Bacterial degradation of bile salts

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Abstract Bile salts are surface-active steroid compounds. Their main physiological function is aiding the digestion of lipophilic nutrients in intestinal tracts of vertebrates. Many bacteria are capable of transforming and degrading bile salts in the digestive tract and in the environment. Bacterial bile salt transformation and degradation is of high ecological relevance and also essential for the biotechnological production of steroid drugs. While biotechnological aspects have been reviewed many times, the physiological, biochemical and genetic aspects of bacterial bile salt transformation have been neglected. This review provides an overview of the reaction sequence of bile salt degradation and on the respective enzymes and genes exemplified with the degradation pathway of the bile salt cholate. The physiological adaptations for coping with the toxic effects of bile salts, recent biotechnological applications and ecological aspects of bacterial bile salt metabolism are also addressed. As the pathway for bile salt degradation merges with metabolic pathways for bacterial transformation of other steroids, such as testosterone and cholesterol, this review provides helpful background information for metabolic engineering of steroid-transforming bacteria in general.

Keywords Biodegradation · Steroids · Bile salts

Introduction

Bile salts are surface-active steroid compounds that occur in the digestive tracts of vertebrates (Hofmann and Mysels

1988; Moschetta et al. 2005; Hagey et al. 2010). Characteristic structural features of many bile salts are 1–3 hydroxyl groups in α -position at C3, C7 and C12, respectively, and a C₅ acyl side chain attached to C17 (Fig. 1a). These structural features are responsible for the high water solubility of bile salts. In addition, bile salts act as detergents because all hydroxyl groups protrude into the same direction thereby creating a hydrophilic and a hydrophobic part (Helenius and Simons 1975; Hofmann and Mysels 1988). The main physiological function of bile salts is to emulsify bile lipids and dietary lipids thereby aiding the digestion of hydrophobic nutrients. In addition, bile salts are involved in several other physiological processes related to digestion and liver functioning as e.g. signalling compounds (Hylemon et al. 2009; Monte et al. 2009). Bile salts are synthesised in the liver and are stored in the gall bladder. Their biosynthesis proceeds via modifications of the steroid skeleton and shortening of the side chain of cholesterol (Russell 2003). In humans and many other mammals, the so-called primary bile salts cholate and chenodeoxycholate are formed and conjugated to taurine or glycine by N-acyl amidation (Fig. 1a); conjugation to glycine lowers the pK of cholate from 6.4 to 4.4 (Russell 2003). These conjugated bile salts are then released into the lumen of the small intestine. A large fraction of bile salts is re-absorbed in the intestinal tract and re-secreted into the bile in the course of the enterohepatic cycle (Ridlon et al. 2006). A considerable amount of bile salts is, however, released into the environment with faeces (300–600 mg per day and human; Ridlon et al. 2006) and urine (4 mg per day and human; Hayakawa 1982).

In bacteria, steroids, and thus also bile salts, appear to occur only as a rare exception. There are only a few reports about steroid biosynthesis in bacteria, e.g. in a methanotrophic bacterium (Bird et al. 1971) and in *Myxobacteria* (Bode et al. 2003 and references therein). Two more reports

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Fig. 1 Structures of different bile salts. **a** Structures of unconjugated and conjugated bile acids occurring in humans; cholate and cheno deoxycholate are primary bile salts that are transformed into the secondary bile salts deoxycholate and lithocholate, respectively, by

intestinal bacteria. **b** Bile salt petromyzonol sulphate of the sea lamprey *Petromyzon marinus* acting as a migratory pheromone (structure redrawn after Sorensen et al. 2005a)

provide indirect evidence of *Myroides* sp. strain SM1 (Maneerat et al. 2005) as well as of other marine bacteria (Kim et al. 2007) producing the bile salts cholate, glycocholate, deoxycholate and glycodeoxycholate.

Nevertheless, the ability of degrading and transforming steroids is widespread among bacteria. A well-studied process is bile salt transformation by anaerobic bacteria in the intestinal tract. Many aerobic bacteria from soil or water are able to grow with bile salts and other steroids as sole source of carbon and energy. For energy conservation, these bacteria oxidise steroid compounds completely to CO_2 . As steroids are abundant natural compounds that are released into the environment through excretion by and the decay of eukaryotic organism, their complete degradation is relevant for the CO_2 -releasing side of the global C cycle. Sterols

serving as membrane constituents, such as cholesterol, ergosterol and phytosterols, are the most abundant steroid compounds in nature. Recently, bacterial degradation of cholesterol has received major attention in clinical microbiology because of the discovery that *Mycobacterium tuberculosis* utilises host cholesterol during lung infections (Pandey and Sasseti 2008; Hu et al. 2010).

