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Functional characterization of 3-ketosteroid 9 α -hydroxylases in *Rhodococcus ruber* strain chol-4

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ABSTRACT

The 3-Ketosteroid-9 α -Hydroxylase, also known as KshAB [androsta-1,4-diene-3,17-dione, NADH:oxygen oxidoreductase (9 α -hydroxylating); EC 1.14.13.142], is a key enzyme in the general scheme of the bacterial steroid catabolism in combination with a 3-ketosteroid- Δ^1 -dehydrogenase activity (KstD), being both responsible of the steroid nucleus (rings A/B) breakage. KshAB initiates the opening of the steroid ring by the 9 α -hydroxylation of the C9 carbon of 4-ene-3-oxosteroids (e.g. AD) or 1,4-diene-3-oxosteroids (e.g. ADD), transforming them into 9 α -hydroxy-4-androsten-3,17-dione (9OHAD) or 9 α -hydroxy-1,4-androstadiene-3,17-dione (9OHADD), respectively.

The redundancy of these enzymes in the actinobacterial genomes results in a serious difficulty for metabolic engineering this catabolic pathway to obtain intermediates of industrial interest.

In this work, we have identified three homologous *kshA* genes and one *kshB* gen in different genomic regions of *R. ruber* strain Chol-4. We present a set of data that helps to understand their specific roles in this strain, including: i) description of the KshAB enzymes ii) construction and characterization of $\Delta kshB$ and single, double and triple $\Delta kshA$ mutants in *R. ruber* iii) growth studies of the above strains on different substrates and iv) genetic complementation and biotransformation assays with those strains. Our results show that KshA2 isoform is needed for the degradation of steroid substrates with short side chain, while KshA3 works on those molecules with longer side chains. KshA1 is a more versatile enzyme related to the cholic acid catabolism, although it also collaborates with KshA2 or KshA3 activities in the catabolism of steroids. Accordingly to what it is described for other *Rhodococcus* strains, our results also suggest that the side chain degradation is KshAB-independent.

1. Introduction

Steroid catabolism is widely spread in Actinobacteria (*Mycobacterium* and *Rhodococcus* spp.) and Proteobacteria (*Comamonas* and *Pseudomonas* spp.) [1]. Besides the general interest in knowing the details of the complex steroid degradation pathways, steroid intermediaries of high economic importance or strong biological activity are produced during the catabolic process [2–5], which is appealing enough to attract biotechnological interests and a great deal of research efforts.

Cholesterol is one of the steroids that have concentrated more studies [6,7]. The proposed catabolic oxidative pathway is formed by a complex set of enzymatic reactions. The first of them are included in the so-called upper pathway, and are: the oxidation of cholesterol to 4-

cholesten-3-one, the carbon side chain cleavage at C17, in a similar way to the β -oxidation of fatty acids, and the polycyclic ring opening [6,8,9].

The cholesterol catabolism combines several activities such as cytochromes P450 [3,10], 3 β -hydroxysterol oxidases or cholesterol oxidases [11–13], dehydrogenases (e.g. 3 β /17 β -hydroxysteroid dehydrogenases and KstDs) [14–18], or monooxygenases (e.g. the two component Rieske non-heme monooxygenase KshAB or the flavin-dependent monooxygenase HsaAB) [19–21]. The complexity of the cholesterol catabolic route lies on the facts that these steps are not always consecutive, that there is a multiplicity of enzymes with the same activity and that different aerobic pathways occur in a strain-dependent way. For instance, although there are a lot of oxygenases involved in this pathway, it has been recently described an aerobic cholesterol

Abbreviations: AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; CHO, cholest-5-en-3 β -ol (cholesterol); KshAB, 3-ketosteroid-9 α -hydroxylase; 9OHAD9, α -hydroxy-4-androstene-3,17-dione; 9OHADD, 9 α -hydroxy-1,4-androstadiene-3,17-dione; Δ^1 -⁴-BNC, 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid; 3-HSA, 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione; DHEA, dehydro-epiandrosterone; ORF, open reading frame; KstD, 3-ketosteroid- Δ^1 -dehydrogenase; LB, Luria-Bertani; CTAB, cetyltrimethyl ammonium bromide

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degradation pathway that it is oxygenase-non dependant [22]. Therefore, this catabolism can be very versatile and a particular compound, ADD for instance, may be produced as a result of different activity sets [23].

