraded prior to the degradation of the ring systems (Wang et al., 2013; Lin et al., 2015). However, the anticipated CoA ester metabolites have not been identified, and the genes and enzymes involved are unknown.

The oxygenolytic cleavage of the AB-rings is no option for anaerobic steroid degradation. Instead, ADD is converted to a 1,3-diketone by a non-studied dehydrogenase and a bifunctional, Mo-cofactor containing hydratase/dehydrogenase that has been characterized in

the testosterone-degrading *Steroidobacter (Sdo.) denitrificans* (Yang et al., 2016). The A-ring is then hydrolytically cleaved to 1,17-dioxo-2,3-*seco*-androstan-3-oic acid (DSAO), the name-giving key intermediate of this 2,3-*seco*-pathway (Wang et al., 2013; Wang et al., 2014). The genes, and enzymes involved in DSAO formation/degradation are unknown.

In this study, we present the metabolic versatility of the steroid-degrading model organism *Stl. denitrificans* by demonstrating degradation of zoosterols, phytosterols and mycosterols in the absence of oxygen. The completed genome sequence allowed an integrative genomic, proteomic and metabolomic study accompanied by the heterologous production of a characteristic enzyme involved in side chain degradation. Our results revealed a patchwork degradation pathway comprising more than 40 gene products from actinobacterial cholesterol degradation, proteobacterial cholic acid degradation, and gene products that are uniquely found in betaproteobacterial anaerobic steroid degradation.

Results and Discussion

MS analysis of metabolites during growth with cholesterol

In previous studies ethyl acetate extractable metabolites were analyzed in *Stl. denitrificans*, and non-CoA-ester intermediates were identified including 25- and 26-hydroxy-cholest-4-en-3-one, C26-, C24- and C22-carboxylic acids, ADD as well as ring-A-cleaved DSAO and 17-hydroxy-2,5-*seco*-3,4-dinorandrost-1,5-dione (2,3-SDAD) (Wang et al., 2013; Lin et al., 2015). These results suggested that cholesterol side chain is cleaved prior to steran ring degradation, and that ring A is opened hydrolytically to DSAO. However, hydrophilic CoA-ester intermediates hypothesized to play a major role in anaerobic cholesterol degradation, escaped detection.

To obtain a complete picture of the anaerobic cholesterol degradation pathway, we analyzed the entire cholesterol-derived catabolite pool by UPLC-ESI-MS/MS using a novel method for extracting/analyzing hydrophilic CoA-ester derived metabolites. For better comparison for all metabolite pools ESI and a single LC-gradient was applied. If applicable, assignment of ion mass spectra was accomplished using authentic standards (deviations of mass parent ion/daughter ions <2 mDa); for CoA ester identification, the detection of the reported characteristic fragment ions was mandatory (Zimmermann et al., 2013).

Cholesterol degradation proceeds via compounds containing either the 4-en-3-one or the 1,4-diene-3-one forms of ring A (Chiang et al., 2007; Chiang et al., 2008b), which makes it impossible to distinguish between dehydrogenations occurring at ring A or at the side chain. To assign detected masses to either of the two forms, we searched for distinguishing mass fragments using androst-4-en-3-one (AD) and androsta-1,4-diene-3-one (ADD) as standards. AD showed next to its parent *m/z* 287.2019 $[M+H]^+$ typical daughter ions at *m/z* = 123.0825, 109.0659 and 97.0664 (Supporting Information Fig. S1). In contrast, ADD provided typical *m/z* of 285.1868 ($[M+H]^+$ parent ion) and daughter ions of *m/z* = 121.0673, 91.0538, and

77.0374. The ion fragmentation pattern of 25-hydroxycholesta-1,4-diene-3-one and 25-hydroxycholest-4-en-3-one standards gave the same distinguishing daughter ion pattern. As a result, the absence/presence of either of the characteristic fragments at *m/z* values of 121.0625 (ADD-form) or 109.0659 (AD-form) were used for the assignment to either of the two forms. Using this setup a total of 38 cholesterol-derived compounds were identified, among 17 being assigned to CoA-esters (Supporting Information Table S1).

Most metabolites of the side chain degradation pathway were detected in both, the AD- and ADD-form. The analysis confirmed all previously detected ethyl acetate extractable intermediates and identified all involved in the conversion of 25-hydroxy-cholest-4-en-3-one to cholest-4-en-26-oic acid (Supporting Information Table S1 and Fig. 3). In addition, many postulated CoA-esters involved in β-oxidation were identified including the C26-, C24- and C22-acyl-CoAs, the C26-, C22-enoyl-CoAs and 24-hydroxy-cholest-4-en-3-one-26-oyl-CoA. However, no masses indicative for a 3-oxoacyl-CoA compound were found. Instead, the aldehydes chol-4-en-24-al-3-one and chola-1,4-dien-24-al-3-one were identified. Together these findings clearly suggest that cholest-4-en-26-oic acid side chain degradation is initiated by thioesterification followed by β-oxidation via aldolytic C–C cleavage that forms aldehydes and acetyl-CoA/propionyl-CoA (Fig. 3).

All predicted ADD-derived non-CoA-ester degradation metabolites were identified including androst-1-en-3,17-dione, its hydrated 1-hydroxy-3-oxo and oxidized 1,3-dioxo product; the latter two have not been identified before (Wang et al., 2013). In agreement with earlier reports, the ring A cleavage product DSAO was detected. The proposed further degradation of the latter via CoA-esters was confirmed by the identification of 2,3-*seco*-androst-1,17-dione-3-oyl-CoA and its dehydrogenated product, both representing unique CoA ester metabolites in anaerobic cholesterol degradation. Hydration of the latter to a 3-hxdroxyacyl-CoA species followed by aldolytic cleavage yields acetyl-CoA and 2,5-*seco*-3,4-dinorandrost-1,5,17-trione (2,5-SDAT), which was identified in this and previous work (Lin et al., 2015, as

17-hydroxy-analogue). Degradation of 2,5-SDAT would plausibly yield 3aα-*H*-4α(3′propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP) and/or the thioesterified HIP-CoA. Indeed, a compound co-eluting with authentic HIP-CoA standard and with identical parent and daughter ion masses was identified indicating that HIP-CoA is a common intermediate of aerobic and anaerobic steroid degradation.