Bacterial steroid transformation and degradation could potentially modulate or interfere with all kinds of eukaryotic signalling, in which steroid pheromones are involved. It is known that teleost fish and sea lampreys release steroid pheromones into the water. In goldfish, these pheromones include androstenedione (Sorensen et al. 2005a, b), which can be easily degraded by bacteria as outlined below. Larvae of the sea lamprey *Petromyzon marinus* use a

combination of three steroid compounds as a migratory pheromone (Fine et al. 2004; Sorensen et al. 2005a, b). One of these steroids is petromyzonol sulphate (Fig. 1b), which is also known as the major bile salt in sea lampreys (Hagey et al. 2010). In the water column, petromyzonol sulphate and the other two steroid compounds are subject to microbial degradation, which could have an impact on signalling between larvae and adults of *P. marinus* under certain conditions (Polkinghorne et al. 2001; Fine and Sorensen 2010). Furthermore, the bile salt cholate has been identified as an elicitor for phytoalexin production in rice plants, thereby conferring increased resistance against plant pathogens (Koga et al. 2006; Shimizu et al. 2008). Bacterial degradation of cholate in soils may therefore influence the fitness of plants. Alternatively, bacterial steroid transformation may convert hormonally inactive into hormonally active steroids. Interesting examples for this are reports that bacterial degradation of phytosterols, which are released in high amounts with effluents of paper mills, leads to the formation of androgenic steroids that cause masculinisation of fish (Carson et al. 2008). Studies on the influence of bacterial degradation of steroids on eukaryotic signalling processes are still rare, but, regarding the great variety of biological functions covered by steroid compounds, they could potentially lead to seminal findings in chemical ecology.

Bacterial transformation of steroid compounds is highly relevant for biotechnology because it is an essential part of the production of steroid drugs. Biotechnological aspects of bacterial transformation of bile salts and other steroids have been reviewed by several authors (Hayakawa 1982; Kieslich 1985; Szentirmai 1990; Mahato et al. 1994; Mahato and Garai 1997; Bortolini et al. 1997; Donova 2007). These reviews deal with the metabolic capacity of production strains and their respective mutants that were isolated in the course of screening procedures while the biochemical and genetic details of the metabolic pathways underlying these biotransformations were neglected. A review focussing on the physiology, biochemistry, genetics and ecology of bacterial steroid degradation is, to our knowledge, still missing. This review aims at filling this gap. For this, the main route for bile acid degradation with cholate as a model compound for steroids will be depicted and the respective enzymes and genes will be described. In addition, physiological properties of bacteria necessary for coping with the toxic effects of bile salts as well as recent biotechnological applications are discussed.

Transformation of bile salts by intestinal bacteria

Transformation of bile salts in the intestinal tract is subject of intense research (for reviews see Hylemon and Harder

1998; Ridlon et al. 2006). This process is not the focus of this review, and is, therefore, only shortly summarised. Intestinal bile salt transformation is mainly performed by strictly anaerobic bacteria of the genera *Bacteroides*, *Eubacterium* and *Clostridium*. The initial step in bile salt transformation is deconjugation of taurine- and glycine-conjugated bile salts to the respective unconjugated free bile salts. This process is catalysed by bile salt hydrolases. Free bile salts are further processed via reductive 7α -dehydroxylation. This process generates the so-called secondary bile salts deoxycholate and lithocholate, which are produced from the primary bile salts cholate and chenodeoxycholate, respectively (Fig. 1a). The benefit of bile salt transformation for the bacteria is believed to be the acquisition of glycine and taurine as substrates and the disposal of electrons from fermentations onto bile salts as electron acceptors; for humans, however, secondary bile salts produced by intestinal bacteria have a negative impact on health (Ridlon et al. 2006). In this respect, deoxycholate has been shown to act as a promoter of colon cancer (Payne et al. 2009). Some aspects of the reductive $7\alpha/\beta$ -dehydroxylation, namely uptake, CoA-activation and redox reactions on the steroid skeleton of bile salts, will be addressed below while describing complete degradation of bile salts.

Complete degradation of bile salts by aerobic bacteria

Complete aerobic bile salt degradation has been reported from various groups of bacteria including *Actinobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. Among the *Actinobacteria*, many genera are capable of bile salt degradation, in particular *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Streptomyces* (Hayakawa 1982; Donova 2007 and references therein). Among the *Betaproteobacteria*, steroid degradation is being intensively studied with different strains of *Comamonas testosteroni* (Horinouchi et al. 2010 and references therein); in addition, cholate degradation has also been reported from the marine psychrophilic bacterium *Pseudoalteromonas haloplanktis* (Birkenmaier et al. 2007). Among the *Gammaproteobacteria*, there are many reports on bile salt degradation by different *Pseudomonas* species (Tenneson et al. 1978a, b, 1979; Leppik 1982, 1983; Philipp et al. 2006 and references therein).

