Minireview

Catabolism and biotechnological applications of cholesterol degrading bacteria

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Summary

Cholesterol is a steroid commonly found in nature with a great relevance in biology, medicine and chemistry, playing an essential role as a structural component of animal cell membranes. The ubiquity of cholesterol in the environment has made it a reference biomarker for environmental pollution analysis and a common carbon source for different microorganisms, some of them being important pathogens such as *Mycobacterium tuberculosis*. This work revises the accumulated biochemical and genetic knowledge on the bacterial pathways that degrade or transform this molecule, given that the characterization of cholesterol metabolism would contribute not only to understand its role in tuberculosis but also to develop new biotechnological processes that use this and other related molecules as starting or target materials.

Introduction

Cholesterol is a steroid, i.e. a class of terpenoid lipids, with a carbon skeleton virtually flat and relatively rigid formed by four fused alicyclic rings (Fig. 1). This polycyclic sterol has a great importance in biology, medicine and chemistry, being one of the most common molecules in nature, as an essential structural component of animal cell membranes (Staytor and Bloch, 1965), although it has been also exceptionally found in some prokaryotes (Hayami *et al.*, 1979).

The metabolism of cholesterol is critical in eukaryotes as precursor of vitamins, steroid hormones and bile acids (Miller, 1988; Björkhem and Eggertsen, 2001) (Fig. 1), but

according to its physiological relevance, an imbalance in its blood level causes serious diseases in humans.

Cholesterol is frequently found in the biosphere, not only because of its natural abundance, but also due to its high resistance to microbial degradation. Cholesterol is a recalcitrant molecule to biodegradation because of its low number of functional groups (one CC double bond and a single hydroxyl group), its low solubility in water $(3 \times 10^{-8}$ M) and its complex spatial conformation constituted by four alicyclic rings and two quaternary carbon atoms. The high hydrophobicity and low volatility of cholesterol leads to a high absorption to solid phases. Because of their high rate of persistence, cholesterol and some derived compounds, such as coprostanol, have been used as reference biomarkers for environmental pollution analysis (Veiga *et al.*, 2005).

Steroids, some of them derived from cholesterol, constitute a new class of pollutants discharged into the environment as a result of human activity (Gagné *et al.*, 2006). The potent metabolic activities of these compounds affect a large number of cellular processes and thus, their presence and accumulation in water wastes and in certain ecological niches can affect the endocrine system of animals and humans (Hotchkiss *et al.*, 2008). Moreover, cholesterol is the main component of lanolin, and this and other related sterols are natural contaminants fairly resistant to the anaerobic treatment that is carried out on the effluents from wool-processing industry (Poole and Cord-Ruwisch, 2004).

The ubiquity of cholesterol and related sterols in the environment has made them a common carbon source for many different microorganisms, some of them being important pathogens as *Mycobacterium tuberculosis*. On the other hand, the bacterial metabolism of steroids has attracted also considerable attention as low-cost natural steroids like cholesterol can be used as starting materials for many bioactive synthetic steroids (Fernandes *et al.*, 2003). Therefore, the characterization of the bacterial metabolism of cholesterol could be useful not only to understand its influence in pathological processes like tuberculosis, but also to develop new organisms by metabolic engineering with potential use as biotechnological tools. In a recent work we reviewed the accumulated
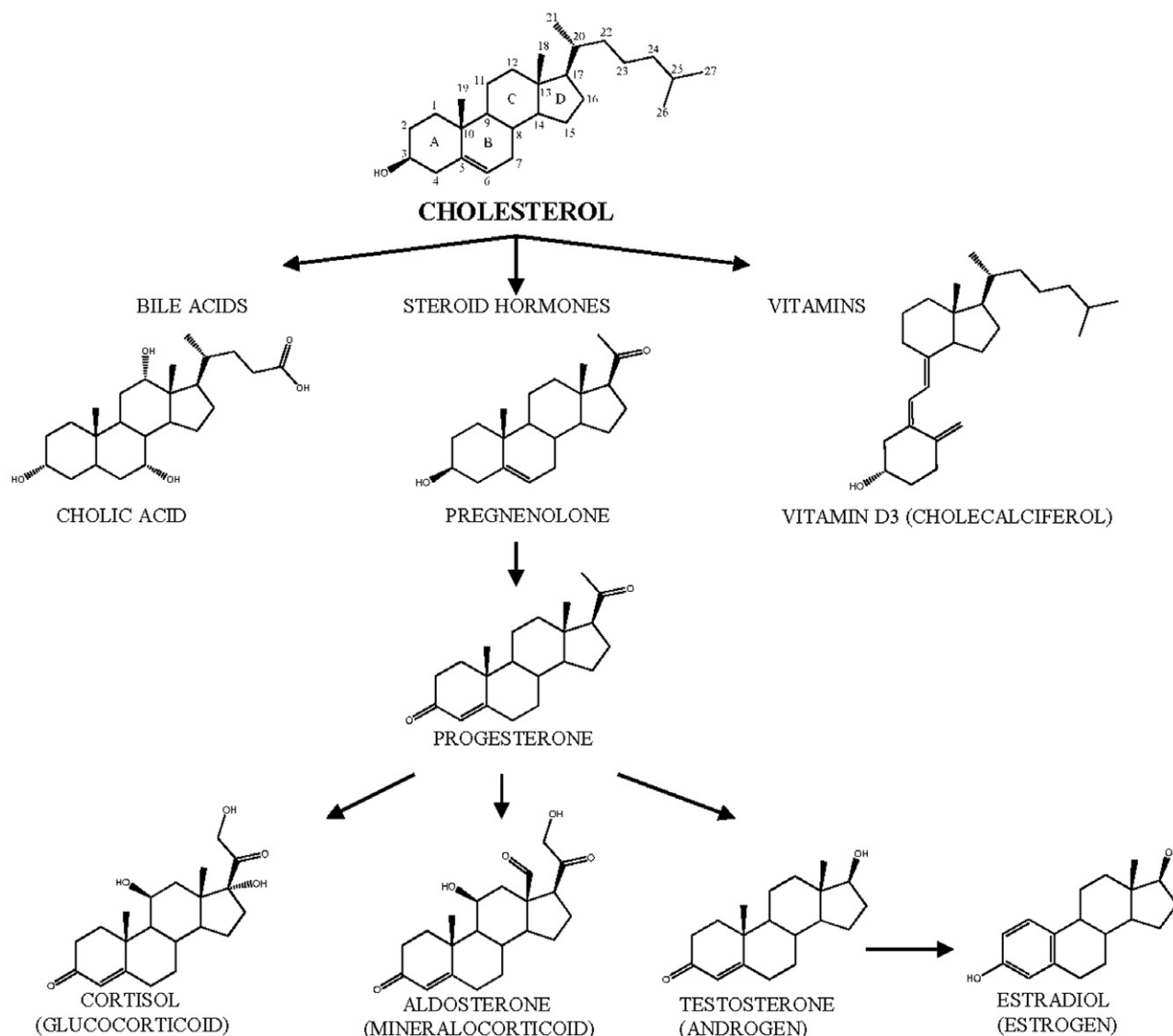
Fig. 1. Chemical structures of cholesterol and some derived natural molecules.

biochemical and genetic information on the bacterial pathways that degrade or transform biochemically different steroids (García *et al.*, 2011). This review is mainly focused in cholesterol catabolism and its biotechnological applications including the development of cholesterol biosensors for clinical diagnosis and food industry, microbial steroid biotransformations for the production of steroid drugs and hormones, probiotics with cholesterol-lowering effects, and enzymes for insecticide and fungicide purposes.

Catabolism of cholesterol

Historical issues

The search for microorganisms capable of degrading cholesterol was started over 70 years ago. In the early 20th

century, it was observed that several species of *Mycobacterium* could use cholesterol as the sole source of carbon and energy (Söhngen, 1913; Tak, 1942). Later on, it was reported that microorganisms belonging to the genus *Proactinomyces*, like *Rhodococcus erythropolis* (formerly *Nocardia erythropolis*), have the ability to degrade cholesterol and a potential capacity for the synthesis of new steroid derivatives (Turfitt, 1944; 1948) Moreover, some species of *Azotobacter* transform cholesterol in cholest-4-en-3-one (cholestenone) or 7-dehydrocholesterol, and have the ability to hydrolyse the side-chain of cholesterol generating methylheptanone (Horvath and Kramli, 1947). Thereafter, other bacteria belonging to the genera *Nocardia*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Streptomyces*, *Microbacterium*, *Serratia*, *Achromobacter*, *Pseudomonas* or *Protaminobacter*,

Table 1. Sequenced genomes of described organisms able to growth in cholesterol as a sole source of carbon and energy.