Complete genome of *Stl. denitrificans*

The genome of *Stl. denitrificans* consists of the 3.02 Mb chromosome with 63.22 % G+C (accession number LT837803 at the European Nucleotide Archive [ENA]) and three plasmids (LT837804 to LT837806). General nucleotide composition features of the main chromosome are described in the Supporting Information Fig. S2 (compositional statistics) and Fig. S3 (coverage biases). The chromosome contains 2691 predicted protein-coding genes, three rRNA operons (5S, 16S and 23S), 43 tRNA genes, and no clear-cut CRISPR gene cluster. Altogether, 2685 protein-encoding genes were classified into COG categories including a strong contingent of about 155 genes affected to lipid metabolism genes (COG I; Supporting Information Table S2). In order to carry out comparative genomics analyses sequences were uploaded in the MicroScope annotation platform (Vallenet et al., 2017). Completeness and contamination of the reconstructed genome was assessed with checkm (Parks et al., 2015) using 540 lineage specific single-copy marker genes organized into 241 sets.

Global synteny statistics based on bi-directional best hits (BBHs) indicated that *Stl. denitrificans* shares more than half of its coding sequences with a dozen of sequenced bacteria (Supporting Information Table S3), most of them affiliated with the *Thauera* genus. Interestingly, about 39% of *Stl. denitrificans* coding sequences were involved in syntenic relationships with Gammaproteobacteria, and another 15% displayed BBH relationships with Actinobacteria. Based on the observation that the enzymatic repertoire acting on side chain containing steroids mainly seems to involve genes found in Gammaproteobacteria and to a lesser extent Actinobacteria, we set out to infer for each *Stl. denitrificans* gene its most

consistent origin among Betaproteobacteria, Gammaproteobacteria or Actinobacteria. This was performed by computing synteny groups [syntons (Boyer et al., 2005)] between the *Stl. denitrificans* genes and the three closest (in terms of % CDS involved in syntons) genomes selected from the three above classes. The results are summarized in Supporting Information Table S4. Interestingly, the majority (20/36) of coding sequences that were exclusively related to the *Rhodococcus* (Actinobacteria) genome appear to be involved in β-oxidation like reactions, as were about one third (22/65) of the genes related to both the *Rhodococcus* and *Thauera* genomes (but not to Gammaproteobacteria). These observations are consistent with a recruitment of enzymatic reactions relevant for the degradation of a large spectrum of side chain containing steroids by *Stl. denitrificans*.

Related sequences in environmental metagenomic datasets

As *Stl. denitrificans* was initially isolated from sewage sludge (Tarlera and Denner, 2003), we searched for related metagenomic sequences in activated sludge from a municipal wastewater treatment plant located in Evry, France (Guermazi et al., 2008). A dozen of 16S sequences displaying more than 96% identity to *Stl. denitrificans'* 16S rRNA gene were recovered, mostly affiliated with *Rhodocyclaceae* and including unclassified *Rhodocyclaceae* nodes for half of them. In these uncultured *Rhodocyclaceae* putative gene products were identified with up to 60% amino acid sequence identities to C25DH-like α-subunits and about 65% identity to 1-testosterone hydratase/dehydrogenase (AtcA) of *Stl. denitrificans*. These results reflect that *Rhodocyclaceae* related to *Stl. denitrificans* play a major role in the degradation of steroid micropollutants in wastewater treatment plants.

General setup for proteogenomic analysis of anaerobic cholesterol degradation

To identify cholesterol-induced genes in *Stl. denitrificans*, the proteome of cells grown with cholesterol was compared to cells grown with testosterone and propionate. A single component analysis of biological quadruplicates for each growth substrate revealed a high reliability of the data obtained (Supporting Information Fig. S5); proteome data are submitted

in Supporting Information Tables S5-S7. For easier discussion, genes involved in cholesterol degradation are functionally categorized according to their consecutive roles in the degradation pathway: (i) uptake of cholesterol, (ii) ring A isomerization/oxidation, (iii) side chain hydroxylation (iv) β-oxidation of the side chain, (v) ring AB degradation, and (vi) ring CD degradation. Characteristic gene clusters involved in anaerobic cholesterol degradation are referred to as *acd*1-*acd*5; their location on the chromosome is depicted in Fig. 2. Genes were assigned to functions based either on their previous experimental identification, or on their induction by cholesterol vs testosterone and/or vs propionate together with clear similarities to reported genes. The best hits with previously identified genes of actinobacterial, gammaproteobacterial or betaproteobacterial (*Thauera* species) origins are summarized in Supporting Information Table S8. The assignment of individual genes to catabolic reactions via detected metabolites is shown in Fig. 3.

(i) Genes involved in cholesterol uptake – A cholesterol-specific outer membrane transport system has been described in *Stl. denitrificans* that showed properties of a FadL-like transporter, though the gene has not been identified (Lin et al., 2015). In this work, two homologs of a FadL-like outer membrane fatty acid transporter were identified in the genome, one of which (SDENv1_21523) was induced during growth with cholesterol. It shows highest similarities to a FadL-like protein of cholic acid-degrading *Pseudomonas* Chol1 (38%), and we conclude that it mediates transport of cholesterol across the outer membrane.

(ii) Genes involved in ring A modification – The initial two-step ring A isomerization/oxidation yielding cholesta-1,4-diene-3-one is catalyzed by AcmA and AcmB (Chiang et al., 2007; Chiang et al., 2008a; Chiang et al., 2008b); the encoding genes SDENv1_10444 and SDENv1_10486 were identified in the genome. There are several additional homologous AcmA-like (up to 33% identity) and AcmB-like (up to 44% identity) gene products, that might

act on steroid substrates with modified side chains. Similar AcmA/B enzymes are present in aerobic steroid degraders (Supporting Information Table S8).

(iii) Genes involved in C-25 hydroxylation – The water-dependent hydroxylation at C25 of cholesta-1,4-diene-3-one by C25DH is a major difference to aerobic cholesterol degradation (Chiang et al., 2007; Dermer and Fuchs, 2012). The genes encoding the three subunits (*sdhABC*) were identified as SDENv1_20804/20461/20462 together with the previously reported seven additional *sdh*ABC-like genes (Fig. 4). Notably, only six putative *sdhBC*- and two *sdhD*-like genes were identified adjacent to *sdhA*-like genes suggesting that putative electron transferring subunits and chaperones are shared by more than one SdhA active site subunit. The induction of the individual C25DH-like gene products during growth with different steroids is presented below.

(iv) Genes involved in side chain degradation/heterologous production of cholesta-1,4-diene-3-one-26-oyl-CoA synthetase – Based on previous studies (Wang et al., 2013; Lin et al., 2015) and the metabolite analyses presented in this work, the degradation of 25-hydroxycholest-4-en-3-one was suggested to involve the isomerization/oxidation of the latter to cholest-4-en-3-one-C26-oic acid (and its ADD-form) and its activation to the corresponding CoA-ester to initiate three consecutive β-oxidation-like reaction sequences yielding ADD, two propionyl-CoA and one acetyl-CoA. Proteogenomic analyses revealed the cholesterol-induced gene clusters *acd*1 and *acd*2, putatively encoding enzymes involved in side chain degradation (SDENv1_10299 to SDENv1_10310 and SDENv1_11188 to SDENv1_11201, Fig. 5). Using BLAST, three cholesterol-induced acyl-CoA synthetases, three acyl-CoA dehydrogenases, two enoyl-CoA hydratases, two aldolases and an aldehyde dehydrogenase with high homologies to enzymes involved in aerobic side chain degradation via aldolytic C-C cleavage were identified. However, genes involved in thiolytic β-oxidation reactions (3-hydroxyacyl-CoA dehydrogenases, thiolases) are missing in these clusters.