KshAB complex is responsible for the C9 α -hydroxylation; it consists of a terminal oxygenase (KshA subunit) which performs the substrate hydroxylation and a ferredoxin reductase (KshB subunit) which mediates the electron transfer. This enzyme initiates the steroid polycyclic ring opening in the steroid catabolism in combination with the KstD activity [24]. KshA contains two domains: a N-terminal Rieske iron-sulfur domain and a C-terminal catalytic domain that utilizes a non-heme Fe(II) to catalyse the addition of one hydroxyl group to the aromatic ring. KshB is similar to the ferredoxin reductase of multi-component oxygenases; it is composed of a NAD(P) binding domain in the N-terminal region and a 2Fe-2S iron-sulfur binding domain in the C-terminal region. The reaction depends on the use of an electron transport chain that transfers electrons from the NADH to KshB and then to KshA, which has an O₂ bounded needed for the hydroxylation process of the substrate [24]. The catalytic mechanism of KshAB is not yet clarified but it follows a pattern similar to other Rieske non-heme oxygenases: the substrate binds to the active site and the non-heme iron is reduced; when the oxygenase is in a reduced state, O₂ can bind to the metal centre and activate the hydroxylation process [24,25].

Particularly, AD is substrate for both KstD and KshAB enzymes that yield ADD and 9OHAD respectively; these products are further transformed with the same combination of enzymes to finally yield the unstable compound 9OHADD that spontaneously – by the break of the B ring- transforms into 3-HSA and enters into the so-called medium pathway that finally leads into the primary metabolism via the tri-carboxylic acid cycle [26]. The above scheme keeps ADD concentration at a low level avoiding cytotoxic effects [27]. The reactions starting from ADD, an intermediate from the degradation of several steroid compounds [28], are also known as 9,10-seco-pathway.

Rhodococcus species have been proved to be robust catabolic strains and potent steroid degraders in nature [4,29]. Among *Rhodococcus* strains, several KstD and KshAB isoenzymes have been reported. The wide enzymatic versatility and catabolic capacities of these activities make difficult to get a complete and detailed map of this catabolism. *Rhodococcus* strains are not yet biotechnologically fully exploited being the knowledge of all the steps of the steroid degradation pathways a serious bottleneck. Only recently, a *Rhodococcus erythropolis* strain got through a suitable combination of mutations in these enzymes has been used as whole-cell industrial steroid biotransformer [30]. *R. ruber*, a strain isolated from a sewage sludge sample, contains a cholesterol oxidase and three KstDs with different functionalities involved in the steroid degradation [13,16,31]. In this work we have identified and studied the three different KshA isoforms of this strain, a necessary step for broadening its biotechnological applications.

2. Material and methods

2.1. Bacterial strains, plasmids and growth conditions

Rhodococcus ruber strain Chol-4 (CECT 7469; DSM 45280) is a sewage sludge sample strain isolated in our laboratory [31]. This strain was routinely grown in LB or minimal medium (M457 of the DSMZ, Braunschweig, Germany) containing the desired carbon and energy source under aerobic conditions at 30 °C in a rotary shaker (250 rpm) for 1–3 days. For the steroids growth experiments, a LB pre-grown culture was washed two times with minimal medium prior to inoculation. Steroids were previously dissolved in 16.5 mM methyl- β -cyclodextrin (CD) to form inclusion complexes following a modification of a previously reported method (Klein et al.) and prepared as described [32]. Stocks of 6 mM cholesterol and 10 mM other steroids (AD, ADD, testosterone, DHEA, cholic acid) were prepared. A mix of phytosterols (Gadea S.A.) was directly added to the culture when required. Cholic

acid and sodium acetate were directly added to the culture to reach the desired concentration in the absence of cyclodextrins. 1.5% (w/v) agar plates were used.

E. coli strains were grown in LB broth at 37 °C, with 250 rpm orbital shaking. Appropriate antibiotics were added when needed at the following concentrations: 100 μ g/mL ampicillin and 50 μ g/mL kanamycin in *E. coli* cultures; 20 μ g/mL nalidixic; 34 μ g/mL chloramphenicol or 200 μ g/mL kanamycin for *Rhodococcus* cultures. Competent cells of *E. coli* DH5 α F' were prepared and transformed by standard protocols [33]. Strains and plasmids used in this study are listed in Table B.1.

2.2. DNA preparation, sequencing and in silico analysis

Chromosomal DNA extraction from *R. ruber* strain Chol-4 was performed using the CTAB procedure as previously published [16]. DNA was suspended in distilled water.