Organisms[a]	GenBank	Chromosome (nt)	Igr[b]	Hsa[b]	Mce[b]
Gordonia neofelifaecis	NZ_AEUD00000000	4 257 286	Yes*	Yes	Yes
Mycobacterium avium	NC_008595	5 475 491	Yes	Yes	Yes
Mycobacterium bovis	NC_008769	4 374 522	Yes	Yes	Yes
Mycobacterium smegmatis	NC_008596	6 988 209	Yes	Yes	Yes
Mycobacterium tuberculosis	NC_000962	4 411 532	Yes	Yes	Yes
Rhodococcus equi	NC_014659	5 043 170	Yes*	Yes	Yes
Rhodococcus erythropolis	NC_012490	6 516 310	Yes*	Yes	Yes
Rhodococcus jostii	NC_008268	7 804 765	Yes*	Yes	Yes
Streptomyces venezuelae	FR845719.1	8 226 158	Yes*	No	Yes
Streptomyces viridochromogenes	NZ_ACEZ00000000	8 548 109	Uncomplete*	No	Yes

a. Only one representative organism has been included in the table.
b. The presence of the *igr, hsa* and *mce* genes has been analysed.
The asterisk (*) means that the Cyp125 encoding gene is not present in the locus.

among others, were reported to accomplish partial or complete cholesterol degradation (Whitmarsh, 1964; Brown and Peterson, 1966; Arima *et al.*, 1969; Nagasawa *et al.*, 1969; Chipley *et al.*, 1975; Martin, 1977; Ferreira and Tracey, 1984; Drzyzga *et al.*, 2009; 2011; Fernández de Las Heras *et al.*, 2009; Ge *et al.*, 2011). Nevertheless, in spite of the large number of described bacteria able to use cholesterol as a sole source of carbon and energy only the genomes of few of them have been completely sequenced (Table 1). When we analysed in the genome of these microorganisms the presence of some of the most representative gene *loci* involved in cholesterol metabolism [e.g. *igr* (side-chain degradation), *hsa* (central pathway) and *mce* (transport)], we have observed that some of them do not contain these genes. This suggests that although these bacteria could transform cholesterol they may not be able to completely mineralize this compound. The relevance of these genes in cholesterol catabolism has been also confirmed recently by high-density mutagenesis and deep-sequencing analysis in *M. tuberculosis* (Griffin *et al.*, 2011). Finally, it is worth to mention that most of these genes are randomly located in different clusters in the bacterial chromosome with the exception of *M. tuberculosis* where it has been assumed that the catabolic predicted genes are mainly located in a single cluster (80 genes in region of about 50 kb) (Van der Geize *et al.*, 2007). However, the recent work of Griffin and colleagues (2011) have shown that while the genes found to be required for growth in cholesterol were clustered in this region, the majority (60%) were distributed elsewhere in the chromosome.

Aerobic degradation of cholesterol

Although the bacterial catabolism of cholesterol has not been fully elucidated in any of the bacterial strains degrading sterols, the metabolic pathway can be postulated by combining the biochemical and genetic studies carried out in different organisms (Martin, 1977; Schoe-

mer and Martin, 1980; Owen *et al.*, 1983; Kieslich, 1985; Sedlaczek and Smith, 1988; Szentirmai, 1990; Yam *et al.*, 2010; García *et al.*, 2011; Griffin *et al.*, 2011; Ouellet *et al.*, 2011) (Fig. 2). Experiments performed with ^{14}C-radiolabelled cholesterol showed that carbon from the sterol ring system was preferentially converted to CO_2 and used for energy generation via tricarboxylic acid cycle, whereas the side-chain is assimilated into the mycobacterial lipids (Cox *et al.*, 1999). To facilitate the comprehension of the catabolic process it can be divided into four major steps as presented below.

Transformation of cholesterol into cholestenone

It is generally assumed that the first reaction in the aerobic metabolism of cholesterol is its oxidation to cholestenone through two sequential reactions. That is, the oxidation of cholesterol to cholest-5-en-3-one [Fig. 2 (2)] followed by its isomerization to cholestenone [Fig. 2 (3)]. The enzymes responsible for this activity are cholesterol oxidases (ChOx), or 3β-hydroxysteroid dehydrogenase/isomerases (HSDs).

ChOx is a monomeric flavoenzyme that catalyses the oxidation and isomerization of cholesterol to cholestenone with a concomitant reduction of FAD. Regeneration of the oxidized cofactor is achieved by the reduction of O_2 to hydrogen peroxide. The enzyme is extracellular and occurs as secreted and/or cell-surface-associated forms, depending on the microorganism and growth conditions (Kreit and Sampson, 2009). The ChOx from *Streptomyces* sp. SA-COO (Yue *et al.*, 1999; Lario *et al.*, 2003) and the BOC1 form of ChOx from *Brevibacterium sterolicum* (Vrielink *et al.*, 1991; Li *et al.*, 1993) have been classified as type I oxidases and belong to the glucose-methanol-choline (GMC) oxidoreductase family of flavoenzymes where the FAD cofactor is non-covalently bound. However, the BOC2 form of ChOx from *B. sterolicum* (Coulombe *et al.*, 2001) has been classified as a type II oxidase belonging to the family of vanillyl-alcohol oxidase
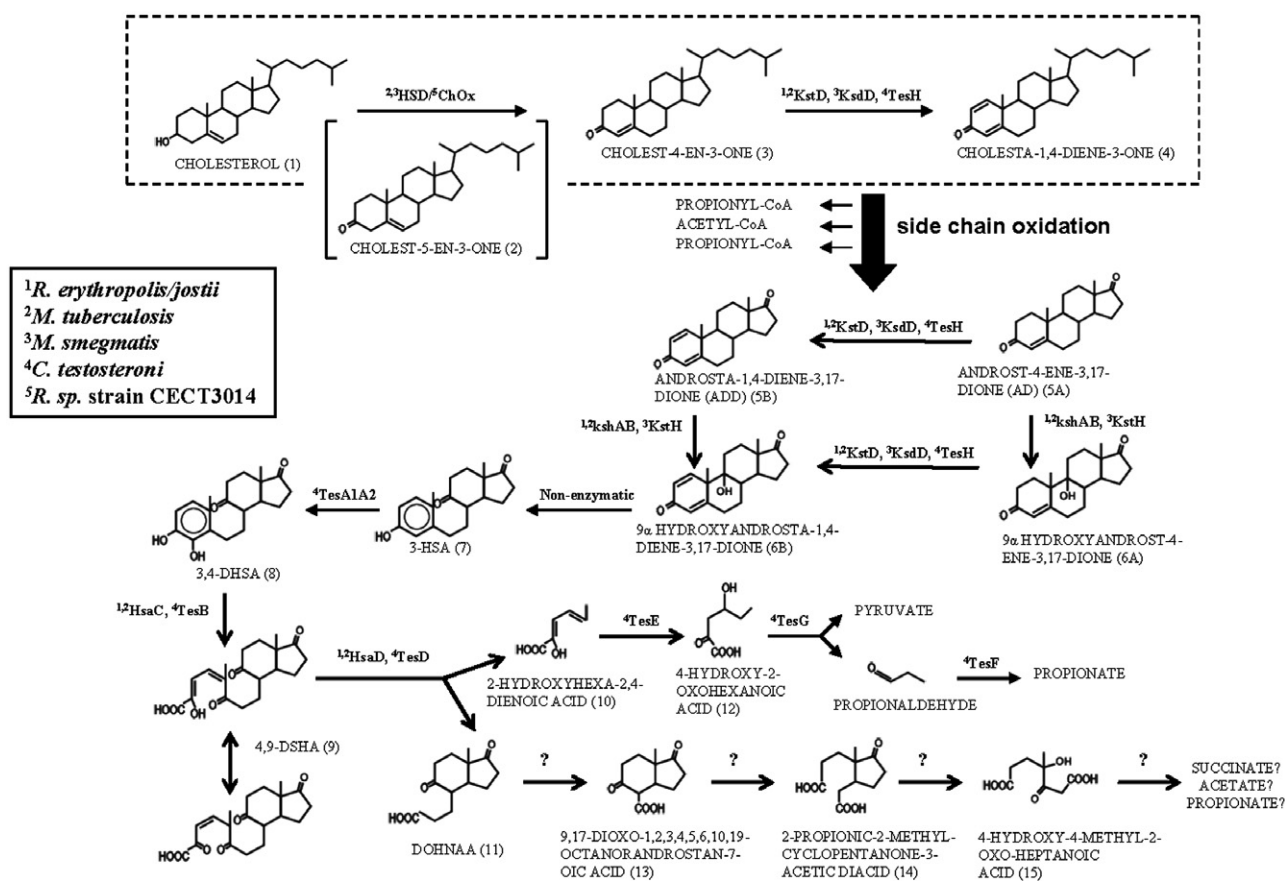
Fig. 2. Proposed pathway for cholesterol degradation under aerobic conditions. Cholest-4-en-3-one or any of the subsequent metabolites from degradation of the side-chain up to (and including) AD may undergo a dehydrogenation reaction to introduce a double bond in the position 1, leading to compound cholest-1,4-diene-3-one in the case of cholest-4-en-3-one, or to the corresponding 1,2-dehydro derivatives for other molecules. The side-chain degradation of this compounds will be identical to that of the cholest-4-en-3-one to the common intermediate 9α-hydroxyandrosta-1,4-diene-3,17-dione. The microorganisms from which enzymes implicated in different steps are indicated by numbers. Numbers in brackets are assigned arbitrarily to facilitate the compound identification in the text.

enzymes, which contain a covalently bound FAD molecule (Motteran *et al.*, 2001). Both classes of ChOxs share the same catalytic activity but show significant differences in their redox and kinetic properties. In general, they exhibit a broad range of steroid specificities and can oxidize a number of hydroxysterols, including sterols, steroid hormones and bile acids, but the presence of a 3β-hydroxyl group is an important requirement for activity (Pollegioni *et al.*, 1999). Remarkably, some ChOxs, like those from *Burkholderia cepacia* ST-200, *Pseudomonas* spp. and *Chromobacterium* sp. DS-1, oxidize cholesterol mainly to 6β-hydroperoxy-cholest-4-en-3-one and hydrogen peroxide instead of cholestenone (Doukyu and Aono, 1999; Doukyu *et al.*, 2008; Doukyu, 2009) but this intermediate has not been associated so far to any cholesterol degradation pathway. Recently, a cholesterol inducible ChOx encoding gene (*choG*) has been identified in *Rhodococcus* sp. strain CECT3014 within a gene cluster involved in the metabolism of cholesterol (Fernández de Las Heras *et al.*, 2011).