The six cholesterol-induced genes (SDENv1_11188-SDENv1_11193) putatively encode a three-subunit homologue of Mo-containing C25DH, an alcohol and an aldehyde dehydrogenase, and an acyl-CoA synthetase. It is tempting to speculate whether these genes are involved in the conversion of 25-hydroxy-cholest-4-en-3-one to the cholest-4-en-3-one-26-oyl-CoA acid via the corresponding C26-alcohol, -aldehyde, and carboxylic acid. Other genes of the *acd*1-cluster putatively encode transporters and regulatory proteins. The cholesterol-induced genes Sdenv1_1198-11201 of *acd*1-cluster putatively encode enzymes that are similar to actinobacterial enzymes converting cholest-4-en-3-one-26-oyl CoA to chol-4-en-3-one-24-oic acid and propionyl-CoA via aldolytic cleavage. Notably, both acyl-CoA dehydrogenases of the *acd*1/*acd*2 clusters are encoded by two different genes unlike the classical homomeric enzymes. A similar two-component acyl-CoA dehydrogenase has been reported to dehydrogenate the CoA ester of pregn-4-en-3-one-20-carboxyl-CoA (Fig. 3) in *M. tuberculosis* which binds only one active-site FAD per heterodimer (Yang et al., 2015).

To provide additional experimental evidence that *acd*1 contains genes involved in the conversion of 25-hydroxy-cholest-4-en-3-one to cholest-4-en-3-one-24-oic acid via a C26 carboxyl-CoA intermediate, we heterologously produced SDENv1_11189, encoding a putative acyl-CoA synthetase in *E. coli* with a C-terminal Strep-tag and purified the product by affinity chromatography (Supporting Information Fig. S6). The 60-kDa-enzyme catalyzed the CoA- and ATP-dependent conversion of cholest-4-en-3-one-26-oic acid to the corresponding cholest-4-en-3-one-26-oyl-CoA with a specific activity of 1.1 µmol min^{-1} mg^{-1} and an apparent K_m-value for the C26-oic acid of 310±50 µM (the ADD-form of the substrate was converted with identical kinetic parameters). Notably, apparent K_m-values determined for steroids in the presence of cyclodextrin are always considered to be highly overestimated (Dermer and Fuchs, 2012; Warnke et al., 2016). Most importantly, the corresponding C24-oic and C22-oic acids were virtually not converted (rate <1% compared to the C26-oic acid). In summary, the results suggest that SDENv1_11189 codes for a specific 26-oyl-CoA

synthetase, and that the genes of *acd*1 are indeed involved in the conversion of the 25-hydroxycholest-4-en-3-one to chol-4-en-3-one-24-oic acid.

The genes SDENv1_10299-10301 of *acd*2 are most highly related to genes encoding a C24-acyl-CoA synthetase and a two-component C24 steroid side chain acyl-CoA dehydrogenase from *Pseudomonas* sp. Chol1. Based on highest similarities to genes from *Pseudomonas* sp. Chol1, but also to genes of the *igr*-operon that are essential for intracellular survival of *M. tuberculosis* in macrophages, SDENv1_10306-SDENv1_10310 are suggested to be involved in the conversion of pregna-1,4-diene-3-one-20-carboxyl-CoA to ADD (Wipperman et al., 2014). The *igr*-operon of *M. tuberculosis* additionally contains a gene encoding CYP125 monooxygenase catalyzing C26 hydroxylation, which is missing in *acd*2. Instead of the gene encoding a classical homomeric *L*-specific enoyl-CoA hydratase (e.g. SDENv1_11199, present in *acd*1), the genes for an *R*-specific, MaoC-like, two-component enoyl-CoA hydratase (Yang et al., 2014) are present in the *acd2-/igr*-cluster. Moreover, genes putatively encoding an acyl-CoA synthetase and an aldolase are located adjacent to the *igr*-like genes in *acd2* (SDENv1_10305/10309). They are likely to be involved in the activation of pregna-1,4-diene-3-one-20-oic acid to its CoA thioester, and aldolytic C–C-bond cleavage yielding propionyl-CoA and ADD.

In summary, the cholesterol-induced gene clusters *acd*1 and *acd*2 contain almost all genes required for cholesterol side chain degradation from cholest-4-en-3-one to ADD, two propionyl-CoA and acetyl-CoA. Whether indeed, the S25DH-homologous enzyme encoded by SDENv1_11191-1193 is involved in the conversion of 25-hydroxy- to 26-hydroxycholest-4-en-3-one remains to be studied.

(v) Genes involved in ring AB degradation – The degradation of ring A in the oxygen-independent 2,3-*seco* pathway involves the Mo-enzyme AtcABC converting androst-1-en-3-one to a 1,3-di-keton (Wang et al., 2014, Yang et al., 2016). Highly homologous genes are

present in the *acd*3 gene cluster (SDENv1_10172-4; Fig. 6) indicating that *acd*3 is involved in ring A cleavage although no clear upregulation of *acd*3 genes by cholesterol was observed. Candidate genes encoding other enzymes of 2,3-*seco*-pathway may be involved in Δ4-reduction of androsta-1,4-diene-4-one (SDENv1_10171) and activation of 1,17-dioxo-2,3-*seco*-androstan-3-oic acid (DSAO, compound 9) to DSAO-CoA by a CoA transferase (SDENv1_10168), whereas no candidate gene for ring cleaving hydrolase acting on DSAO was identified. DSAO-CoA was identified by mass spectrometry and initiates a new round of β-oxidation to the identified 2,5-SDAT (compound 10) and acetyl-CoA. Degradation of 2,5-SDAT to HIP (compound 11) proceeds via ring B cleavage. No reliable candidate genes could be identified for this reaction sequence because (i) they are not differentially regulated, (ii) they are scattered in the genome, and (iii) no model enzyme involved in such a reaction sequence has been described so far.