Manipulation of genomic DNA was carried out according to standard protocols [33]. The BioEdit program vs 7.1.3.0 [34] was used to blast a known ORF against the published genome of *R. ruber* [35].

The GenBank accession number for the *R. ruber* genome nucleotide sequences reported in this paper corresponds to NZ_ANGC000000000.1. The ORF finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>), the RAST server [36] and the pDraw32 (<http://www.acaclone.com/>) programs were used to analyse the ORFs contained in the corresponding contigs. Putative domains in the ORFs were searched with the BLASTs server (<http://expasy.org/prosite/>). DNASTAR (Lasergene) programs were used to analyse sequences, to elaborate cladograms (MegAlign program using the alignment with the Clustal W method) and to design primers.

For the protein modelling, I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>) was used for an approach to protein structure and function prediction [37] while PredictProtein (www.predictprotein.org/home) was used for the secondary structure, solvent accessibility and transmembrane helix predictions [38]. COBALT was used as a multiple sequence alignment tool to find similarities among the catalytic residues (www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi) [39] and PyMOL (www.pymol.org/) as a molecular visualization system (PyMOL Molecular Graphics System, Version 0.99, Schrödinger).

2.3. PCR and RT-PCR analysis

For PCR analysis, Chol-4 cultures were grown on 50 mL of 457 DSMZ medium with either 0.6 mg/mL cholesterol (added as a powder) or 2 mg/mL sodium acetate. Cells were harvested in mid-exponential growth phase (OD_{600nm} = 0.6) after addition of 5 mL of a 9:1 (v/v) mix of ethanol/phenol and further centrifugation at 5000 rpm, 4 °C for 15 min. Afterwards, cells were stabilized by addition of RNA protect Bacteria Reagent (Qiagen) following the manufacturer's instructions. Pellets were kept at –80 °C until being used.

Total RNA was prepared with the RNeasy Mini Kit (Qiagen) following the manufacturer's indications with the following modification: 50 mg of acid-washed glass beads (150 μ m diameter) were added in the first step and each sample was shaken at maximum speed in a Bullet Blender for 1 min. The cell debris was removed by centrifugation. The supernatant was treated according to the RNeasy Mini Kit (Qiagen) protocol. The total RNA obtained (0.5–1 μ g) was treated once with 5 U of Turbo DNase RNase-Free (Ambion) in a 700 μ L total volume for 2 h at 37 °C. RNA samples were extracted with 1 vol of acid phenol (Sigma), vigorously shaken and incubated at room temperature for 15 min. After a 15 min centrifugation, the upper phase was precipitated by addition of 0.12 vols of 5 M NH₄Ac, 0.02 vols of glycogen (5 mg/mL) and 1 vol of isopropanol, washed twice in 70% ethanol and dissolved in water. To completely remove DNA contamination samples were treated with DNase until no DNA was detected by PCR.

cDNA was synthesized using SuperScript III Reverse Transcriptase

(Invitrogen) following the manufacturer's indications. cDNA was used as template (25 ng) for PCR reactions (20 μ L final volume). Controls without reverse transcriptase (RT-) were used to detect any contamination of undigested DNA in the RNA preparations.

PCR was performed under standard conditions using Expand High Fidelity PCR System Enzyme Mix (Roche). For amplifications of Chol-4 DNA, glycerol at a final concentration of 5% was also added to the reaction mix. Primers, dimethyl sulfoxide, dNTPs and template DNA were added at a final concentration of 0.4 μ M, 4% (v/v), 200 μ M and 10 ng/ μ L respectively. PCR conditions were: 30 cycles of 1 min at 95 °C, 1 min at the desired T_m and 0.5–3 min at 72 °C unless stated otherwise. PCR primers used in this work are listed in Table B.2.