The 3β-hydroxy-Δ5-steroid HSD belongs to a family of enzymes that catalyse the conversion of 3β-hydroxy-5-ene-steroids to 3-oxo-4-ene-steroids through consecutive oxidation and isomerization reactions, using NAD or NADP as cofactors. Gene database analysis revealed the existence of a protein superfamily that in addition to the bacterial HSDs includes mammalian 3β-hydroxysteroid dehydrogenases, plant dihydroflavonol reductases, bacterial UDP-galactose-4-epimerases and viral 3β-hydroxysteroid dehydrogenase (Baker and Blasco, 1992). All of them present a glycine-rich sequence (Gly-X-X-Gly-X-X-Gly) similar to the Rossman fold sequence and an atypical active site motif (Tyr-X-X-X-Lys). The NAD(P)-dependent HSD from *Nocardia* sp. has been well characterized and appears to be responsible for the transformation of cholesterol to cholestenone in this cholesterol degrader organism (Horinouchi *et al.*, 1991). Recent genetic and biochemical studies have also demonstrated the direct involvement of a NAD-dependent HSD in mycobacterial cholesterol catabolism (Yang *et al.*,

2007; Griffin *et al.*, 2011; Thomas *et al.*, 2011; Uhía *et al.*, 2011a). In fact, it has been demonstrated that the ChOx analogue (ChoD) found in Mycobacteria does not seem to play a role in cholesterol degradation (Griffin *et al.*, 2011; Uhía *et al.*, 2011a).

Cholesterol side-chain degradation

It has been assumed that simultaneously and independently of the first oxidative process, the long aliphatic chain of cholesterol is removed via a process similar to the β-oxidation of fatty acids, leading to the formation of a steroid C22-oic acid intermediate with the concomitant release of two molecules of propionyl-CoA and one acetyl-CoA (Fig. 2). This process has been elucidated at the biochemical level in strains of *Mycobacterium* and *Nocardia* and proceeds differently for cholesterol and β-sitosterol (Sih, 1968; Martin, 1976; Wilbrink *et al.*, 2011).

Recent studies have pointed out that the Cyp125 cytochrome from *Rhodococcus jostii* RHA1 (*ro04679* coding gene) and *M. tuberculosis* (*Rv3545c* coding gene) initiates the side-chain degradation of cholesterol (Capyk *et al.*, 2009a; Rosłoniec *et al.*, 2009). This cytochrome belongs to the P450 family and catalyses the hydroxylation at carbon C-26 yielding 26-hydroxycholest-4-en-3-one. Cyp125 from *M. tuberculosis* CDC1551 was proved to be involved not only in the hydroxylation of cholesterol but also in its further oxidation to cholest-4-en-3-one-27 oic acid (Ouellet *et al.*, 2010). The crystallographic structure of Cyp125 from *M. tuberculosis* has been recently determined (McLean *et al.*, 2009).

Driscoll and colleagues (2010) and Johnston and colleagues (2010) have demonstrated that Cyp142 is a cytochrome that is able to replace the activity of Cyp125 in *M. tuberculosis* H37Rv, explaining why a Cyp125 deletion mutant of this strain is still able to grow using cholesterol as a carbon source. Furthermore, Δ*cyp125* mutant strains of *Mycobacterium bovis* and *M. bovis* BCG (Driscoll *et al.*, 2010) and *M. tuberculosis* CDC1551 (Johnston *et al.*, 2010) could not grow on cholesterol because they have a defective *cyp142* gene. In addition, its crystal structure was very similar to that of Cyp125 (Driscoll *et al.*, 2010). Although Griffin and colleagues (2011) have identified Cyp125 as the sole monooxygenase significantly required for growth on cholesterol the involvement of Cyp142 cannot be completely discarded.

After the formation of the carboxylic acid intermediate, the cholesterol side-chain is shortened by a β-oxidation-like process initiated by an ATP-dependent sterol/steroid CoA ligase catalysing the CoA activation of the C27 carboxylic acid intermediate (Sih, 1968; Sih *et al.*, 1968). Subsequent cleavage of the side-chain to 17-ketosteroid takes place stepwise in three consecutive cycles and two molecules of propionyl-CoA and one molecule of acetyl-

CoA are formed from the side-chain (Fig. 3). This process involves two initial steps of typical β-oxidation with the participation of one or two different sets of four enzymes, i.e. acyl-CoA dehydrogenases, enoyl-CoA-hydratases, hydroxyacyl-CoA dehydrogenases and thiolases. These steps will release one propionyl-CoA and one acetyl-CoA. The additional propionyl-CoA cannot be released by a conventional β-oxidation due to the presence of the cyclopentane ring D. Although the two following steps would proceed by the action of an acyl-CoA dehydrogenase and an enoyl-CoA-hydratase, as occurs in a typical β-oxidation, they will render a tertiary alcohol, which is not susceptible to dehydrogenation to form the keto group. Therefore, the last step will require the participation of a hydroxyacyl-CoA lyase similar to other CoA-lyases (e.g. malyl-CoA lyase, citramalyl-CoA lyase or 3-hydroxy-3-methylglutaryl-CoA lyase) that will render androst-4-ene-3,17-dione (AD) and propionyl-CoA. A similar mechanism has been proposed for the metabolism of cyclohexaneacetate in *Arthrobacter* sp. CA1 (Ougham and Trudgill, 1982). A number of genes related to β-oxidation have been recently identified in *M. tuberculosis* by transposon mutagenesis and suggested to be involved in cholesterol side-chain degradation (Griffin *et al.*, 2011). Some of these genes were located inside the large cholesterol degradation gene cluster (*hsd4A, ltp2, fadE29, fadE28, fadA5, fadE30, fadE32, fadE33, fadE34 and hsd4B*), but others were found outside of the cluster (*fadE5, echA9*, *fadD36* and *fadE25*).

Our knowledge of genes involved in microbial degradation of cholesterol side-chain is still very limited, but a catabolic gene cluster was identified in *R. jostii* RHA1 and *Mycobacterium smegmatis*, encoding several enzymes predicted to be involved in the β-oxidation process (van der Geize *et al.*, 2007; Uhía *et al.*, 2012) (Fig. 4). Recently, the *fadD19* gene product of this cluster has been identified as a steroid-CoA ligase with an essential role in the degradation of C-24 branched-chain-sterols (Wilbrink *et al.*, 2011). In addition, the *fadA5* (*Rv3546*) gene from *M. tuberculosis* H37Rv has been proposed to encode a β-ketoacyl-CoA thiolase that functions in cholesterol side-chain β-oxidation (Nesbitt *et al.*, 2010). This gene is upregulated by cholesterol and repressed by KstR (see below), and is required for utilization of cholesterol as a sole carbon source *in vitro* and for full virulence of *M. tuberculosis* in the chronic stage of mouse lung infection (Nesbitt *et al.*, 2010).