(vi) Genes involved in ring CD degradation – In aerobic steroid degrading organisms HIP is activated by a specific acyl-CoA synthetase (Casabon et al., 2013; Barrientos et al., 2015). *Stl. denitrificans* contains a homologue to this synthetase (SDENv1_10766) encoded by a gene located in the cholesterol-induced cluster *acd5* (Fig. 6). Based on high homologies to genes clusters from other organisms that are involved in ring CD degradation (Horinouchi et al., 2012; Yang et al., 2016; Crowe et al., 2017), the two cholesterol-induced gene clusters *acd4* and *acd5* contain almost all genes required for β-oxidation and hydrolytic cleavage of the CD-rings of HIP-CoA to 4-methyl-5-oxooctanedioyl-CoA (MOODA-CoA, compound 14 in Fig. 3), (Fig. 6). The two candidates genes for the hydrolases involved in cleavage of ring C (SDENv1_10317-18, annotated as two-component *ipd*AB-like CoA transferase) and ring D (SDENv1_10322, annotated as enoyl-CoA hydratase) are most similar to the corresponding enzymes from *Pseudomonas* sp. Chol1 (60-69% identity). Other upregulated genes of the *acd4*- and *acd5*-clusters are homologous to classical β-oxidation enzymes.

Growth of *Stl. denitrificans* with different steroids and fatty acids

Stl. denitrificans has a very narrow growth substrate spectrum restricted to cholesterol, cholest-4-en-3-one, 3β-hydroxy-5α-cholestane, androstane-3,17-dione analogues and the fatty acids palmitate and stearate (Tarlera and Denner, 2003); growth with propionate was shown in this work. The identification of eight gene clusters encoding C25DH-like genes suggests that S25DH isoenzymes may hydroxylate different side chain containing steroid growth substrates. We therefore tested the possibility, whether *Stl. denitrificans* uses globally abundant zoo-, phyto- and mycosterols as carbon and energy source under denitrifying conditions. Next to cholesterol, *Stl. denitrificans* used the branched side chain containing phytosterols β-sitosterol and stigmasterol as well as the mycosterol ergosterol; no growth was observed with estrogen (Fig. 7a-d); growth was also observed with testosterone, palmitate and propionate (Supporting Information Fig. S7e-h). Growth rates with side chain containing steroids were generally higher than with fatty acids (for exact doubling-times see Supporting Information Table S9). In all cases, anaerobic growth was coupled to nitrate reduction that was added in 5 mM portions to avoid nitrite accumulation.

Protein levels of S25DH-like and other proteins during anaerobic growth with different steroids and fatty acids

The proteomes of *Stl. denitrificans* during growth with cholesterol, β-sitosterol, stigmasterol, ergosterol, testosterone, palmitate, propionate under denitrifying conditions and with cholesterol under aerobic conditions were compared. Fig. 8 shows the relative abundancies of the eight putative active site subunits of S25DH isoenzymes (S25dA1-8, for exact abundance values see Supporting Information Table 7). With S25dA1-7 a higher protein level was observed during growth with all side chain containing steroids; only S25dA8 was merely regulated under any condition. Highest abundancy of C25DH (S25dA1) was observed during growth with cholesterol, fitting to the described specificity of the enzyme for cholesterol-derived intermediates (Dermer and Fuchs, 2012). For S25dA3, a higher protein abundance level was observed in ergosterol-grown cells suggesting a specific role in C25 hydroxylation

of steroids with structural features of ergosterol side chain. During growth with the phytosterols β-sitosterol or stigmasterol two different isoenzymes (S25dA2 and S25dA4) were induced, suggesting a specificity of the gene products for ethyl-branched steroids. In contrast the S25dA5-7 proteins were merely differentially abundant. Interestingly, all S25DH-like enzymes were significantly less abundant during aerobic growth with cholesterol. This finding was not expected, as *Stl. denitrificans* was reported to use the same oxygenase-independent steroid degradation pathway under denitrifying and aerobic conditions, and the growth rate was similar under aerobic/anaerobic conditions (Fig. 7, Chiang et al., 2008b; Wang et al., 2013). The lack of genes encoding oxygenases in the genome is in agreement with a common pathway during aerobic and anaerobic steroid degradation.

Conclusions and outlook

The integrated omics study of a side chain degrading anaerobic model organism now allows a complete view on the oxygen-independent degradation of globally abundant zoo-, phyto- and mycosterols. It helps to identify steroid degradation capacities at anoxic sites including wastewater-treatment plants where steroids belong to an emerging class of micropollutants. The degradation pathway of side chain containing steroids represent a patchwork of alternate CoA-ester dependent/independent reaction sequences that are known from aerobic cholesterol or cholic acid degradation in Actinobacteria or Gammaproteobacteria, together with enzymatic steps that are uniquely found in anaerobic steroid degrading Betaproteobacteria. *Stl. denitrificans* apparently uses different S25DH isoenzymes for C25-hydroxylation of side chain containing steroids that differ in the presence/absence of methyl-/ethyl-branches or a double-bond in the side chains. The subsequent shift of the C25-hydroxy group to C26 by an unknown enzyme is probably one of the most intriguing reactions of anaerobic cholesterol degradation. It cannot be explained by standard dehydration/hydration reactions because the pK_a of the non-activated C26-proton is >30. A radical-based mechanism would be plausible, especially the cholesterol-induced S25dA7 would be a likely candidate, as the encoding genes are located in a cluster adjacent to those encoding

cholest-4-en-3-one-26-oyl-CoA synthetase and alcohol and aldehyde dehydrogenases putatively involved in the oxidation of the C26-alcohol to the corresponding carboxylic acid. Molybdopterin-containing enzymes typically catalyze oxo-/hydroxy-transfer reactions (Hille et al., 2014) but are also capable of catalyzing single electrons/hydrogen atom transfers (Unciuleac et al., 2004; Weinert et al., 2015); thus they principally serve as candidates for a radical-based hydroxyl-group shift.

While the enzymatic reactions proposed for the degradation of rings A, C, and D of the steran skeleton can plausibly be assigned to gene products; the enzymes involved in ring B degradation are still elusive, probably because they are not organized in an induced gene cluster. The conversion of compound 10 to 11 (Fig. 3) involving ring B cleavage is is most likely catalyzed by a hydrolase.

Experimental procedures

Materials

If not otherwise stated steroids and fatty acids for growth experiments and analytical purposes were purchased from Sigma-Aldrich (Darmstadt, Germany), Cayman Chemicals (Ann Arbor, Michigan, USA) or Santa Cruz Biotechnology (Heidelberg, Germany) at appropriate purity. HIP was kindly provided by Prof. L. Eltis (University of British Columbia, Canada) and Prof. B. Philipp (University of Münster, Germany).

Bacterial strains

Stl. denitrificans Chol1S (DSM 13999) was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany). It was anaerobically grown under denitrifying conditions on steroids (4 mM), propionate (65 mM), or palmitate (8 mM), as described for cholesterol previously (Dermer and Fuchs, 2012; Warnke et al., 2016). Steroids were added as solid compounds to the medium without addition of detergents or cyclodextrin. Growth was monitored by measuring the protein content using the BCA protein assay kit (Roth, Karlsruhe, Germany) according to the manufacturer's instructions.