2.4. Mutagenesis of the *kshA* and *kshB* genes of *R. ruber* strain chol-4

Primers and conditions used in this experiment are summarized in Table B.2. Unmarked gene deletions were carried out as described previously in *R. erythropolis* SQ1 involving conjugative transfer of a mutagenic plasmid carrying the *sacB* selection system [40]. Specific pairs of primers were designed from up and downstream sequences of the terminal regions of each ORF in order to get PCR amplicons from isolated *R. ruber* strain Chol-4 genomic DNA. The primers also contained suitable restriction sites to allow the later cloning and fusion of both terminal regions of every ORF. The amplified *kshA1* terminal regions contained *EcoRI-XbaI* and *XbaI-HindIII* restriction sites in the up and down terminal regions respectively; *XbaI-EcoRV* (up) and *EcoRV-HindIII* (down) were in the amplified *kshA2* terminal regions; *HindIII-BglII* (up) – *BglII-XbaI* (down) were in the amplified *kshA3* terminal regions and *EcoRI-HindIII* (up) – *HindIII-PstI* (down) in the amplified *kshB* terminal regions. These amplicons were cloned into pGEM-T-Easy vectors and every pair was combined in order to get a DNA fragment containing a truncated *kshA* or *kshB* ORF that keeps the right reading frame to avoid polar effects (Fig. 1). These DNA fragments were cloned into the pK18mobsacB plasmid [41] to construct the mutagenic plasmids pK18(*kshA1U* + D), pK18(*kshA2U* + D), pK18(*kshA3U* + D) and pK18(*kshBU* + D), which were used to delete *R. ruber* strain Chol-4 *kshA1*, *kshA2*, *kshA3* and *kshB* genes, respectively.

The mutagenic plasmids were introduced into *E. coli* S17.1 and mobilized to *R. ruber* strain Chol-4 by conjugation as already published [16].

kshA and *kshB* ORF truncation were then achieved as a result of a spontaneous double homologous recombination process within the genome of *R. ruber* strain Chol-4. Deletions were checked by colony PCRs. Double or triple mutations were generated taking the single or double mutant strain as a new recipient, respectively.

2.5. Monitoring of steroids intermediaries by thin layer chromatography (TLC) in Δ *kshA* and Δ *kshB* on growing or resting-cells

The protocol for resting cells was as followed. Chol-4 wild-type and Δ *kshA* and Δ *kshB* strains were grown in 100 mL of LB medium in 500 mL flasks at 30 °C in a rotary shaker (250 rpm) for 2 days. One hour before harvesting, chloramphenicol was added to a final 34 μ g/mL. The equivalent of cells to 30 mL of culture at OD_{600nm} of 18, were harvested at 4 °C by centrifugation at 10,000 $\times$ g for 15 min, washed twice with 0.85% NaCl, and suspended in 25 mM of HEPES (Sigma) pH 7. Cholesterol (1.7 mg/mL) and AD (1 mg/mL) were added as powder. The resting-cell reaction was allowed to proceed at 30 °C and 150 rpm. Aliquots of 500 μ L were taken at fixed times and their lipid fraction was obtained by extraction twice with 1 mL chloroform and let drying at 65 °C.

50 μ L of chloroform were added to the samples; an aliquot of 10–20 μ L (in the case of cholesterol) or 10 μ L (in the case of AD) of the solution were applied on aluminium TLC Silica gel 60 F254 sheets (Merck).

Chromatography was performed using a mix of hexane:ethylacetate

(10:4, v/v). Spots were revealed by UV exposure (λ_{254nm}). Afterwards, the TLC plate was dunked in a sulfuric acid:methanol solution (1:9, v/v), dried with warm air and heated 2 min at 100 °C. 1 μ g of cholesterol, AD, ADD and 9OHAD standard control samples were also included. To detect additional oxidation, a control experiment was performed: the steroid fractions of AD grown or cholesterol grown cultures were extracted with chloroform and dried at 15 °C in the presence of Argon for 30 min. These samples were immediately dissolved on 50 μ L chloroform and 10 μ L of it were applied on TLC plates. Chromatography results confirmed that no accidental oxidation had occurred in any case.

To monitor intermediary production in culture, mutants were grown in 3 mL LB for two days. Then cells were washed 2–3 times with minimal medium, 50 μ L of the washed cells suspended in 50 mL of minimal medium supplemented with cholesterol (1.7 mg/mL) or AD (1 mg/mL) and kept growing at 30 °C, 250 rpm. Aliquots of 0.5 mL were taken from the culture at fixed times after OD_{600nm} was measured. Their lipid fraction was obtained by extraction and TLC analysis was done as previously described.

3. Results and discussion

3.1. In silico analysis and identification of the putative *kshA* and *kshB* ORFs of *R. ruber* strain chol-4

3-ketosteroid 9 α -hydroxylase (KshAB) and 3-ketosteroid- Δ^1 -dehydrogenase (KstD) are good examples of the high enzymatic redundancy that occur in Rhodococci. In particular, a deep search in the NCBI database led to find several strains that contain a multiplicity of KshA proteins: there are at least three putative isoforms of KshA in *R. pyridinivorans* KG-16 (AZXY01000000); four KshA subunits in *R. erythropolis* PR4, *R. qingshengii* BKS 20–40 and *R. aetherivorans* strain BCP1 (AP008957; NZ_AODN00000000; NC_008269; AVAE01000000); five KshAs in *R. rhodochrous* and *R. jostii* RHA1 [19] and seven KshAs in *R. equi* NBRC 101255 = C7 (BCRL00000000).