It is worth to mention that cholesterol catabolism would follow an obligatory order for side-chain and ring structure modifications depending of the microorganism. In this sense, while the Δ*cyp125* mutants of BCG (*Bacillus* Calmette-Guerin, an inactivated form of *M. bovis*) and *Rhodococcus rhodochrous* RG32 transformed cholesterol to cholestenone, the corresponding mutant of *R. jostii*
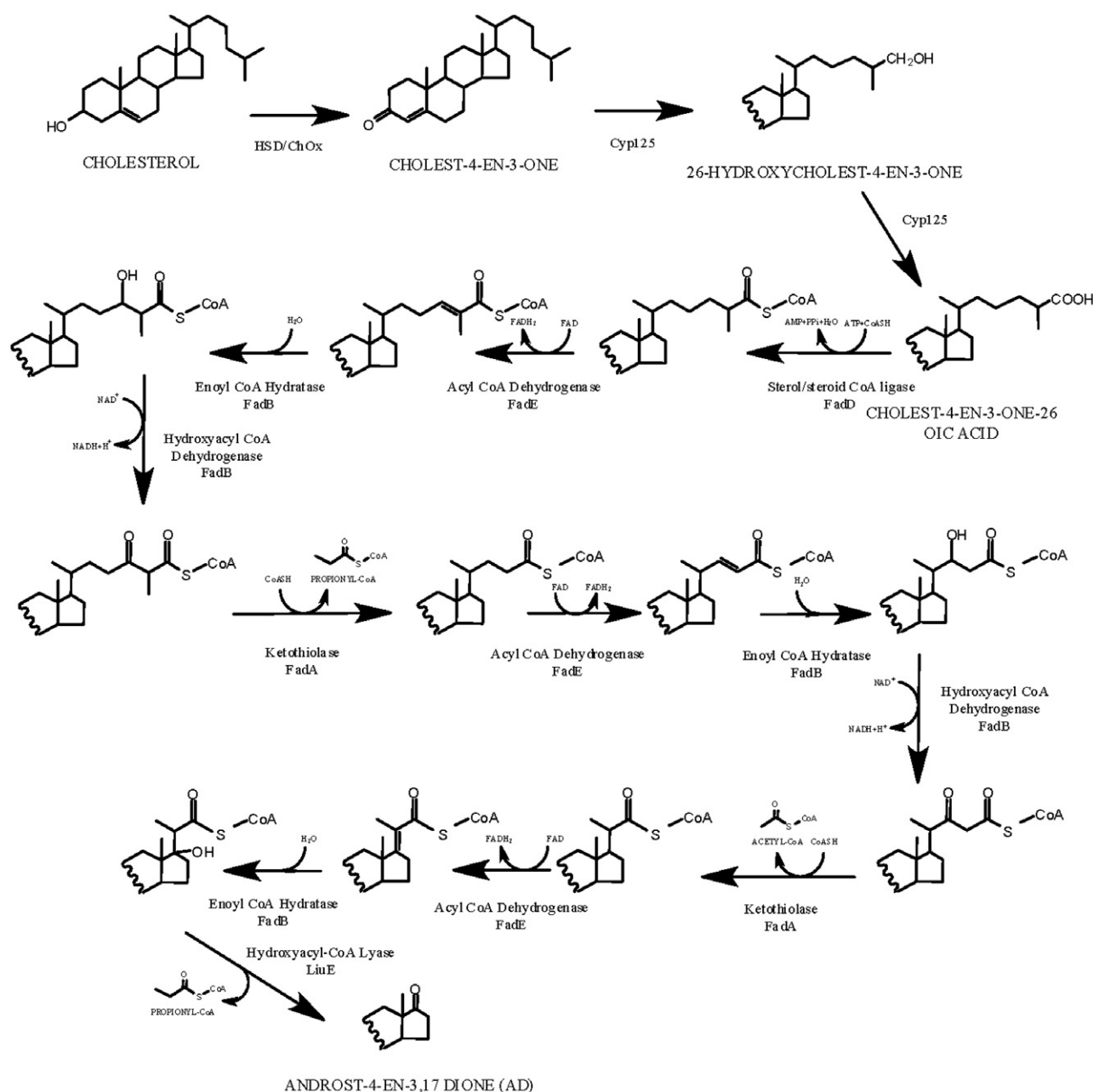
Fig. 3. Proposed β-oxidation-like reactions for cholesterol side-chain degradation. The Fad proteins have been assigned according to the nomenclature of the *E. coli* genes involved in the β-oxidation of fatty acids. LiuE is the name assigned to 3-hydroxy-3-methylglutaryl-coenzyme A lyases.

RHA1 did not (Capyk *et al.*, 2009a). Furthermore, ring degradation cannot proceed with substrates having a full-length aliphatic side-chain in the BCG mutant as it is not able to grow or further transform cholestenone, while *R. jostii* RHA1 mutant grows in this compound (Capyk *et al.*, 2009a).

The combination of these two first enzymatic steps, i.e. the oxidation of cholesterol together with the degradation of its lateral chain, renders the central intermediate AD

[Fig. 2 (5A)] that is further metabolized by the central catabolic pathway.

Central catabolic pathway

Once the aliphatic chain has been oxidized, cholesterol catabolism appears to follow the common pathway described for C19 steroids. Briefly, 3-ketosteroid-Δ^1-dehydrogenases, as KsdD in *M. smegmatis* (Brzostek
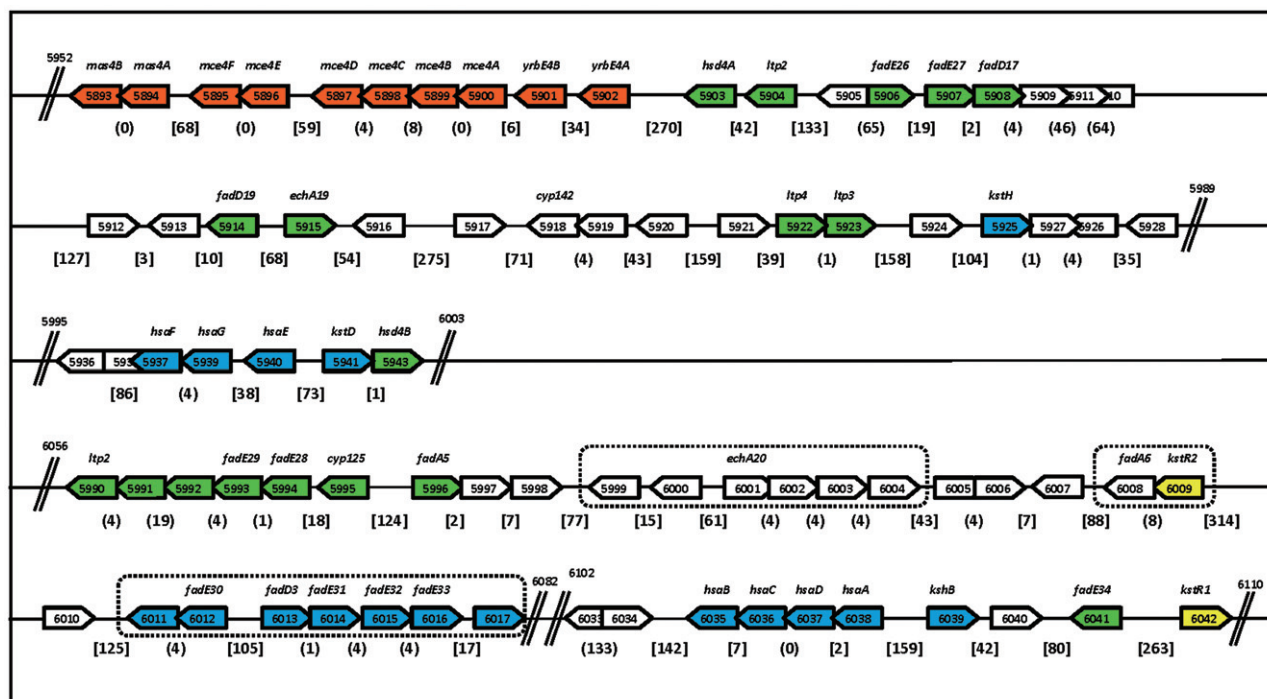
Fig. 4. Organization of the main gene clusters implied or suggested to be involved in the degradation of cholesterol in *M. smegmatis* mc²155. The identity number for each MSMEG gene is indicated within the arrows. The name of some genes of interest is written above them. Numbers below genes indicate the number of bp between adjacent genes; numbers in brackets indicate separation and numbers in parentheses indicate overlap. Numbers above diagonal lines indicate the genomic position in kb. Orange: *mce* cluster. Green: genes suggested and/or proved to participate in the side-chain degradation. Blue: genes suggested and/or proved to participate in the central or lower catabolic pathway. Yellow: genes coding the transcriptional repressors KstR and KstR2. Genes surrounded by a dashed line are controlled by KstR2, the rest of the genes showed in this figure are controlled by KstR (except for MSMEG_5905, 5909, 5910, 5912, 5916, 5917, 5924, 5926, 5928, 5936, 5938, 6005, 6006, 6007, 6010, 6034, which could not be proved to be controlled by any of both repressors) (Kendall *et al.*, 2007; 2010; Uhía *et al.*, 2012).

et al., 2005), KstD in *R. erythropolis* (van der Geize *et al.*, 2000; 2001; 2002a) and *M. tuberculosis* (Knol *et al.*, 2008) that are equivalent to TesH of *Comamonas testosteroni* (Horinouchi *et al.*, 2003a) transform AD into androsta-1,4-diene-3,17-dione (ADD) [Fig. 2 (5B) and Fig. 4]. Later on, a 9α-hydroxylation takes place catalysed by KshAB in *R. erythropolis* (van der Geize *et al.*, 2001; 2002b; 2008a), KshAB (Rv3526/Rv3571) in *M. tuberculosis* H37Rv (Capyk *et al.*, 2009b), and KstH (KshA-like) in *M. smegmatis* (Fig. 4) (Andor *et al.*, 2006), followed by a non-enzymatic transformation of the 9α-hydroxyandrosta-1,4-diene-3,17-dione [Fig. 2 (6B)] to 3-hydroxy-9,10-secoandrosta-1,3,5 (10)-trien-9,17-dione (3-HSA) [Fig. 2 (7)]. The crystal structure of KshA of *M. tuberculosis* H37Rv has been recently elucidated revealing that it is a trimer (Capyk *et al.*, 2009b). Five *kshA* homologues have been identified in *R. rhodochrous* DSM43269 showing different substrate specificity (Petrusma *et al.*, 2011).