Aerobic bacterial bile salt degradation was intensely investigated in the 1970s and 1980s. These studies were focused on the identification of steroid degradation intermediates that were extracted from bacterial cultures growing with bile salts as carbon and energy source while physiological, biochemical and genetic aspects were mainly neglected (as an example see Tenneson et al. 1979). Based on the structures of degradation intermediates, a general

scheme for bacterial bile salt degradation by environmental bacteria was proposed by Hayakawa (1982). Briefly, bile salt degradation starts with oxidation of the hydroxyl group at C3, followed by desaturation of the A-ring, leading to 3-keto-1,4-diene structures. Concomitant with or after A-ring oxidation the acyl side chain is shortened and finally removed from the steroid skeleton yielding a 1,4-androstadiene-3,17-dione (ADD) derivative with hydroxyl groups at C7 and/or C12 (such as compound XIII in Fig. 2), depending on the bile salt being degraded (Kieslich 1985). ADDs are further degraded by hydroxylation at C9 resulting in cleavage of the B-ring between C9 and C10 with concomitant aromatisation of the A-ring. The resulting steroids with an opened ring are termed seco-steroids (such as compound XIV). In the next step, the aromatic A-ring is cleaved, and the resulting di-seco steroids (such as compound XVI) are hydrolytically cleaved between the former rings A and B yielding a C₆-acid (XVII) and an acidic perhydroindane derivative (such as compound XIX). The pathway starting from ADD is also referred to as the 9,10-seco-pathway for aerobic degradation of steroid compounds (Hayakawa 1982).

In the following chapters, the recent physiological, biochemical and genetic knowledge on bile salt degradation is summarised and illustrated by a plausible degradation pathway for cholate (compound I in Fig. 2). Most studies referred to were actually not on the degradation of cholate itself but of cholesterol and testosterone by actinobacteria (mainly *M. tuberculosis* and *Rhodococcus* spp.) and *C. testosteroni*, respectively. Consequently, most enzymes and genes described originate from these bacteria. There is, however, evidence that the degradation pathways for cholesterol and testosterone merge with the degradation pathway for C₂₄ bile salts. First, degradation of the C₈ alkyl side chain of cholesterol proceeds via β -oxidation yielding C₂₄ steroids with a C₅ acyl side chain (Sih et al. 1968; Rosloniec et al. 2009). Second, the pathways for testosterone, cholesterol and bile salts merge at ADD and its respective derivatives. As the degradation pathways for several steroid hormones (Horinouchi et al. 2010) also lead to ADDs, these compounds can be considered as central intermediates in aerobic degradation of steroid compounds (Fig. 3). The further degradation of ADDs proceeds via the 9,10-seco-pathway for all steroid-degrading bacteria analysed so far.

Uptake of bile salts

The only described bile salt transporter is BaiG from the intestinal cholate-dehydroxylating bacterium *Clostridium scindens* (Mallonee and Hylemon 1996; Ridlon et al. 2006). This 50-kDa protein with 14 membrane-spanning domains catalyses the proton-motive-force-dependent uptake of bile

salts. A gene for another putative bile salt transporter has been found in *Lactococcus johnsonii* (Elkins and Savage 1998). Both BaiG and the putative bile salt transporter have only insignificant similarities to putative transporters in the cholate-degrading bacteria *C. testosteroni*, *Rhodococcus jostii* strain RHA1 and *P. haloplanktis* strain TAC125. In *R. jostii* strain RHA1, the Mce4 system has been identified as a transporter for cholesterol and β -sitosterol (Mohn et al. 2008). While the *mce4* locus occurs in diverse *Actinobacteria*, it is not found in Gram-negative steroid-degrading bacteria. With *R. jostii* strain RHA1, it was shown that *mce4*-knockout mutants were not affected in growth with cholate indicating that other systems might be involved in cholate uptake. Taken together, specific transporters for bile salt uptake in bile salt-degrading bacteria are still unknown.

For Gram-negative bacteria, it has been shown that the general bacterial porin OmpF is involved in bile salt uptake across the outer membrane (Thanassi et al. 1997). As unconjugated bile acids have a low pK value, bile diffusion across the membrane might also contribute to bile salt uptake.

A-ring oxidation

Most of the studies on A-ring oxidising enzyme activities have been performed with testosterone as substrate. In general, oxidation of the 3 α -hydroxyl group catalysed by 3 α -hydroxysteroid dehydrogenases is considered as the initiating step in bile salt degradation. This reaction is catalysed by NAD(P)-dependent enzymes related to the short chain dehydrogenase/reductase superfamily (Hoffmann and Maser 2007). The 3 α -hydroxysteroid dehydrogenase HsdA from *C. testosteroni* strain ATCC11996 has been described in great detail including its crystal structure (Oppermann and Maser 1996; Grimm et al. 2000). The 3 α -hydroxysteroid dehydrogenase BaiA from *C. scindens* acts on free bile salts as well as on bile salt CoA-esters (Ridlon et al. 2006). The gene for the 3 α -hydroxysteroid dehydrogenase has recently been identified in *C. testosteroni* strain TA441 as well (Horinouchi et al. 2010).