Synthesis, preparation and purification of steroid compounds

26-hydroxycholesterol, cholest-4-en-3-one-26-oic acid and cholest-4-en-26-al-3-one were chemically synthesized (Williams et al., 2002; Martin et al., 2009) and kindly provided by Prof. B. Breit (Freiburg, Germany). Steroid compounds without a 3-keto-4-en moiety were converted by cholesterol oxidase (Sigma-Aldrich). Conversion of steroid compounds to a 3-keto-1,4-diene was as described elsewhere (Chiang et al., 2008a). Production and purification of 25-hydroxy compounds was achieved as described (Chiang et al., 2007; Dermer and Fuchs, 2012). Synthesis of androst-1-en-3-one was as described (Leu et al., 2011).

Assembly of *Stl. denitrificans* genome

Sequencing was performed with Roche's 454 technology. The raw reads were quality-checked using custom scripts, assembled and scaffolded with newbler (Roche), and gap closed with SOAP (Luo et al., 2012) using an independent subset of shorter reads generated with the Illumina technology, yielding a 3,023,724 bp scaffold encompassing the complete *Stl. denitrificans* genome, together with three additional smaller (19-42 kbp) scaffolds corresponding to plasmid sequences. Completeness and contamination of the reconstructed genome was assessed with checkm (Parks et al., 2015) using 540 lineage specific single-copy marker genes organized into 241 sets.

Extraction of steroid and steroid-CoA-ester intermediates

For extraction of metabolites, cells were harvested by centrifugation at a protein content of 80-100 µg mL^{-1}, frozen in liquid nitrogen and directly used or stored at –20°C. Extraction of non-CoA ester steroids was as described (Lin et al., 2015). In the case of steroid CoA esters, cells were gently resuspended in 1 mL 100% acetonitrile and mixed with 1 mL 30% aqueous acetonitrile (pH 2, adjusted by formic acid). After centrifugation (10 min, 14.000 rpm) the supernatant was purified by solid phase extraction to enrich the steroid-CoA esters as described elsewhere (Gan-Schreiner et al., 2005). Lyophilized samples were dissolved in 200-500 µL methanol or 30% aqueous methanol and subjected to MS analysis.

LC-UV-vis and LC-ESI-MS/MS analyses of steroid compounds

Metabolites were analyzed by a Waters Acquity I-class ultra-performance-liquid-chromatography (UPLC®) system using Knauer Bluespher 100-2 C18 column (2 mm x 100 mm, 2 µm particle size, Knauer, Berlin, Germany) coupled to a Waters Synapt G2-S*i* HDMS electrospray ionization (ESI)/quadrupole-time-of-flight (Q-TOF) system (Waters, Eschborn, Germany). A gradient of 15–90% acetonitrile in 10 mM ammonium acetate buffer and a flow rate of 0.3 mL min^{-1} were used for separation. Samples were measured in positive mode with a capillary voltage of 3 kV, 150 °C source temperature, 450°C desolvation

temperature, 1000 L min^{-1} N_2 desolvation gas flow and 100 L min^{-1} N_2 cone gas flow. Collision induced dissociation of precursor ions was performed at 20 V collision energy. LC-UV-vis analyses were conducted on a Waters Acquity H-class UPLC system using a Knauer Eurospher C18 column and the same gradient as described above. Evaluation of LC-MS metabolite data was performed using MassLynx (Waters); for evaluation of LC-UV-vis data MassLynx or Empower (Waters) was used. Metabolites were identified by their retention times, UV-vis spectra and *m/z*-values compared to standards if available. CoA esters were verified by detection of their characteristic fragment ion at *m/z* = 428.0367 (Gan-Schreier et al., 2005).

Proteome analyses

Stl. denitrificans was grown in biological quadruplicates in 2-L sealed bottles as described above. Cells were harvested in the exponential growth phase (80-100 µg protein mL^{-1}). Detailed description of sample preparation and mass spectrometric analysis using a nano-LC-Orbitrap Fusion MS are provided as Supporting Information.

Enzyme assays

Activity of heterologously produced acyl-CoA synthetase was determined using a spectrophotometric assay as described (Casabon et al., 2013). Reactions were performed in 0.1 mL with 0.1 M HEPES buffer (pH 8), containing 1 mM CoA, 1.5 mM ATP, 1 mM PEP, 4 units pyruvate kinase, 4 units adenylate kinase, 4 units lactate dehydrogenase, 500 µM of NADH, 2.5 mM $MgCl_2$, 2 mM DTE, 7.8% (w/v) 2-hydroxypropyl-β-cyclodextrin, approximately 10 nM purified acyl-CoA synthetase and 300 µM of the respective steroid substrate (from 5-10 mM stock, dissolved in 70% isopropanol).

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Figure legends

Figure 1 Bacterial cholesterol and cholic acid degradation pathways. **A/D** Aerobic degradation of cholesterol in Actinobacteria; **B/D** Aerobic cholic acid degradation in Proteobacteria; **C/E** Anaerobic cholesterol degradation in *Stl. denitrificans*. The gene products involved that have experimentally been verified are indicated.

Figure 2 Circular chromosome map of *Stl. denitrificans*. The genes potentially involved in uptake (FadL-like), initial oxidation of ring A (*acmA*, *acmB*), C25-hydroxylation (S25dA1 and S25dBC1), and β-oxidation like reactions in the degradation of the side chain (clusters *acd*1-2) and the sterane skeleton (*acd*3-5) are displayed according to their respective locations; location of genes encoding homologues to C25DH subunits are indicated (S25dABC 2-8). Circles display (from the outside) correspond to: (1) GC percent deviation (GC window - mean GC) in a 1000-bp window, (2) predicted CDSs transcribed in the clockwise direction, (3) predicted CDSs transcribed in the counterclockwise direction, (4) GC skew (G+C/G-C) in a 1000-bp window, (5) rRNA (blue), tRNA (green), misc_RNA (orange), transposable elements (pink) and pseudogenes (grey).