The three KshAs (GeneBank: ACD11366, BAH31462, AAL96829) identified in *R. erythropolis* SQ1 [27] and the five isoforms (GeneBank: KshA1: ADY18310, KshA2: ADY18316, KshA3: ADY18318, KshA4: ADY18323, KshA5: ADY18328) of *R. rhodochrous* [19] were blasted against the *R. ruber* strain Chol-4 genome [35]. Three different *kshA* ORFs were found in different genomic contexts (Fig. 2). They were named as *kshA1* (GeneBank: KXF8587), *kshA2* (GeneBank: KXF84594) and *kshA3* (GeneBank: KXF84022) on the basis of their similarities to the *R. rhodochrous* isoforms.

Supplementary Table B.3 collects the identity data of the protein coded by these ORFs and their genomic surroundings. The cladogram (Fig. 3) shows that all KshAs could be grouped into three branches. KshA1 and KshA3 of *R. ruber* are in the first two branches and show a 97% and a 95% of amino acid (aa) identity when compared to the homologues of *R. aetherivorans* GenBank: AKE91471 and AKE88516, respectively. In contrast, *R. ruber* KshA2 has a relatively low aa identity with the rest of KshA proteins. The three *R. ruber* KshAs show a 63–65% aa identity among them.

The different Rhodococci known genomes code for a less variable number of KshB copies: one isoform in *R. erythropolis* PR4 (AP008957) and *R. equi* NBRC 101255 = C7 (BCRL00000000); two isoforms in *R. rhodochrous* [19] and *R. opacus* (AP011115.1); and three isoforms in *R. jostii* RHA1, although one of them is associated to a plasmid and presents a higher divergence (Fig. 3).

The KshB proteins from *R. erythropolis*, *R. rhodochrous* and *R. jostii* RHA1 [19,27,42,43] were used to blast against the *R. ruber* Chol-4 genome. Only one *kshB* ORF could be found (GeneBank: KXF85152) (Fig. 2) that keeps a 99% of aa identity with one homologue of *R. rhodochrous* (see Table B.3). Its genomic location is not near any KshA, similarly to the cases of *Mycobacterium* and other *Rhodococcus* [7,15]. The lesser multiplicity of KshB respect to KshA subunits may be due to its role in the electron transfer to the KshA subunit: the same KshB

Fig. 1. Scheme of the *kshA* and *kshB* ORFs deletion in the *R. ruber* strain Chol-4 genome. Solid lines indicate co-transcription of the ORFs confirmed by RT-PCR. HP: hypothetical protein; DH: dehydrogenase; ox: oxidase; MFS: major facilitator superfamily transporter; *padR* and *lcrI*: transcriptional regulators; *hsa*: flavin-dependent mono-oxygenase that hydroxylates 3-hydroxy-9,10-secoandrosterone-1,3,5(10)-triene-9,17-dione (3-HSA) to a catechol.

molecule can work with several different KshA subunits whose catalytic domains will finally define the substrate specificity. In fact, the same KshB reductase was functional with KshA1 and KshA2 subunits from *R. erythropolis* SQ1 [27]; one KshB reductase was positively coupled to the five KshA homologues of *R. rhodochrous* DSM43269 [19]; and a quimeric *Mycobacterium* KshA – *Gordonia* KshB was proved to be functional [44]. Furthermore, there are evidences that KshB can also act as a reductase for other oxygenases as well as for KshAs [45].

Multiplicity of these steroid catabolic enzymes seems to be a general characteristic of actinobacteria, as *Nocardioideis simplex* VKM Ac-2033D contains in its genome two genes orthologous to *kshA* and two genes orthologous to *kshB* [46] and *Mycobacterium abscessus* contains at least two *kshA* ORFs (NCIB: EIV82964 and EIV71174).

3.2. Molecular modelling of KshAs of *R. ruber* strain chol-4

In an attempt to go deeper inside the characterization of these enzymes, we performed protein sequence analysis and modelling studies on the *R. ruber* KshAs using different approaches. The published three-dimensional structure and the catalytic mechanism proposed for KshA from *Mycobacterium tuberculosis* and KshA5 from *R. rhodochrous* have been used as suitable models [47,48].