Subsequently 3-HSA is hydroxylated by enzymes similar to the two-component oxygenase TesA1A2 from *C. testosteroni* (Horinouchi *et al.*, 2004) leading to the production of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (3,4-DHSA) [Fig. 2 (8)]. *Mycobacterium*

tuberculosis and *R. jostii* RHA1 have a similar enzyme named HsaAB (Dresen *et al.*, 2010). The crystal structure of HsaA from *M. tuberculosis* revealed that the enzyme is very similar to *p*-hydroxyphenylacetate hydroxylase (Dresen *et al.*, 2010). The last catechol derivative is cleaved by a *meta* extradiol dioxygenase similar to TesB in *C. testosteroni* (Horinouchi *et al.*, 2001), which was named HsaC in *R. jostii* RHA1 (van der Geize *et al.*, 2007) or *M. tuberculosis* (Yam *et al.*, 2009) yielding 4,5,9,10-*diseco*-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) [Fig. 2 (9)]. This compound is then hydrolysed by enzymes similar to TesD from *C. testosteroni* (Horinouchi *et al.*, 2003b) named HsaD in *M. tuberculosis* (Lack *et al.*, 2008; 2010) or *R. jostii* RHA1 (van der Geize *et al.*, 2007) yielding 2-hydroxyhexa-2,4-dienoic [Fig. 2 (10)] and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic (DOHNAA) acids [Fig. 2 (11)].

Lower catabolic pathway

The lower metabolic steps of the cholesterol catabolic pathway should degrade the 2-hydroxyhexa-2,4-dienoic and DOHNAA acids leading to metabolites able to enter

the central metabolic pathways (Kieslich, 1985), but the enzymes involved in this downward pathway have not been yet investigated in detail.

Most likely the catabolism of the 2-hydroxyhexa-2,4-dienoic acid involves genes similar to the *tesE*, *tesF* and *tesG* testosterone catabolic genes from *C. testosteroni* (Horinouchi *et al.*, 2005). In this sense, the 2-hydroxyhexa-2,4-dienoic acid can be metabolized to 4-hydroxy-2-oxo-hexanoic acid by the TesE-like hydratase [Fig. 2 (12)] that eventually will be transformed by the action of TesG-like aldolase into pyruvic acid and propionaldehyde that is further transformed into propionic acid by the action of TesF-like aldehyde dehydrogenase (Horinouchi *et al.*, 2005). Although it has been suggested that TesF could render propionyl-CoA, the similarity of TesF with CoA acylating aldehyde dehydrogenases is very low and therefore, the most probably end-product should be propionic acid.

DOHNAA would be further transformed to succinic acid and it is assumed that the first step in the degradation of this compound could be the elimination of a propionyl moiety, to produce 9,17-dioxo-1,2,3,4,5,6,10,19-octanorandrostan-7-oic acid [Fig. 2 (13)] through a typical β-oxidation (Kieslich, 1985). This reaction is carried out in two steps, i.e. the activation of DOHNAA by ATP and coenzyme A, followed by a reduction of DOHNAA-CoA by an NADPH-dependent dehydrogenase (Miclo and Germain, 1990). The enzymes specifically involved in these catabolic steps have not been precisely identified nor characterized so far.

Anoxic metabolism of cholesterol

Taylor and colleagues (1981) showed for the first time that bacteria were capable of degrading cholesterol under denitrifying conditions. The most frequent transformation of cholesterol in anoxic habitats is a reduction of its double bond yielding coprostanol (Li *et al.*, 1995; Harder and Probian, 1997). Many intestinal fermenting bacteria are able to catalyse this reaction (Groh *et al.*, 1993; Freier *et al.*, 1994), and the reductase responsible for this reaction has been characterized in *Eubacterium coprostanoligenes* (Li *et al.*, 1995).

A denitrifying β-proteobacterium, strain 72Chol, was the first bacterium isolated and cultured on cholesterol under strictly anoxic conditions able to mineralize cholesterol to carbon dioxide (Harder and Probian, 1997; Hylemon and Harder, 1998). However, the anoxic metabolism of cholesterol has been mainly studied at the biochemical level in the denitrifying β-proteobacterium *Sterolibacterium denitrificans*, now used as a model organism (Tarlera and Denner, 2003). Interestingly, this bacterium was also able to catabolize anaerobically testosterone (Chiang *et al.*, 2010).

The first steps of the anoxic catabolism of cholesterol are similar to those described for the aerobic process and thus, first the hydroxyl group at C-3 is oxidized into the keto group leading to cholest-5-en-3-one followed by a subsequent isomerization to yield cholestenone (Chiang *et al.*, 2007). These two reactions are catalysed by the bifunctional enzyme AcmA that belongs to the short-chain dehydrogenase reductase (SDR) superfamily (Chiang *et al.*, 2008a). The oxidation of cholestenone to cholesta-1,4-diene-3-one, is catalysed by AcmB, a cholest-4-en-3-one-Δ¹-dehydrogenase (Chiang *et al.*, 2008a,b). The enzyme is very similar to the flavin adenine dinucleotide-dependent 3-ketosteroid-Δ¹-dehydrogenase from *Nocardia corallina* (Itagaki *et al.*, 1990).

In contrast with the aerobic pathway where cholestenone is hydroxylated by a monooxygenase at C-27 to produce the corresponding primary alcohol, the anaerobic hydroxylation of the cholesterol side-chain is oxygen-independent (Chiang *et al.*, 2007). This step is catalysed by an enzyme belonging to the molybdenum-containing hydroxylase family and involves the hydroxylation of C-25 of cholestenone or cholesta-1,4-diene-3-one using water as a source of the oxygen atoms (Hille, 2005).

The cholesterol uptake system

Cholesterol as other steroids does not appear to be able to diffuse to the bacterial cytoplasm and needs to be transported by specific uptake systems before being metabolized. A pioneering work in steroid uptake systems was carried by Watanabe and Po (1974) demonstrating the presence of an inducible transport system for testosterone in *Pseudomonas testosteroni*.

Mce-family proteins are closely involved in the invasion and prolonged existence of *M. tuberculosis* in host macrophagues. The first *mce* gene to be discovered was found to promote the uptake of bacteria into nonphagocytic cells and was thus designated to function in mycobacterial cell entry ('*mce*') (Arruda *et al.*, 1993). The genome of *M. tuberculosis* contains four *mce* operons (*mce1-4*) each including 9–13 genes with a similar arrangement encoding two transmembrane proteins with homology to the permease subunits of ABC transporters, along with several putative secreted or cell-surface proteins (Pandey and Sassetti, 2008). Recently, it was reported that the mycobacterial gene cluster *mce4* encodes a cholesterol import system that enables *M. tuberculosis* to derive both carbon and energy from this component of host membranes (Pandey and Sassetti, 2008). The energy for the substrate translocation by all four Mce systems in *M. tuberculosis* is generated by a common ATPase, named MceG, belonging to the Mlk family (Joshi *et al.*, 2006). Interestingly, the *mceG* gene is located far away from the four *mce* operons in *M. tuberculosis* (Mohn *et al.*, 2008). Genome sequence analyses revealed that diverse actinobacteria, including members

of the *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Streptomyces* genera, harbour *mce* transporter operons involved in steroid uptake (Casali and Riley, 2007; García *et al.*, 2011). The reason why these Mce systems require many more proteins than classical ABC transporters remains unclear. It has been suggested that these proteins might form a large complex necessary for the movement of the substrate across cell walls (Mohn *et al.*, 2008).

The *mce4* locus also encodes a system involved in steroid uptake in *R. jostii* RHA1 (Mohn *et al.*, 2008) and in *Rhodococcus equi* (van der Geize *et al.*, 2008b). The *mce4* locus of *R. jostii* RHA1 is upregulated by cholesterol, and mutants with deletions in this operon lost their ability to grow in this compound (van der Geize *et al.*, 2007). A deletion of *supAB* genes, which form part of the *mce4* operon in *R. equi*, confirmed that these genes are essential for growth of this organism on cholesterol (van der Geize *et al.*, 2008b). Therefore, up to now, the *mce4* locus has been associated with steroid or cholesterol metabolism in all annotated bacterial genomes.

On the other hand, the Mce4 uptake system is also essential for growth of *R. jostii* RHA1 in cholesterol related compounds such as 5α-cholestanol, 5α-cholestanone and β-sitosterol (Mohn *et al.*, 2008). Nevertheless, mutants of this uptake system were unaffected for growth on steroid compounds having shorter polar side-chains (Mohn *et al.*, 2008). These data indicate that only side-chains of at least eight carbons are recognized by Mce4 steroid uptake system.