Figure 3 Genes and metabolites involved in anaerobic cholesterol degradation in *Stl. denitrificans*. Gene products are presented with abbreviations and gene identification numbers. For simplicity, "SDENv1_" was omitted from identification numbers. Metabolites identified by LC-MS analyses in this work are depicted in red; for MS analyses of these compounds see Supporting Information Table S3. Reactions without a clear assignment to a gene are marked with a question mark. Enzyme abbreviations: AcmA = cholesterol dehydrogenase/isomerase; AcmB = cholest-4-en-3-one-Δ1-dehydrogenase; C25DH = cholesterol C25 dehydrogenase; ACS = acyl-CoA synthetase; ACAD = acyl-CoA dehydrogenase; ECH = enoyl-CoA hydratase; ALD = aldolase; ALDH = aldehyde

dehydrogenase; Δ4R = ketosteroid-Δ4-reductase; AtcABC = 1-testosterone dehydrogenase/hydratase; αβHL = α,β-hydrolase; TL = thiolase. Selected structures: 1 = cholesterol; 2 = cholesta-1,4-diene-3-one; 3 = 25-hydroxycholesta-1,4-diene-3-one; 4 = cholesta-1,4-diene-3-one-26-oic acid; 5 = chola-1,4-diene-3-one-24-oic acid; 6 = pregna-1,4-diene-3-one-20-oic acid; 7 = androsta-1,4-diene-3-one (ADD); 8 = androst-1-en-3-one (1-AD); 9 = 1,17-dioxo-2,3-*seco*-androstan-3-oic acid (DSAO); 10 = 2,5-*seco*-3,4-dinorandrost-1,5,17-trione (2,5-SDAT); 11 = 3aα-*H*-4α(3'-propanoate)-7aβ-methylhexahydro-1,5-indanedione (HIP); 12 = 3aα-*H*-4α(carboxylic acid)-5-hydroxy-7aβ-methylhexahydro-1-indanone (5OH-HIC); 13 = (*R*)-2-(2-carboxyethyl)-3-methyl-6-oxocyclohex-1-ene-1-carboxyl-CoA (COCHEA-CoA); 14 = 4-methyl-5-oxooctanedioyl-CoA (MOODA-CoA). For intermediates with intact ring A, identified metabolites can either have 4-en-3-one or 1,4-diene-3-one moiety. The last three structures refer to acetyl-CoA, propionyl-CoA and succinyl-CoA.

Figure 4 Arrangement of genes encoding molybdopterin containing steroid C25 dehydrogenases (S25DH) in the genome of *Stl. denitrificans*. The genes encoding experimentally verified cholesterol C25 dehydrogenase (C25DH) that converts cholesterol/cholest-4-en-3-one/cholesta-1,4-diene-3-one to the corresponding 25-hydroxy compounds are shown in bold arrows. Color code: red, genes encoding molybdopterin-containing active site subunits; orange, genes encoding electron-transferring FeS-cluster subunits; yellow, genes encoding heme B containing electron-transferring subunits; blue, genes encoding putative chaperones.

Figure 5 Proposed cholesterol-induced gene clusters *acd1* and *acd2* involved in isoprenoid side chain degradation. Genes involved in reactions with C26-, C24-, and C22-substrates are clustered as indicated; genes numbers assigned to reactions shown in Fig. 3 are highlighted in green. The numbers below the gene arrows refer to log2 ratios of differential protein abundancies during growth with cholesterol vs propionate and cholesterol vs testosterone; n.d. = not detected (detection of proteins was below the quality criteria of MS analyses); n.r.

= non-differentially regulated (log2 ratios were below 1.25). The gene SDENv1_11189 was heterologously expressed and the purified product was identified as a specific cholest-4-en-3-one-26-oyl-CoA synthetase. The three genes shown in purple encode a putative S25DH isoenzyme (SDENv1_11191-93; S25dABC7) that is hypothetically involved in the isomerization of 25- to 26-hydroxy-cholest-4-en-3-one; genes with high similarities to those of the *igr*-operon of *M. tuberculosis* involved in the conversion of 22-oic acid to ADD are highlighted. The location of the cluster regions in the genome of *Stl. denitrificans* is indicated. ACS: acyl-CoA synthetase; ALD: aldolase; ALDH: aldehyde dehydrogenase; ACAD: acyl-CoA dehydrogenase; ECH: MaoC-like enoyl-CoA hydratase. Genes in grey code for putative proteins involved in transport, regulation or are assigned to unknown function.

Figure 6 Proposed gene clusters *acd*3-5 involved in steran skeleton degradation. Clusters *acd*4 and *acd*5 are cholesterol-induced (vs propionate), whereas *acd3* showed no differential regulation. Genes assigned to code for enzymes involved in ring A and ring CD-degradation are clustered as indicated; no distinct putative gene cluster involved in ring B degradation was identified. Gene numbers assigned to reactions shown in Figure 3 are highlighted in green. The numbers below the gene arrows refer to log2 ratios of differential protein abundancies during growth with cholesterol vs propionate and cholesterol vs testosterone; n.d. = not detected (detection of proteins was below the quality criteria of MS analyses); n.r. = non-differentially regulated (log2 ratios were below 1.25).The location of the cluster region in the genome of *Stl. denitrificans* is indicated. ACS: acyl-CoA synthetase; ALD: aldolase; ALDH: aldehyde dehydrogenase; ACAD: acyl-CoA dehydrogenase; ECH: MaoC-like enoyl-CoA hydratase. Genes in grey code for putative proteins involved in transport, regulation or are assigned to unknown function.

Figure 7 Growth of *Stl. denitrificans* with various steroids. The mean of five biological replicates for total protein content [µg/ml] (●) are shown. (a) cholesterol, (b) β-sitosterol, (c)

stigmasterol, (d) ergosterol. The apparent biphasic growth curves in a and d are due to the consumption and stepwise addition of nitrate (5 mM) to avoid toxic nitrite accumulation.

Figure 8 Abundance of eight homologous putative active site subunits of C25DH (S25dA1-8) from *Stl. denitrificans* during growth under different conditions. The heatmap (green = upregulation, red = downregulation) shows the log2 ratio of S25dA1-8 on steroid substrates compared to **A** propionate and **B** testosterone. For exact abundance values see Supporting Information Table S7.