The three KshAs found in *R. ruber* contain the Rieske Fe-S binding motif DAYCRHMGGLsQGeiKGDSVACPFHDWR and the non-heme iron binding motif ELiD*NvVDMAHFFyVHYsf reported for other KshA homologues: the consensus among the three *R. ruber* KshAs are written in capital letters [24]; the asterisk marks the conserved aspartic acid Asp₁₇₈ that is involved in catalysis and that also facilitates the electron transfer between adjacent subunits not only in KshAB but also in similar enzymes such as phthalate dioxygenase [47,49–51]; in *Mycobacterium*

tuberculosis there are two histidines (His₁₈₁, His₁₈₆) and a Asp₃₀₄ that are involved in the iron coordination at the core of the catalytic domain [47].

Similarly, KshB from *R. ruber* contains the flavin binding motif SVARCYSLASS, the NADH binding motif LFGAGSGITPVVS and the plant-type Fe₂-S₂ binding motif PYSQCQEGECGSCACTVLDGKVEME HCEILDPEIDIEAGYVLGCQAR. In both KshA and KshB regions, the conserved residues in actinobacteria described by Petrusma et al. [24] appear in black and underlined.

I-TASSER and PredictProtein programs predicted that none of the three *R. ruber* KshAs contain sulfur bridges or transmembrane segments. The prediction of the secondary structure and solvent accessibility of the three KshAs from *R. ruber* are shown in Table B.4. Table 1 collects the described residues involved in the active site of *R. rhodochrous* KshA5 [48] and the homologous residues of the three KshAs of *R. ruber* found by COBALT program.

The eight key residues (Val₁₈₂, Gln₂₁₀, Tyr₂₃₉, Met₂₄₅, Asp₂₄₇, Asn₂₆₄, Phe₃₀₈ and Trp₃₁₅) of the *R. rhodochrous* KshA5 active site have counterpart residues in the *R. ruber* KshAs. However, the I-TASSER model prediction of these *R. ruber* enzymes shows that the orientation of the key residues within the catalytic pocket differs, being specific of each enzyme (Fig. 4). Particularly, the orientation of KshA1 Tyr₂₃₃ benzene ring differs in about 45° from that of its KshA2 and KshA3 homologues, a difference that may cause an impact in the anisotropic properties of the catalytic pocket. The comparison of the relative position of several homologous residues of the three KshAs *R. ruber* models has showed differences that may be meaningful. This is the case of F₃₀₁, M₂₃₉, Y₂₃₃, W₃₀₈ KshA1 residues and their respective KshA2 and KshA3 homologous residues (see Table 1).

Moreover, KshA1 has an Ile₁₇₆ where its KshA2 and KshA3

Fig. 2. Scheme of *R. ruber* *kshAB* genomic contexts. A comparison with *R. jostii* Rha1 (GeneBank: NC_008268.1) and *R. rhodochrous* gene homologues is included. Abbreviations: *kstD*: 3-ketosteroid Δ^1 -dehydrogenase; *hsaC*: 2,3-dehydroxyphenyl dioxxygenase; *hsaD*: 4,5:9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oate hydrolase; *hsaA*: 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione monooxygenase, subunit oxygenase; *kshA*: 3-ketosteroid 9 α -hydroxylase; *tetR*, *padR*, *lclR*: transcriptional regulators; *HP*: hypothetical protein; *fadA5*: acetyl-CoA acetyltransferase; *fadE*: acyl-CoA dehydrogenase; *ox*: oxygenase; *CO DH*: Carbon monoxide dehydrogenase.

homologues have a Val. Possibly, some or most of these differences will finally be related to the different substrate preference among these KshAs. At the moment, the current shortage of crystalline structures of these proteins greatly limits more detailed conclusions.

3.3. *KshAB* ORFs co-transcription studies

The *R. ruber* *kshAB* genes surroundings contain ORFs that could be also involved in the steroid metabolism (Fig. 2). Some of these ORFs may be expressed in a coordinated way or even co-transcribed with *kshA* genes. RT-PCR confirm that all *R. ruber* *kshA* genes are transcribed together with adjacent ORFs giving rise to long transcripts (*kshB* gene is flanked by opposite ORFs preventing co-transcription) (Fig. 1).

kshA1 ORF is co-expressed with an hypothetical protein (Fdx) that keeps a 58% of amino acid identity with a ferredoxin (ADY18311.1) of *R. rhodochrous* and with an ORF that has 98% aa identity with a short chain dehydrogenase of *R. rhodochrous* (Table B.3). The cluster *kshA1*-

fdx-short chain dehydrogenase might be conserved within the *Rhodococcus* genus (Fig. 2).