Regulation of the cholesterol catabolism

From the very beginning it was recognized that expressions of several genes/enzymes involved in steroid degradation were induced by their respective steroid substrates (Marcus and Talalay, 1956; Möbus *et al.*, 1997; Skowasch *et al.*, 2002), but very few data are still available upon the regulation of genes involved in cholesterol degradation. Kendall and colleagues (2007) have recently identified a repressor, named KstR, belonging to the TetR family of transcriptional regulators, which is involved in the regulation of 83 genes in *M. smegmatis* related with cholesterol and lipid metabolism (Fig. 4). The operator motif of the KstR dimer was predicted *in silico* as a 14 bp conserved sequence (TnnAACnnGTTnnA, where n is any nucleotide) located in the upstream region of the KstR-regulated genes (Kendall *et al.*, 2007; Uhía *et al.*, 2011b). The KstR-dependent promoter of the *MSMEG_5228* gene of *M. smegmatis*, which encodes an HSD responsible for the first step in the cholesterol degradative pathway, has been characterized in more detail (Uhía *et al.*, 2011a). DNaseI footprint assays have experimentally demonstrated that KstR specifically binds to an operator region

of 31 nucleotides containing the predicted palindromic sequence AACTGgAACgtGTTtCAGTT overlapping the −10 and −35 boxes of the P_{5228} promoter, suggesting that KstR represses the *MSMEG_5228* transcription by preventing the binding of RNA polymerase. The repression mediated by KstR is alleviated by the binding of an inducer molecule that still remains unknown.

Using the microarray data from *M. smegmatis*, a KstR regulon was also deduced for the human pathogen *M. tuberculosis* due the high degree of sequence similarity. In this bacterium, KstR is involved in the transcriptional repression of 74 genes (Kendall *et al.*, 2007). The KstR regulon in *M. tuberculosis* contains a large number of genes that have been found to be essential for the survival of this pathogen in both macrophages and mice (Schnappinger *et al.*, 2003). The 3D-structure of KstR from *M. tuberculosis* has been already determined (PDB ID: 3MNL).

More recently, another transcriptional repressor has been described as KstR2, which controls the expression of other additional 15 genes related to the lower cholesterol catabolic pathway in *M. tuberculosis* and *M. smegmatis*, suggesting that this complete pathway is controlled by at least two regulatory proteins (Fig. 4) (Kendall *et al.*, 2010).

The transcriptome of *M. smegmatis* growing in cholesterol has been compared with that of cells growing in glycerol as the sole carbon and energy sources (Uhía *et al.*, 2012). The results supported the role of KstR and KstR2 as autoregulated repressors of cholesterol catabolism. Microarray analyses revealed that 89 genes were upregulated at least threefold during growth on cholesterol. These upregulated genes are scattered throughout the *M. smegmatis* genome and likely reflect a general physiological adaptation of the bacterium to grow on this highly hydrophobic polycyclic compound. The catabolic genes are mainly organized in three large *clusters* that are putatively controlled by KstR and KstR2. Most of the cholesterol upregulated genes within these clusters appear to be responsible for the uptake of cholesterol, the β-oxidation of the branched side-chain at C17, and the catabolism of the rings to central metabolites. However, the transcriptome analysis also revealed the presence of other cholesterol upregulated genes that were not clustered. Within these genes, some are putatively controlled by the KstR repressor whereas others do not contain binding motifs for KstR or KstR2 in their promoter regions. This finding suggests the putative existence of additional regulatory systems different to KstR and KstR2.

The KstR binding motif identified in mycobacteria is also present in the genome of *R.* RHA1 (van der Geize *et al.*, 2007; Kendall *et al.*, 2007). Moreover, the *kstR* and *kstR2* orthologues in *R. jostii* RHA1, i.e. *ro04482* and *ro04598*, respectively, are induced by cholesterol (van der Geize

et al., 2007). In *R. jostii* RHA1 several cholesterol-induced genes do not seem to be regulated by KstR or KstR2, which could also indicate the presence of additional regulators of the catabolic pathway (Uhía *et al.*, 2012).

Pathogenic implications of cholesterol metabolism in mycobacteria

In contrast to previous thoughts (Av-Gay and Sobouti, 2000), it has been recently demonstrated that *M. tuberculosis* can grow using cholesterol as a sole carbon and energy source (Pandey and Sassetti, 2008). In this sense, cholesterol has recently been identified as an important lipid for mycobacterial infection (de Chastellier and Thilo, 2006; Ouellet *et al.*, 2011). The relatively abundant cholesterol present in host cells is an important growth substrate for these bacteria in different infection stages (e.g. intracellular growth or intracellular persistence).

Mycobacterium tuberculosis growing in the human cells appears to obtain the energy from host lipids rather than carbohydrates, supported by the finding that numerous genes involved in lipid metabolism are upregulated during growth in the mammalian macrophage and the mouse lung (Dubnau *et al.*, 2005). Some genes that are critical for survival of *M. tuberculosis* in macrophages are involved in cholesterol degradation (Rengarajan *et al.*, 2005; van der Geize *et al.*, 2007). The Mce4 transport system that, at stated above, is required for cholesterol import into bacterial cells, plays an important role in pathogenesis because a mutant lacking this transporter fails to persist in the lungs of chronically infected mice and cannot grow in (IFN-γ)-activated macrophages (Pandey and Sassetti, 2008). Furthermore, it has been proposed that the ability of Mce4 proteins to bind cholesterol could act as a signal allowing the pathogen to interact with the host (Mohn *et al.*, 2008).

The *igr* locus, which was identified because it is necessary for intracellular growth of *M. tuberculosis* in macrophages and mice, is absolutely required for cholesterol metabolism, because *igr*-deficient bacteria cannot grow using cholesterol as a primary carbon source (Chang *et al.*, 2007; Miner *et al.*, 2009). This locus consists of a single operon containing genes for a putative cytochrome P450 (*igrA*), two acyl coenzyme A dehydrogenases (*igrBC*), a conserved hypothetical protein (*igrD*), a putative enoyl coenzyme A hydratase (*igrE*) and a lipid carrier protein (*igrF*) (Chang *et al.*, 2007). A homologous operon is present in *R. jostii* RHA1 and its expression is shown to be induced during growth in cholesterol (van der Geize *et al.*, 2007). The *igr* cluster is encompassing the *mce4* genes, which encode the uptake system for cholesterol and related steroids (Mohn *et al.*, 2008). Interestingly, the *igrA* gene is homologous to the *Rv3545* and *ro04679* genes from *M. tuberculosis* and *R. jostii* RHA1, respec-

tively, and encode the Cyp125 cytochrome involved in cholesterol C27 (or C26) hydroxylation described above (Capyk *et al.*, 2009a; Rosłoniec *et al.*, 2009).

Mycobacterium tuberculosis lacking the *kshA* and *kshB* genes, which encode the two-component system catalysing the NADH dependent 9α-hydroxylation of 3-ketosteroids (Petrusma *et al.*, 2009), failed to use cholesterol and 4-androstene-3,17-dione as carbon sources and its growth and survival is attenuated in macrophages and mice (Hu *et al.*, 2009).

On the other hand, ChOxs have been considered as potential pathogenic determinants. In this sense, the ChOX (ChoE) from *R. equi*, a primary pathogen of horses and an opportunistic pathogen of humans, has been described as the major membrane-damaging factor (Navas *et al.*, 2001), although its role in pathogenesis is controversial (Pei *et al.*, 2006). The ChOx (ChoD) from *M. tuberculosis* also appears to be important for the growth of the pathogen in peritoneal macrophages and lungs of mice (Brzostek *et al.*, 2007). *Mycobacterium leprae*, an obligate intracellular pathogen that shows a dramatic reduction of functional genes when compared with *M. tuberculosis*, still conserves and expresses the *choD* orthologue (Marques *et al.*, 2008) and although this pathogen cannot catabolize cholesterol, it is a necessary factor for growth in macrophages (Kato, 1978). Nevertheless, the relevance of ChoD in cholesterol metabolism has been recently questioned (Griffin *et al.*, 2011; Uhía *et al.*, 2011a).

Interestingly, a mutation in the HSD (Rv1106c) of *M. tuberculosis* that is required for growing in cholesterol does not reduce its growth in infected macrophages or guinea pigs suggesting that cholesterol is not a sole nutrition source *in vivo* (Yang *et al.*, 2011), consistently with the idea that there are multiple lipids in the host that *M. tuberculosis* would be able to catabolize for surviving (de Carvalho *et al.*, 2010).

As mentioned above, the FadA5 thiolase involved in the side-chain metabolism of cholesterol is required for full virulence of *M. tuberculosis* as a *fadA5* mutant showed attenuated phenotype and at 8 weeks after infection, the number of cfu in the mouse lungs decreased about 10-fold compared with the level in the wild-type strain (Nesbitt *et al.*, 2010).

Finally, the development of chemical inhibitors targeting cholesterol catabolic enzymes has been proposed as a novel route for designing new therapeutics to deal with the re-emergent pathogen *M. tuberculosis* (Ouellet *et al.*, 2011).

Biotechnological applications of cholesterol degrading bacteria

Microorganisms capable of degrading and/or transforming cholesterol have been widely used for many years in

biotechnological applications. These applications include the development of cholesterol biosensors for clinical diagnosis, microbial steroid biotransformations for the production of steroid drugs and hormones, probiotics with cholesterol-lowering effects, and enzymes for insecticide and fungicide purposes.