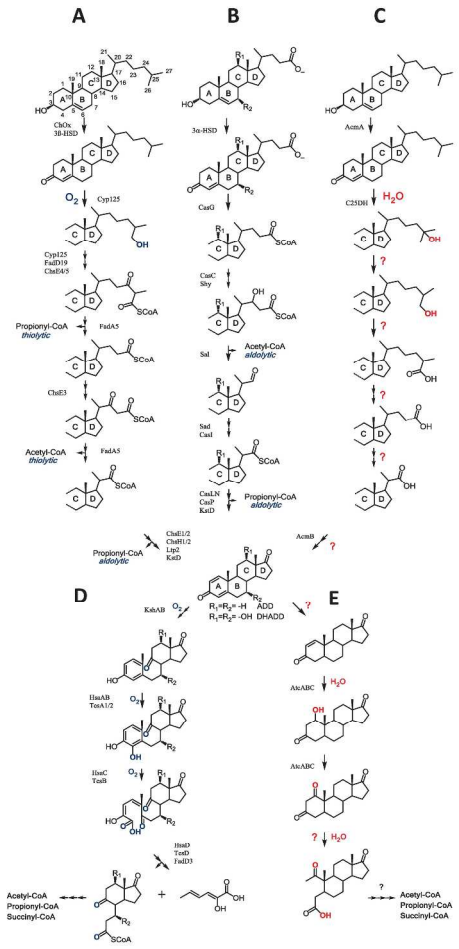

Fig 1

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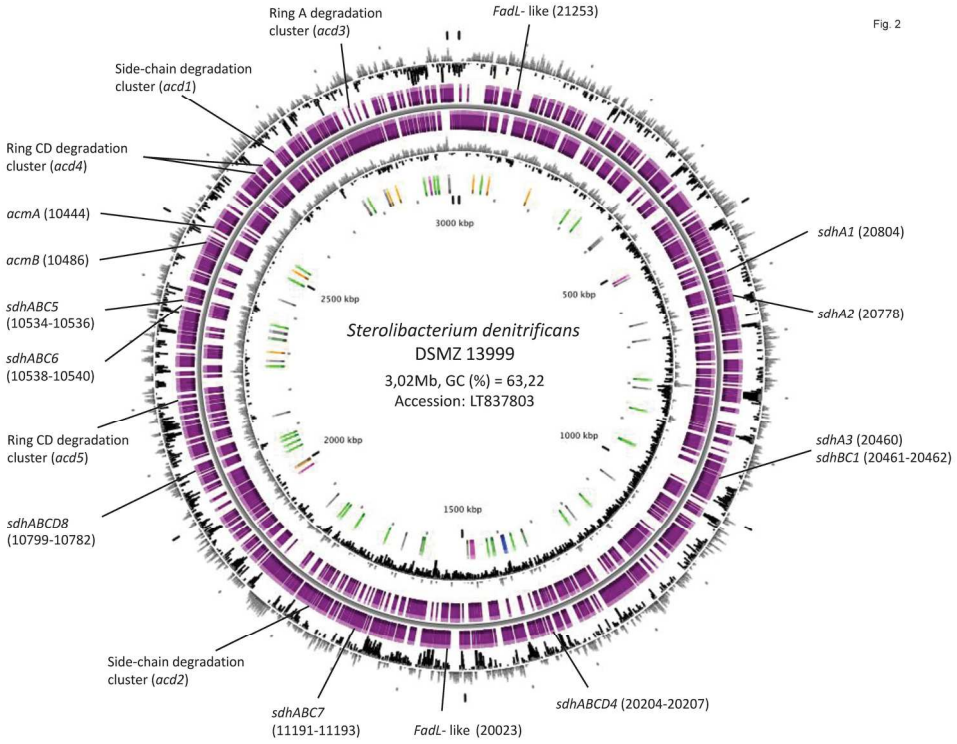

Fig 2

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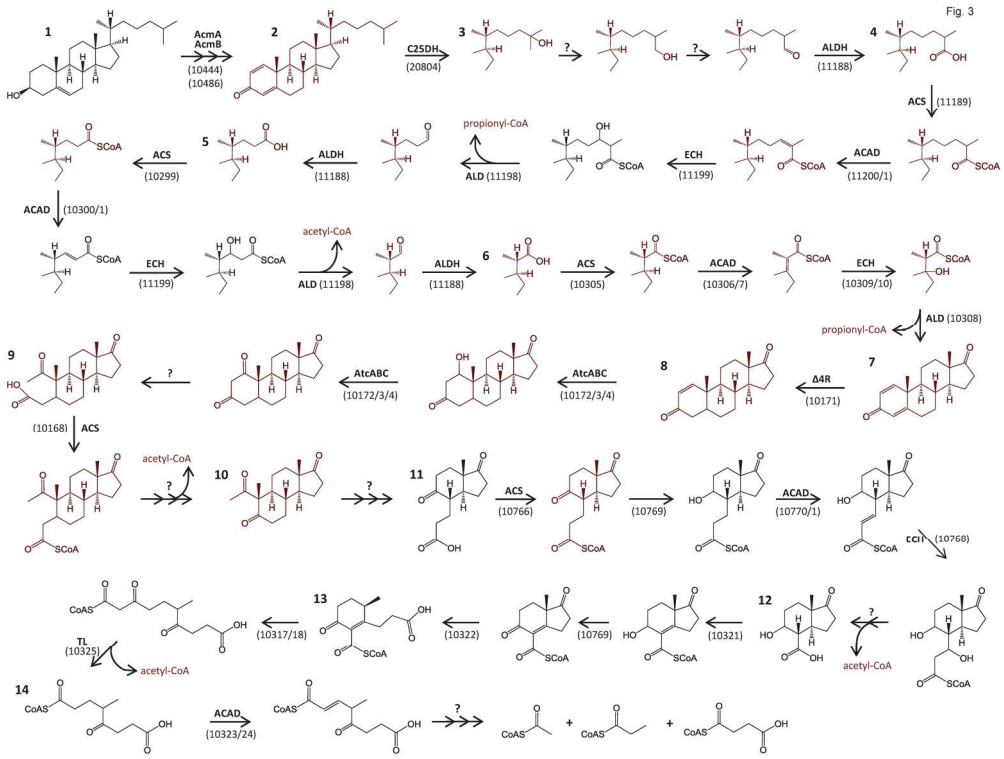

Fig

190x142mm (300 x 300 DPI)