kshA2 ORF is co-expressed with an ORF that shows a 98% of aa identity with a hydroxylase of *R. rhodochrous* (WP_054372665), another ORF 94% similar to an 2,3-dihydroxybiphenyl 1,2-dioxxygenase of *R. rhodochrous* (WP_054372664) and a third ORF 95% similar to an *IclR* family transcriptional regulator (*R. rhodochrous*, WP_054372663) (Table B.3). This gene cluster organization is identical to that of *R. jostii* Rha1 but something different to that of *R. rhodochrous* BKS6-46 (GeneBank:NZ_AGVW02000046) (Fig. 2).

Lastly, *kshA3* ORF is co-expressed with two ORFs that may code for two hypothetical proteins that do not contain conserved domains. The first ORF keeps a 63% of aa identity with a putative ferredoxin (ADY18319) of *R. rhodochrous*. The second ORF has a 99% aa identity with a putative protein (WP_054371650) of *R. rhodochrous*. The putative ferredoxin is conserved in both *R. jostii* Rha1 and *R. rhodochrous* but the second ORF is different in each strain. The presence of a putative

Fig. 3. Cladogram of *Rhodococcus* KshBs and KshAs. Putative substrate preference of KshAs is indicated. Arrows indicate the *Rhodococcus ruber* Ksh subunits.

ferredoxin near a *kshA* ORF could be related to the proposed role of ferredoxin as an enhancer of the electron transfer between KshA and KshB [19].

3.4. Construction of *R. ruber* strain *chol-4* mutants

KshAB plays a role at different steps of the steroids degradation pathway, as they can act on a variety of substrates with several A-

steroid ring configurations and also with different C17 side chains [19,24,52]. Only few studies on the physiological roles of the *kshA* isoforms have been reported to date. In this piece of work we have made $\Delta kshA$ and $\Delta kshB$ *R. ruber* strains and studied their growth on different substrates in order to clarify the role of every isoform in this complex metabolic pathway.

Once identified the putative *kshA* and *kshB* genes in the genome of *R. ruber*, we have proceeded to build single, double and triple *kshA*

Table 1

Homologous amino acid residues implicated in the catalytic pocket of KshAs. Grey lines contain the key residues of the KshA5 active site of *R. rhodochrous* (Rrho) and its homologues in *R. ruber* given by COBALT.

Rrho	KshA1	KshA2	KshA3
I ₁₇₈	I ₁₇₂	I ₁₇₆	I ₁₆₆
N ₁₈₁	N ₁₇₅	N ₁₇₉	N ₁₆₉
V ₁₈₂	I ₁₇₆	V ₁₈₀	V ₁₇₀
H ₁₈₇	H ₁₈₁	H ₁₈₅	H ₁₇₅
F ₁₈₈	F ₁₈₂	F ₁₈₆	F ₁₇₆
H ₁₉₂	H ₁₈₆	H ₁₉₀	H ₁₈₀
S ₁₉₄	S ₁₈₈	S ₁₉₂	A ₁₈₂
Q ₂₁₀	Q ₂₀₄	Q ₂₀₈	Q ₁₉₈
M ₂₁₂	M ₂₀₆	M ₂₁₀	L ₂₀₀ *
S ₂₁₄	G ₂₀₈	F ₂₁₂	T ₂₀₂
L ₂₃₃	S ₂₂₇	–	L ₂₂₀
S ₂₃₅	S ₂₂₉	S ₂₃₂	S ₂₂₂
A ₂₃₇	A ₂₃₁	A ₂₃₄	A ₂₂₄
Y ₂₃₉	Y ₂₃₃	Y ₂₃₆	Y ₂₂₆
M ₂₄₅	M ₂₃₉	M ₂₄₂	M ₂₃₂
D ₂₄₇	D ₂₄₁	D ₂₄₄	N ₂₃₄
L ₂₄₉	L ₂₄₃	L ₂₄₆	L ₂₃₆
L ₂₆₂	L ₂₅₆	L ₂₅₉	L ₂₄₉
N ₂₆₄	N ₂₅₈	N ₂₆₁	N ₂₅₁
V ₃₀₄	I ₂₉₇	T ₃₀₁	I ₂₉₁
F ₃₀₈	F ₃₀₁	F ₃₀₅	F ₂₉₅
D ₃₁₁	D ₃₀₄	D ₃₀₈	D ₂₉₈
W ₃₁₅	W ₃₀₈	W ₃₁₂	W ₃₀₂