Desaturation of the A-ring is catalysed by Δ -3-ketosteroid dehydrogenases. The Δ -3-ketosteroid dehydrogenases KSTD1, KSTD2 and KSTD3 from *Rhodococcus erythropolis* strain SQ1 as well as the Δ^4 -3-ketosteroid dehydrogenases from *C. testosteroni* ATCC 17410 and from *Rhodococcus rhodochrous* have been identified as flavoproteins (Florin et al. 1996; Morii et al. 1998; Knol et al. 2008). In *C. testosteroni* strain TA441, the Δ^4 - and the $\Delta^{1,4}$ -3-ketosteroid dehydrogenases are encoded by the genes *tesH* and *tesI*, respectively (Horinouchi et al. 2003b). In cell extracts of cholate-grown cells of *Pseudomonas* sp. strain Choll, cholate 3 α -dehydrogenase and $\Delta^{1,4}$ -3-

Fig. 2 Hypothetical degradation pathway for cholate (I) based on compounds, genes and proteins identified in different steroid degrading bacteria. The following compounds have been identified: II, 3 ketocholelate; III, $\Delta^{1,4}$ 3 ketocholelate; IV, V, VI, CoA esters of I, II and III, respectively; X, CoA ester of DHOPDC (7 α ,12 α dihydroxy 3 oxopregna 1,4 diene 20 carboxylate); XIII, DHADD (7,12 dihydroxy 1,4 androstadiene 3,17 dione); XIV, THSATD (3,7,12 trihydroxy 9,10 secoandrosta 1,3,5(10)triene 9,17 dione); XVI, 7,12 dihydroxy derivate of 4,9 DSHA (4,5 9,10 diseco 3 hydroxy 5,9,17 trioxoandrosta 1(10)2 diene 4 oate); XVII, HHD (2 hydroxy hexa 2,4 dienoate); XVIII, 4 hydroxy 2 oxohexanoate; XIX, 7,12 dihydroxy DOHNAA (9,17 dioxo 1,2,3,4,10,19 hexanorandrostan 5 oate). Compounds in *brackets* are plausible intermediates of

cholate degradation but have not been detected yet; the free bile salts of CoA esters VII and XI lacking the hydroxyl group at C7 have been detected in degradation of deoxycholelate; 3,4 DHSA (3,4 dihydroxy 9,10 secoandrosta 1,3,5(10) triene 9,17 dione), an analogue of compound XV lacking the hydroxyl groups at C7 and C12, has been detected in degradation of testosterone and cholesterol. Enzymes and genes related to cholate degradation are *underlined*; *non underlined* enzymes and genes are involved in analogous steps in the degradation of testosterone or cholesterol. The genes and enzymes originate from the following bacteria: ¹*C. testosteroni* strain ATCC11996; ²*C. scindens*; ³*R. erythropolis*; ⁴*C. testosteroni* strain TA441; ⁵*Pseudomonas* sp. strain Choll; ⁶*M. tuberculosis*

Fig. 3 The central role of androstadienediones (ADDs) in aerobic bacterial degradation of steroid compounds

ketocholelate dehydrogenase activities were detected with NAD⁺ and potassium hexacyanoferrate as electron acceptors, respectively, (Birkenmaier et al. 2007). These reactions lead to the formation of 3-ketocholelate (II), Δ^1 or Δ^4 -3-ketocholelate and $\Delta^{1,4}$ -3-ketocholelate (III), respectively.

Degradation of the acyl side chain

The degradation of the steroid acyl side chain by bacteria was studied mainly on the phenomenological level (Kieslich 1985; Szentirmai 1990) and has, to our knowledge, so far not been shown *in vitro*. Theoretically, the degradation of the C₅-acyl side chain of bile salts should proceed via β -oxidation causing the consecutive release of an acetyl and a propionyl residue.

A prerequisite for β -oxidation of the acyl side chain is its activation with coenzyme A (CoA). CoA-activation has been reported from intestinal bacteria as part of 7 α -dehydroxylation. BaiB has been identified as a bile acid CoA ligase in *C. scindens* strain VPI 12708 (Mallonee et al. 1992; Ridlon et al. 2006). This enzyme is also activating other C₂₄ bile acids (Mallonee et al. 1992). Formation of cholyl-CoA (IV) has been detected in extracts of cholate-grown cells of *Pseudomonas* sp. strain Choll (Birkenmaier

et al. 2007). In these assays, the CoA-esters of 3-ketocholelate (V) and $\Delta^{1,4}$ -3-ketocholelate (VI) were also detected. It is, however, still unclear whether A-ring oxidation precedes CoA-activation or whether CoA-esters are substrates for A-ring oxidation.