Biosensors for cholesterol analysis in biological samples

The increase of cardiovascular disorders mainly due to hypercholesterolemia make the quantification of cholesterol levels an important clinical target and therefore several biosensors have been developed for this purpose (Arya *et al.*, 2008; Jubete *et al.*, 2009; Solanki *et al.*, 2011). Biosensors for cholesterol are also useful in the food industry to maintain quality control (Bragagnolo and Rodriguez-Amaya, 2002; Hwang *et al.*, 2003; Jubete *et al.*, 2009). Some of these cholesterol biosensors are based on cholesterol degrading enzymes (Table 2).

ChOxs are widely utilized for the enzymatic analyses of cholesterol. The most common source of ChOxs are from *Streptomyces hygroscopicus*, *B. sterolicum* and *Pseudomonas fluorescens* (Arya *et al.*, 2008; Nien *et al.*, 2009; Singh *et al.*, 2009). Because 70% of cholesterol exists in ester form and 30% as free form in blood samples, cholesterol esterases are also included in bio-sensors together with ChOxs (Arya *et al.*, 2008; Shih *et al.*, 2009). In addition, horseradish peroxidase has been used in the fabrication of photometry based biosensors (Arya *et al.*, 2008). This enzyme can be also coupled to 4-aminoantipyrine and phenol to determine the hydrogen peroxide released in the ChOx reaction, a method routinely used in clinical laboratories (Pollegioni *et al.*, 2009). To avoid the interference of blood pigments in colorimetric assays some electrochemical methods based on oxygen consumption by ChOx reaction have been also developed (Cheillan *et al.*, 1989; Pollegioni *et al.*, 2009).

Microbial ChDHs, which catalyse the conversion of 3β-hydroxy-5-ene-steroids to 3-oxo-4-ene-steroids using NAD or NADP as cofactors, are also utilized in cholesterol biosensors (Ishige *et al.*, 2009; Wallace-Davis *et al.*, 2011).

Several authors have also shown that the P450scc cytochrome, an isoform of the P450 (CYP) cytochrome superfamily, which catalyses the side-chain cleavage of cholesterol to pregnenolone in the presence of molecular oxygen and NADPH-ferrihaemoprotein reductase (Carrara *et al.*, 2008), can be utilized for determination of cholesterol with high sensitivity (Antonini *et al.*, 2004; Paternolli *et al.*, 2004; Shumyantseva *et al.*, 2004; 2005). The highly specific conversion of steroid hormones by cythocromes makes feasible a specific substrate

Table 2. Biosensors based on cholesterol degrading enzymes.

Sensing element (Transductor)	Range	Reference
Cholesterol oxidase		
Spectrophotometric	25–400 mg dl^{-1}	Dhand and colleagues *(*2007)
Electrochemical	25–400 mg dl^{-1}	Solanki and colleagues (2007a)
Surface plasmon resonance	50–500 mg dl^{-1}	Solanki and colleagues (2007b)
Amperometric	6–30 mg dl^{-1}	Özer and colleagues (2007)
Nano-Amperometric	0.1–50.0 µg dl^{-1}	Umar and colleagues (2009)
Flow injection chronoamperometric	50–400 mg dl^{-1}	Wisitsoraat and colleagues (2010)
Voltametry	0.1–10.0 µg dl^{-1}	Norouzi and colleagues (2010)
Cholesterol oxidase + cholesterol esterase		
Colorimetric	1200–3600 mg dl^{-1}	Law and colleagues (1997)
Potentiometric	3–200 mg dl^{-1}	Situmorang and colleagues *(*1999)
Amperometric	65–520 mg dl^{-1}	Singh and colleagues (2004)
Electrochemical	50–400 mg dl^{-1}	Arya and colleagues (2007)
Spectrophotometric	12–780 mg dl^{-1}	Singh and colleagues (2007)
Voltammetric	1–6 mg dl^{-1}	Aravamudhan and colleagues (2007)
Polarographic	2–50 mg dl^{-1}	Basu and colleagues (2007)
Cholesterol oxidase + cholesterol esterase + horseradish peroxidase		
Acoustic	3–20 mg dl^{-1}	Martin and colleagues (2003)
Spectrophotometric	100–400 mg dl^{-1}	Singh and colleagues (2006)
Amperometric	0.1–65.0 mg dl^{-1}	Salinas and colleagues (2006)
Electrochemical	0–300 mg dl^{-1}	Ahmadalinezhad and Chen (2011)
Cholesterol oxidase + horseradish peroxidase		
Amperometric	130–780 mg dl^{-1}	Kumar and colleagues (2006)
Cholesterol dehydrogenase		
Potentiometric	33–233 mg dl^{-1}	Ishige and colleagues (2009)
Electrochemical	65–500 mg dl^{-1}	Wallace-Davis and colleagues (2011)
Cytochrome P450		
Electrochemical	7–50 mg dl^{-1}	Paternolli and colleagues (2004)
Amperometric	0.7–5.0 mg dl^{-1}	Shumyantseva and colleagues (2005)

determination in complex media by using CYP based sensors (Bistolasa *et al.*, 2005)

Biotransformations

The synthesis of the complex steroid molecules requires very specific reactions, and the utilization of microorganisms should enable the performance of reactions of high regio- and stereo-selectivity. Moreover, the mild conditions required for bioconversions as well as the more ecological processes compared with chemical synthesis make the high yield biological production an interesting field to develop.

The importance of microbial biotransformations in the production of steroid drugs and hormones became a reality when the 11α-hydroxylation of progesterone by a *Rhizopus* species was patented in 1952 (Hogg, 1992). Since then, microbial reactions for the transformation of steroids have proliferated and specific transformation steps have been incorporated into numerous partial syntheses of steroids with therapeutic use and commercial value (Mahato and Garai, 1997). Although some of these bioconversions are well-established, increasing the efficiency of the existing processes as well as identifying new potentially useful bioconversions, are two current objectives of the steroid industry (Fernandes *et al.*, 2003).

Microbial bioconversion has been focused mainly in steroid hydroxylation, Δ^1-dehydrogenation and sterol side-chain cleavage that associated to chemical synthesis steps, have enhanced the large-scale production of natural and modified steroid analogues. The use of whole cells instead of enzymes is preferred as the production costs are lower and it is possible to perform multi-steps conversions with a single biocatalyst (Bortolini *et al.*, 1997). The manufactured steroid compounds have a wide range of therapeutic applications as anti-inflammatory, immunosuppressive, diuretic, anabolic, contraceptive agents, breast and prostate cancer and anti-obesity agents, among others. The therapeutic action of steroid hormones has been traditionally associated to their binding to the respective intracellular receptors, which act as transcription factors in the regulation of gene expression. Some steroid molecules are also called neurosteroids due to their role as memory enhancers, inducers of endocrine response to stress, anxiolytic agents, anticonvulsants, antidepressives and neuroprotective effect (Fernandes *et al.*, 2003).

Many different steroids have been used for biotransformations, but specifically cholesterol obtained from animal fats and oils, such as lard, beef tallow, milk fat or fish oil has been also employed as starting raw material. It has been biotransformed into different compounds such as 17-ketosterols, AD, 9α-OH-AD and ADD (Fernandes *et al.*, 2003). Microorganisms capable of oxidizing cholesterol have a serious draw-back for steroid synthesis, as they do not only degrade the side-chain but usually also cause the complete undesirable fission of the steroid skeleton (Kieslich, 1980). Some of the microorganisms capable of cleavage the side-chain of cholesterol and used by their potential interest as biocatalysts are *R. coralline*, *Arthrobacter simplex*, *R. equi*, *Mycobacterium fortuitum*, *R. erythropolis*, *M. neoaurum* or *Micrococcus roseus* (Liu and Lee, 1992; Shi *et al.*, 1992; Ahmed and Johri, 1993; Ahmed *et al.*, 1993; Lee *et al.*, 1993; Smith *et al.*, 1993; Srivastava and Patil, 1994; Liu *et al.*, 1994a; Mahato and Garai, 1997; van der Geize and Dijkhuizen, 2004; Sripalakit *et al.*, 2006; Molchanova *et al.*, 2007). *Mycobacterium* sp. NRRL B-3805 (Liu *et al.*, 1994b; Liu and Lo, 1997) and *Lactobacillus bulgaricus* (Kumar *et al.*, 2001) have been used to produce testosterone from cholesterol using a single strain. Production of ADD from cholesterol using two-step microbial transformation has been reported (Lee *et al.*, 1993). First, cholesterol is converted to cholestenone by the inducible ChOx of *A. simplex*, and second, cholestenone is transformed into ADD by *Mycobacterium* sp. (Mahato and Garai, 1997). Some of the microorganisms used to produce cholestenone from cholesterol are *Mycobacterium* sp. (Smith *et al.*, 1993), *R. equi* (Ahmed and Johri, 1993), *Escherichia* (Panchishina, 1992), *Nocardia rubra* (Osipowicz *et al.*, 1992), *A. simplex* (Liu *et al.*, 1994a), *R. erythropolis* (Jadoun and Bar, 1993), *R. equi* (Myamato and Toyoda, 1994), *Agrobacterium* sp. M4 (Mahato and Garai, 1997; Yazdi *et al.*, 2000).