Fig. 4

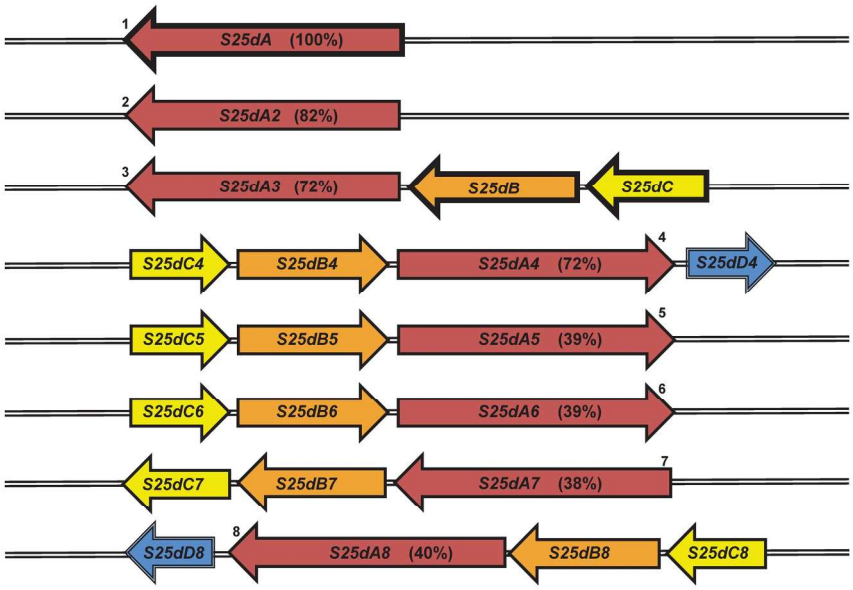

Fig 4

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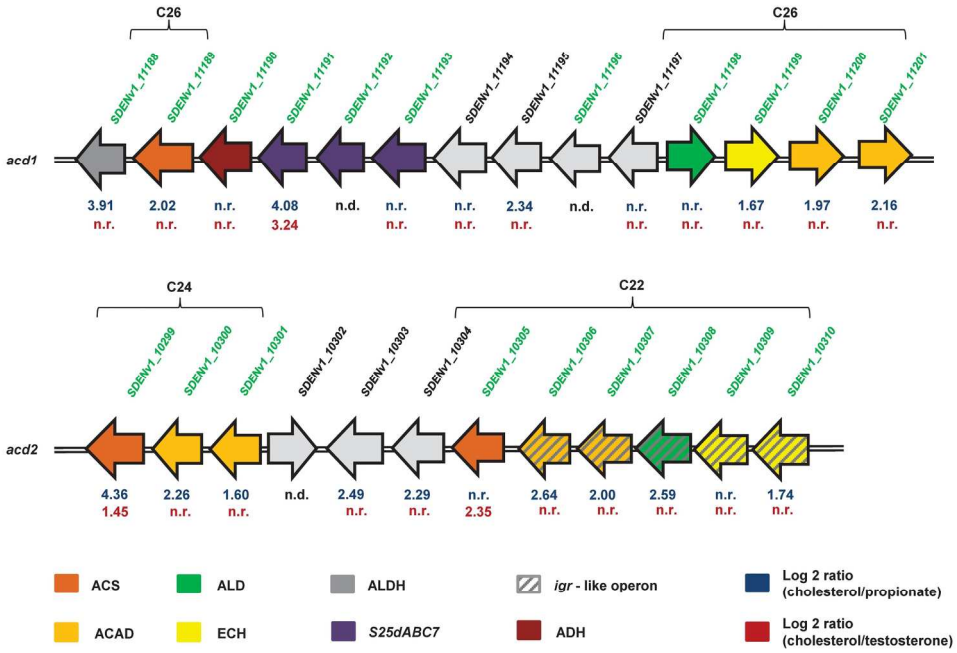

Fig 5

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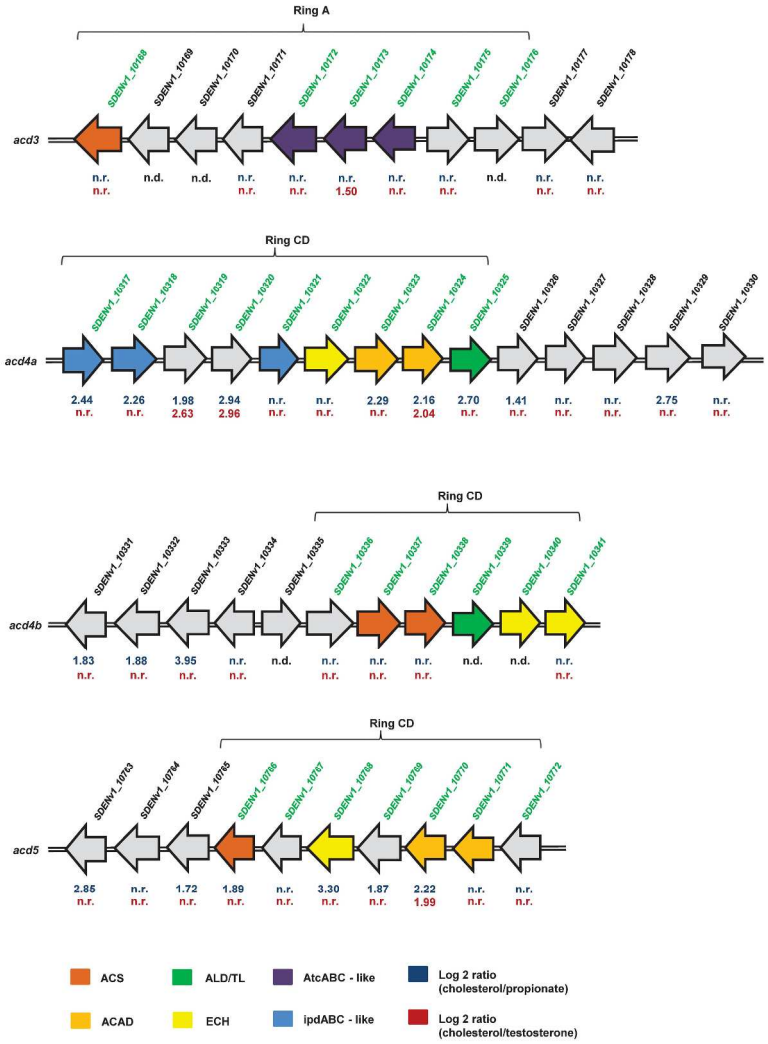

Fig 6

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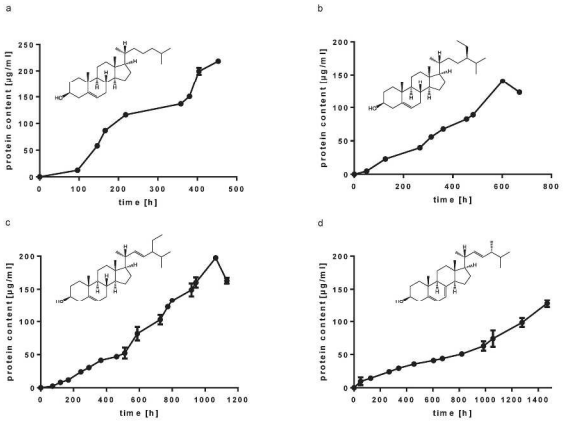

Fig 7

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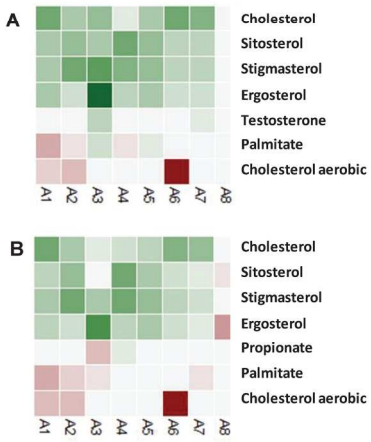

Fig 8

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