In studies with different *Pseudomonas* sp. on degradation of cholate (Tenneson et al. 1979), deoxycholate (Leppik 1982, 1983), lithocholate (Tenneson et al. 1978a) and taurocholate and glycocholate (Tenneson et al. 1978b), steroid compounds were isolated and identified that are in agreement with degradation of the acyl side chain via β -oxidation. The first genetic and biochemical studies on β -oxidation of bile salts were performed with *Pseudomonas* sp. strain Choll. The mutant strain R1 of *Pseudomonas* sp. strain Choll, which is unable to grow with cholate, transforms cholate into 7 α ,12 α -dihydroxy-3-oxopregna-1,4-diene-20-carboxylate (DHOPDC) (Birkenmaier et al. 2007). DHOPDC can be formed from cholate by the release of an acetyl residue from the acyl side chain. The disrupted gene *acad* encodes a putative acyl-CoA-dehydrogenase. Its physiological function is to insert a double bond into the side chain of DHOPDC. Formation of DHOPDC-CoA (X), which is the prerequisite for this oxidation, was detected in extracts of cholate-grown cells of strain Choll. The two

following CoA-esters (XI and XII) have not been detected yet but an analogue of the free bile salt of XI has been identified in the degradation of deoxycholate (Leppik 1983). The release of a C₃ residue as propionyl-CoA from compound XII is believed to proceed via aldol cleavage (Kieslich 1985). Accumulation of DHOPDC has also been reported from a mutant of *C. testosteroni* strain TA441 with a transposon insertion in a gene that has about 70% amino acid identity to *acad* of *Pseudomonas* sp. strain Chol1 (Horinouchi et al. 2010). Of the compounds preceding DHOPDC, the free bile salt of compound VII lacking the hydroxyl group at C7 has been detected in the degradation of deoxycholate (Leppik 1983). In addition, the free bile salts of CoA-esters VII and VIII have been identified in the degradation of cholate by a mutant of *Pseudomonas* sp. strain Chol1 (Birkenmaier, Möller, Philipp unpublished).

Although some of the intermediates of β -oxidation of the acyl side chain have not been detected yet, the compounds identified so far strongly support the proposed pathway. In addition, two genes for proteins similar to that encoded by *acad* in strain *Pseudomonas* sp. strain Chol1 were found to be upregulated in cholesterol-grown cells of *R. jostii* strain RHA1 (van der Geize et al. 2007).

Breakdown of steroid skeleton: 9 α -hydroxylation and cleavage of B-ring

The final products of A-ring oxidation and acyl side chain degradation are ADDs, and in the case of cholate 7,12-dihydroxy-1,4-androstadiene-3,17-dione (DHADD (XIII); Philipp et al. 2006). ADDs are subject to hydroxylation at C9 in the B-ring. This reaction is catalysed by 3-ketosteroid-9 α -hydroxylases. This enzyme, KshAB, has been isolated from *M. tuberculosis* and from *R. rhodochrous*, and has been shown to be a two-component Rieske oxygenase (Capyk et al. 2009; Petrusma et al. 2009). While the reductase component KshB appears to interact with other oxygenases and is also involved in various cellular processes, KshA is the specific oxygenase component for ADDs (Hu et al. 2010). So far, 3-ketosteroid-9 α -hydroxylases have not been purified from Gram-negative bacteria, and BLAST analysis (Altschul et al. 1997) does not indicate predicted proteins with significant similarity to the oxygenase subunit KshA in Gram-negative steroid-degrading bacteria.

If the A-ring has a $\Delta^{1,4}$ -3-one-structure, 9 α -hydroxylation leads to the cleavage of the B-ring between C9 and C10 concomitant with the aromatisation of the A-ring by a spontaneous chemical reaction via a retro-aldol rearrangement (Kieslich 1985; Park et al. 1986). The resulting compounds are 3-hydroxy-9,10-secoandrosta-1,3,5(10)-9,17-

diones (HSAs). In the case of cholate, 9 α -hydroxylation of DHADD leads to the formation of 3,7,12-trihydroxy-9,10-secoandrosta-1,3,5(10)triene-9,17-dione (THSATD (XIV); Philipp et al. 2006).

In *C. testosteroni* strain TA441, the 12 α -hydroxyl group of DHADD (XIII) is epimerised to a 12 β -hydroxyl group by enzymes encoded by *steA* and *steB* (Horinouchi et al. 2008). This epimerisation is believed to facilitate 9 α -hydroxylation of DHADD. In earlier reports on cholate degradation by different *Pseudomonas* sp., 12 β -hydroxyl groups have been found in DHADD and THSATD (XIV) (Tenneson et al. 1979; Park et al. 1986). As it is not known whether this epimerisation occurs in all cholate-degrading bacteria, the stereochemistry of the 12-hydroxyl group in DHADD (XIII) and its further degradation intermediates (XIV, XV, XVI, XIX, XX) is not indicated in Fig. 2.