Microbial transformation of steroids can also be an environmentally beneficial process. Dias and colleagues (2002) studied the composition of different industrial waste materials for their steroid/cholesterol content in order to find out if these industrial by-products were suitable as steroid sources for the microbiological production of AD and ADD. They were used as a substrate for microbial degradation by a *Mycobacterium* sp. strain and proved to be easily converted to AD and ADD. Thus, a profitable application of these wastes could be the production of sterol-rich fractions, reducing the pollution loads.

Probiotics

Probiotics are living microorganisms, which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition (Guarner and Schaafsma, 1998). Although many *in vitro* and *in vivo* studies have shown that the administration of probiotics reduces serum/plasma total cholesterol, LDL-cholesterol and triglycerides or increases HDL-cholesterol, their hypocholesterolemic effects remain controversial (Ooi and Liong, 2010). The species with cholesterol-lowering effects

studied include genera as *Lactobacillus, Lactococcus, Enterococcus*, *Streptococcus* or *Bifidobacterium* (Pereira and Gibson, 2002).

Probiotics have been suggested to reduce cholesterol via various mechanisms that do not necessarily imply the transformation of cholesterol by the probiotic strains, e.g. enzymatic deconjugation of bile acids by bile-salt hydrolases of probiotics (Lambert *et al.*, 2008), assimilation of cholesterol (Pereira and Gibson, 2002), co-precipitation of cholesterol with deconjugated bile (Liong and Shah, 2006), cholesterol binding to cell walls of probiotics (Liong and Shah, 2005), incorporation of cholesterol into the cellular membranes of probiotics during growth (Lye *et al.*, 2010a), production of short-chain fatty acids upon fermentation by probiotics in the presence of prebiotics (De Preter *et al.*, 2007) and conversion into coprostanol (Lye *et al.*, 2010b).

As stated above, the most frequent transformation of cholesterol in the intestinal anoxic habitats is a biogenic reduction of its double bond yielding coprostanol, which is directly excreted in faeces (Li *et al.*, 1995; Harder and Probian, 1997). Many intestinal fermenting bacteria are able to catalyse this reaction (Groh *et al.*, 1993; Freier *et al.*, 1994) decreasing the amount of absorbed cholesterol and leading to a reduced concentration in the physiological cholesterol pool (Ooi and Liong, 2010). Lye and colleagues (2010b) evaluated the conversion of cholesterol to coprostanol by strains of lactobacilli such as *L. acidophilus, L. bulgaricus* and *Lactobacillus casei* detecting both intracellular and extracellular cholesterol reductases in these strains, indicating possible intracellular and extracellular conversions of cholesterol to coprostanol. Cholesterol reductase has been also directly administered to humans to convert cholesterol to coprostanol in the small intestines to reach a bloodstream cholesterol-lowering effect (Ooi and Liong, 2010).

One of the best-studied mechanisms proposed for the cholesterol-lowering effects of probiotics is the enzymatic deconjugation of bile acids by bile salt hydrolases (Begley *et al.*, 2006). Bile, a water-soluble end-product of cholesterol in the liver that consists of cholesterol, phospholipids, conjugated bile acids, bile pigments and electrolytes, is released into the duodenum upon ingestion of food. When it is deconjugated by bile salt hydrolases-producing strains, bile acids are less soluble, being no absorbed by intestines and eliminated in the faeces. Cholesterol is then used to synthesize new bile acids as a homeostatic response, resulting in a hypocholesterolemic effect of probiotics (Begley *et al.*, 2006; Ooi and Liong, 2010).

The cholesterol-lowering effects are also attributed to the ability of probiotic strains to bind cholesterol, a process that is growth-dependent and strain-specific. However, although growing cells remove more cholesterol than dead cells, the heat-killed cells can still remove cholesterol from media, indicating that some cholesterol is bound to the cellular surface (Usman and Hosono, 1999). During the probiotic growth, the presence of cholesterol increases the concentration of saturated and unsaturated fatty acids, leading to increased membrane strength and subsequently higher cellular resistance towards lysis (Lye *et al.*, 2010a; Ooi and Liong, 2010).

Microbial enzymes for insecticides and fungicides

Besides the nutritional role of ChOx, other roles as biological weapons have been ascribed to these enzymes (Ghoshroy *et al.*, 1997; Martín and Aparicio, 2009). Purcell and colleagues (1993) discovered a highly efficient protein that killed boll weevil (*Anthonomus grandis grandis Boheman*) larvae in *Streptomyces* culture filtrates and identified the protein as ChOx. Purified enzyme was active against boll weevil larvae at a concentration comparable with the bioactivity of *Bacillus thuringiensis* proteins against other insect pests. The enzyme is involved in the lysis of the midgut epithelial cells of the larvae and also exhibits insecticidal activity against lepidopteran cotton insect pests, including tobacco budworm (*Heliothis virescens*), corn earworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) (Corbin *et al.*, 2001). Recently, it was reported that *Chromobacterium subtsugae* has insecticidal properties (Martin *et al.*, 2007). ChOx might be involved in this insecticidal activity because it was recently found that *Chromobacterium* strains produce this enzyme (Doukyu *et al.*, 2008). Some insecticide proteins are vital for pest control strategies employing transgenic crops. The ChOx encoding gene has been expressed in tobacco cells (Corbin *et al.*, 1994; Cho *et al.*, 1995; Doukyu, 2009).

Future prospects

Despite the large number of works carried out during the last century, the bacterial catabolism of cholesterol and other steroids is still far for being completely understood. For instance, the enzymes involved in the degradation of the steroid side-chain as well as those responsible for the last steps of the catabolic pathway remain to be precisely established. Moreover, the physiological role played by bacterial ChOxs is still unclear. Very few data are available about the proteins responsible for the bacterial uptake of steroids and this field will require supplementary attention as the possibility to create by metabolic engineering heterologous recombinant bacteria able to transform steroids for biotechnological purposes will depend on the possibility of conferring these bacteria-specific steroid transport capacities. As stated above, only a limited number of regulatory proteins involved in the metabolism of cholesterol have been identified and

partially characterized and the real inducers of these regulators remain to be elucidated as it has not been determined yet whether the cholesterol or their catabolic intermediates are their effectors. Taking into account that the availability of commercial pathway intermediates is very limited the identification of such putative inducers appears to be a difficult task.

Although cholesterol is equally disseminated both in oxic and anoxic environments only two bacterial strains were reported so far to mineralize cholesterol to carbon dioxide under anoxic conditions. Therefore, the anoxic catabolism of cholesterol remains challenging because compared with the oxic metabolism of cholesterol, the metabolism of this substrate in the absence of oxygen is still very poorly understood (Chiang *et al.*, 2008a).

While powerful technological approaches based on modern *omic* technologies (genomics, metagenomics, transcriptomics, etc.) will be useful to solve many of the questions raised here, the complexity of the pathways and their intermediates still demand to carry out huge efforts by using conventional biochemical and genetic tools to be able to decipher all the steps and processes involved in steroid catabolism.

In spite of the fact that the increment of cardiovascular diseases makes the availability of cholesterol biosensors of outstanding interest, few have been successfully commercialized. Optimization of critical parameters, such as enzyme stabilization, quality control and instrumentation design is needed. Increased understanding of the immobilized bioreagents, improved techniques for immobilization and technological advances in the microelectronics will lead to the rapid development of more accurate cholesterol biosensors (Arya *et al.*, 2008).

Expected developments in biological production of steroid compounds range from the identification of novel biocatalysts or the improvement of existing ones to the enhancement of the biotransformation processes. The application of genetic engineering and systems biology tools for the upgrading of steroid-transforming microorganisms; solubility improvement of substrates that are sparingly soluble in water; immobilization of enzymes or whole cells in a suitable matrix for repetitive economic utilization of enzymes and development of a continuous process for economic product recovery are some of the biotransformation fields of research.

Lamb and colleagues (1998) have proposed the existence of a sterol biosynthetic pathway in *M. smegmatis*, after ascertaining a significant number of homologies between the genome sequence of *M. tuberculosis* and the sterol biosynthetic enzymes of *Saccharomyces cerevisiae*. Therefore, the development of a steroid cell-factory in *M. smegmatis* based on the approach followed by Duport and colleagues (1998), who engineered six genes to construct a yeast strain that produces pregnenolone and progesterone from galactose, could be envisaged (Fernandes *et al.*, 2003).

The knowledge of microbial cholesterol catabolic pathways could lead to the introduction of several changes in probiotic organisms thus transforming them in strains capable of reducing cholesterol levels in foods and intestines. In this regard, Park and colleagues (2008) constructed a constitutive high-level expression vector for the genus *Bifidobacterium* and used it to express ChOx from *Streptomyces coelicolor*. *In vivo* studies of the cholesterol-lowering effects of this genetically modified organism as well as of the hypocholesterolemic properties of other modified strains that could be constructed in the future would be of outstanding interest.

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