Cleavage of A-ring and further degradation

The next step is the hydroxylation at C4 in the A-ring, yielding 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-diones (3,4-DHSAs, such as XV). HsaAB from *M. tuberculosis* catalyses this reaction and was shown to be a two-component flavin-dependent monooxygenase (Dresen et al. 2010). In *C. testosteroni* strain TA441, this reaction is catalysed by a two-component flavin monooxygenase encoded by *tesA1A2* (Horinouchi et al. 2004a).

The vicinal hydroxyl groups at the aromatic ring render 3,4-DHSAs substrates for ring-cleaving dioxygenases via *meta*-cleavage. HsaC from *M. tuberculosis* catalyses this reaction and was shown to be an extradiol dioxygenase with a mononuclear iron centre (Yam et al. 2009). In *C. testosteroni* strain TA441, this reaction is catalysed by a *meta*-cleaving dioxygenase encoded by *tesB* (Horinouchi et al. 2001). The reaction products are 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10)2-diene-4-oates (4,9-DSHAs, such as XVI).

The 4,9-DSHAs are further degraded by a hydrolytic cleavage between the former rings A and B. HsaD from *M. tuberculosis* catalyses this reaction and was shown to be a carbon-carbon hydrolase (Lack et al. 2010). In *C. testosteroni* strain TA441, this reaction is catalysed by a protein encoded by *tesD* (Horinouchi et al. 2003a). The reaction products are the aliphatic 2-hydroxy-hexa-2,4-dienoate (HHD (XVII)) and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostano-5-oates (DOHNAAAs, such as XIX). DOHNAAAs are acidic perhydroindane derivatives.

A *tesD* mutant of *C. testosteroni* strain TA441 accumulates bile-salt-specific 4,9-DSHA derivatives, e.g. a 7,12-dihydroxy derivative of 4,9-DSHA (XVI) for cholate, indicating that bile salts are channelled into the same degradation pathway as testosterone (Horinouchi et al.

2010). Thus, the hydroxyl groups at C7 and C12 do not hinder the degradation of the steroid skeleton via this pathway, and the 7,12-dihydroxy derivatives of 3,4-DHSA (XV) is also likely to be an intermediate of cholate degradation.

The further degradation of the C6-compound HHD has been investigated in *C. testosteroni* strain TA441; HHD is hydrated by a protein encoded by *tesE* to give 4-hydroxy-2-oxohexanoate (XVIII), which is presumably converted into propionyl-CoA and pyruvate via unknown reactions involving the genes *tesGF* (Horinouchi et al. 2005).

The further degradation of acidic perhydroindane DOH-NAA is still unknown (Lack et al. 2010). A mutant of *C. testosteroni* strain TA441 disrupted in a gene encoding a putative CoA-transferase (ORF18) accumulates perhydroindane derivatives, such as 7,12-dihydroxy-DOHNAA (XIX), during degradation of bile salts indicating that DOHNAA degradation is initiated by activation with CoA (Horinouchi et al. 2006). Studies from the 1960s and 1970s describe some intermediates that originate from perhydroindane degradation, but the respective biochemical reactions are not known (Schubert et al. 1968; Hashimoto and Hayakawa 1977; Hayakawa and Fujiwara 1977).

Taken together, the shortest route for degradation of cholate (I) to HHD (XVII) and 7,12-dihydroxy-DOH-NAA (XIX) takes 15 enzymatic steps (Fig. 2). However, it must be emphasised that the course of reactions involved in oxidation of the steroid rings and of the acyl side chain may vary between different bacteria. For some *Actinobacteria*, it is reported that they perform the cleavage of rings A and B before oxidation of the acyl side chain (Hayakawa 1982).

Organisation of genes and gene regulation

Many of the genes necessary for the shortest route for degradation of a bile salt to the level of the perhydroindane derivative are known and orthologs can be found within the genomes of the following bacteria, for which bile salt degradation has been shown: *C. testosteroni* strains TA441 (Horinouchi et al. 2010) and KF-1 (Rösch et al. 2008), *R. jostii* strain RHA1 (Mohn et al. 2008) and *P. haloplanktis* strain TAC125 (Philipp et al. 2006). In addition, orthologs of these genes can be found in several genomes of steroid-degrading *M. tuberculosis* strains such as strain H37Rv (van der Geize et al. 2007). As a general tendency, genes involved in steroid degradation are clustered within these genomes. However, it must be emphasised that many genes within these clusters encode hypothetical proteins, for which a function in steroid degradation is likely but has not been proven yet. This accounts particularly for genes encoding enzymes for the degradation of steroid side chains.

Detailed studies on the regulation of genes involved in steroid degradation have been performed with *C. testosteroni* strain ATCC11996. TeiR has been identified as a positive regulator of steroid degradation (Pruneda-Paz et al. 2004; Linares et al. 2008; Göhler et al. 2008). TeiR is a membrane-associated protein with a steroid-binding domain as well as with sensing/signalling domains. It can act as a kinase but its downstream targets are not known. Furthermore, TeiR can bind to promoters for transcription of genes involved in steroid degradation. Interestingly, TeiR is localised at the cell poles and has also a function in chemotaxis for steroid compounds. In addition, testosterone metabolism in *C. testosteroni* strain ATCC11996 is also regulated by two negative regulators RepA and Rep B that act on transcription and translation, respectively, of *hsdA* (Xiong et al. 2003). In *C. testosteroni* strain TA441, the gene *tesR* has been identified to encode a positive regulator of steroid degradation, which is almost identical to TeiR (Horinouchi et al. 2004b). For strain TA441, it has been shown that bile salts can induce genes involved in testosterone metabolism, too (Horinouchi et al. 2003b). Cholesterol metabolism in *M. tuberculosis*, *Mycobacterium smegmatis* and *R. jostii* strain RHA1 is regulated by TetR-type repressors (Kendall et al. 2007, 2010; van der Geize et al. 2007). Enzyme activities involved in cholate degradation by strain Choll were not induced in succinate-grown cells indicating the induction of steroid degradation in this model organism as well (Birkenmaier et al. 2007).

So far it is not known whether steroid compounds induce the complete pathway for their degradation as a whole or whether genes required for different modules of steroid degradation are sequentially induced.

Anaerobic degradation of bile salts

Anaerobic transformation of bile salts exceeding the formation of secondary bile salts has been reported for *Pseudomonas* sp. (Barnes et al. 1975; Owen and Bilton 1983; Philipp et al. 2006), *Bacteroides* sp. (Owen et al. 1977) and for *Escherichia coli* (Tenneson et al. 1977). *Pseudomonas* sp. strain Choll can grow by degrading cholate to DHADD with nitrate as electron acceptor (Philipp et al. 2006). Further degradation of the steroid skeleton does not occur because the next step is catalysed by an oxygen-dependent 3-ketosteroid-9 α -hydroxylase. In principle, complete oxidation of steroids under anoxic conditions with nitrate as electron acceptor is possible. This has been shown for cholesterol (Hylemon and Harder 1998; Chiang et al. 2007), testosterone (Fahrbach et al. 2010) and 17 β -estradiol (Fahrbach et al. 2006). There, the oxygenase-dependent reactions are replaced

by other enzymes. The only anaerobic steroid hydroxylation reaction known so far has been detected in degradation of cholesterol by *Sterolibacterium denitrificans* (Chiang et al. 2007). There, the O₂-independent hydroxylation of C25 of the alkyl side chain of cholesterol has been shown with ¹⁸O-labelled water. In the absence of molecular oxygen, hydroxylation reactions are usually accomplished by molybdenum-containing enzymes (Hille et al. 1998). Such enzymes are presumably also present in *S. denitrificans*.

Physiological adaptations for growth with bile salts

Bile salts are toxic to bacterial cells. Due to their amphiphilic character, they perturbate the structure of biological membranes and can cause cell lysis (Helenius and Simons 1975). The toxicity of bile salts is utilised in microbiology laboratories to select for enteric bacteria on McConkey agar, which contains about 2 mM deoxycholate. In the duodenum, bacteria face bile salts at concentrations of 20 mM (Thanassi et al. 1997), and, as expected, bacteria living in this environment have protection mechanisms against bile salts (Gunn 2000; Begley et al. 2005). These include the outer membrane as a diffusion barrier (Hancock 1997; Nikaido 2003) and efflux pumps for the extrusion of bile salts, such as the proton-motive-force-dependent efflux pumps of the resistance-nodulation-division (RND) family (Bina and Mekalanos 2001; Bina et al. 2006). The maintenance and the operation of these protection mechanisms require energy, and the intestinal environment provides sufficient nutrients to cover this extra energy investment. The situation is fundamentally different for environmental bacteria growing with bile salts as their carbon and energy source. As for these bacteria, bile salts are a nutrient and a stress factor at the same time; they need to find a trade-off for energy investment. To conserve energy for growth, they have to take up bile salts, thereby risking to be injured. For protection and repair, they have to invest part of this energy, which is consequently not available for growth. The toxicity is not restricted to bile salts because also steroid compounds formed in the course of the 9,10-seco-pathway have toxic effects. For *Pseudomonas* sp. strain Choll, it has been shown that DHADD (XIII) and THSATD (XIV) were both toxic to the cells in the presence of EDTA (Philipp et al. 2006). The toxicity of ADDs has been described before but the physiological basis for the toxicity is unclear (Lee and Liu 1992; Perez et al. 2003).

The difficult energetic situation for steroid-degrading bacteria calls attention to further adaptive strategies. One

possibility is that the degradation pathway is organised in such a way that the cells minimise their exposure to the toxic steroid substrates. This could be achieved by the transient extracellular accumulation of degradation intermediates to keep the intracellular level of toxic metabolites low. This hypothesis is supported by the fact that many studies on bile salt (Smith and Park 1984; Philipp et al. 2006; Birkenmaier et al. 2007) and testosterone degradation (Horinouchi et al. 2001, 2003b) report that intermediates accumulate transiently outside the cells in culture supernatants.

It is not known whether the extracellular accumulation of steroid compounds is the result of diffusion or of active efflux, but several reports demonstrate that efflux pumps, such as those of the RND-type, are important for the removal of bile salts in bacteria (Plesiat and Nikaido 1992; Thanassi et al. 1997; Yokota et al. 2000; Bina et al. 2006). A bile salt exporter has also been postulated for intestinal bacteria performing 7 α -dehydroxylation, but no respective gene or protein has been identified so far (Ridlon et al. 2006). In addition, two major multidrug efflux systems of *E. coli* were shown to pump non-charged steroids, such as estradiol and progesterone (Elkins and Mullis 2006). The transient extracellular accumulation of steroid compounds would also imply uptake of degradation intermediates for further conversion. Bile salt transport has been described above, and active transport of testosterone has been demonstrated in a steroid-degrading *Pseudomonas* strain (Watanabe and Po 1974).

The energetic costs of such an active efflux strategy would be high because in addition to the energetic investment for the postulated transport processes, intermediates of β -oxidation after their re-uptake would repeatedly require ATP-dependent activation with CoA. However, energy investment for CoA-activation could be reduced if these compounds can be activated via CoA-transferases. The gene *baiF* in *C. scindens* encodes a candidate for such a bile salt CoA-transferase (Ridlon et al. 2006).

Biotechnological applications of bacterial bile salt transformation

The most important biotechnological application of bacterial bile salt metabolism is the production of pharmaceutically active steroid compounds (for reviews see Hayakawa 1982; Kieslich 1985; Mahato et al. 1994; Bortolini et al. 1997). For this process, bile salts are extracted from cattle bile and subjected to a combination of chemical and biological transformations. Deoxycholate from ox bile used to be the precursor for the production of cortisol because the 12-hydroxyl group could be easily transformed into the

essential 11-oxygen function of corticoids (Kieslich 1985). Nowadays, corticoids and other steroid hormones are mainly produced via biotransformations of low-cost plant sterols as main substrates. An important milestone in steroid biotransformation was the discovery of 11 α -hydroxylation of progesterone by the mould *Rhizopus* (Weaver et al. 1960). In the meantime, hydrocortisone production from ethanol has been accomplished in engineered cells of *Saccharomyces cerevisiae* (Szczebara et al. 2003; Dumas et al. 2006).

A direct application of bile salts as pure compounds is their use as pharmaceuticals for the dissolution of gallstones (Konikoff 2003). For this treatment, the bile salts chenodeoxycholate and ursodeoxycholate are orally administered. These bile salts and their precursors can be extracted from bile of cattle and sheep. The precursors are further processed to the desired bile salts by biotransformation (Mahato et al. 1994; Bortolini et al. 1997). Bile salts have also an increasing importance in supramolecular chemistry and nanotechnology (Nonappa and Maitra 2008). They are, for example, used as building blocks for crown ethers and for molecular tweezers. Bacterial transformation could be used for shaping bile salts for these purposes.

The detailed molecular and biochemical knowledge about reactions, enzymes and genes that is currently being generated will pave the way to optimise bacterial strains for steroid transformation by metabolic engineering. Studies on the degradation of the easily available and highly water-soluble bile salts are an appropriate model system to identify genes involved in bacterial steroid metabolism. Metabolic engineering of steroid-degrading bacteria could also be applied to bioremediation, e.g. with bioreactors containing engineered strains for degrading hormonally active steroid compounds in wastewater that are only poorly degraded in sewage treatment plants (Combalbert and Hernandez-Raquet 2010). Further future aspects of bacterial steroid metabolism could be in biological pest control through the transformation or degradation of steroid pheromones.

An important aspect in these applications is to increase the resistance of bacteria towards the toxic effects of bile salts and steroids in general. This is particularly interesting for a completely different application, namely the development of probiotic bacteria. A key property of probiotic bacteria is to survive the contact to bile salts in the duodenum; thus, engineering of bile salt resistance could be very useful for improving probiotic strains. This approach has been tested for two strains of lactic acid bacteria (Watson et al. 2008). One goal of probiotics is to introduce bile salt-transforming bacteria into the intestine for lowering the concentration of secondary bile salts that are presumptive colon cancer promoters (Ridlon et al. 2006). Increasing bacterial bile salt deconjugation in this way could also lower the re-absorption of bile salts in the

enterohepatic system, thereby increasing the rate of cholesterol degradation for the formation of cholate and chenodeoxycholate (Kaushik et al. 2009).

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