Fig. 4. Modelling of the active site of KshAs. The orientation of some of the catalytic residues in the KshA binding pocket is shown. They are superposed and depicted in different colours: blue for KshA1, red for KshA2 and green for KshA3. Only KshA1 residues are named, their counterpart homologues residues of KshA2 and KshA3 being listed in Table 1.

mutants and the single *kshB* mutant of this strain following the unmarked gene deletion procedure described before. Care has been taken to keep the reading frame after truncation of the *kshA* and *kshB* genes, in order to avoid possible polar effects in the expression of the *kshA* and *kshB* operons. Single mutants ($\Delta kshA1$, $\Delta kshA2$, $\Delta kshA3$, $\Delta kshB$), double mutants ($\Delta kshA1,2$, $\Delta kshA2,3$, $\Delta kshA1,3$) and the triple mutant $\Delta kshA1,2,3$ were obtained for this work. None of these mutants were impaired to growth in rich medium or showed growth differences in minimal medium supplemented with sodium acetate.

3.5. Growth of *R. ruber* strain *chol-4* $\Delta kshA$ and $\Delta kshB$ strains in different media

The capability of the *R. ruber* mutants to grow on different steroid

media was checked by cultivating them in 457 DSMZ minimal medium supplemented with the desired carbon source (Fig. A.1). Steroids were prepared in 16 mM cyclodextrins as indicated in Section 2.1 (*R. ruber* was not able to grow in cyclodextrins as single carbon source). Table 2 shows the data obtained after a 48 h growth of the wild type and the mutant strain cultures.

These results show that KshA1 is the only KshAB enzyme involved in the cholic acid catabolism while it also participates in the degradation of other substrates used in this study. On the other hand, KshA2 is mainly involved in the catabolism of AD, ADD, testosterone and DHEA, while KshA3 is exclusively related to the cholesterol, cholestenone and phytosterol degradation.

Growing behaviour of the *R. ruber* wild type and *kshAB* mutant strains on molecules most related to the cholesterol catabolic pathway was studied deeper. Growth curves of these strains are shown in Fig. 5.

Maximum absorbance at 600 nm and doubling time of cultures of *R. ruber* wild type (WT) and *kshAB* mutants on different substrates are shown in the table. ng: no growth

The growth parameters of the $\Delta kshA2,3$ strain growing in cholesterol are similar to those of the wild type (Fig. 5), but its doubling time in AD or in ADD is 10 times that of the wild type (22 h versus 2.2 h) and the maximum absorbance at 600 nm reached when growing in ADD is a 33% lower than the maximum got by the wild type (2.0 versus 3.0, Fig. 5). It seems that KshA1 works very well in the cholesterol degradation pathway and rather worse in the AD or ADD catabolism. KshA3 shows a more strict behaviour, as the $\Delta kshA1,2$ strain can only grow on cholesterol and phytosterol substrates, but not in AD or ADD; its growing parameters on cholesterol are the same as those of the wild type strain, suggesting that KshA3 is mainly devoted to long chain steroid degradation. When KshA3 is lacking, KshA1 helps to do the work.

The presence of just the KshA2 activity in the $\Delta kshA1,3$ *R. ruber* strain gave growth parameters in cholesterol similar to those obtained from the *R. ruber* $\Delta kshA1,2,3$ or from the $\Delta kshB$ strain (a maximum absorbance at 600 nm of 0.3 unit and more than 15 h of doubling time), suggesting that cholesterol is not a direct substrate of the KshA2 activity. The small growth detected when some mutants grow in cholesterol can be attributed to the degradation of the side chain of the substrate molecule: growth can occur until the side chain is completely gone (see later).

On the other hand, the only presence in the cell of the KshA2 activity in the $\Delta kshA1,3$ strain is enough to obtain growth parameters on AD or ADD similar to those of the wild type. It can be concluded that the growth in AD and ADD is mainly mediated by KshA2 activity in *R. ruber* although KshA1 activity can also contribute as we have mentioned earlier.

R. ruber strains devoid of any KshAB activity ($\Delta kshB$ or $\Delta kshA1,2,3$ strains) were able to grow in cholesterol although 10 times slower than the wild type strain (Fig. 5). An impaired growth on cholesterol has been also described for *R. rhodochrous* RG32 mutant (devoid of their five KshAs) [19,53]. This growth is due to the degradation of the side chain, a process that has been described to occur at different steps of the pathway being independent of the polycyclic ring opening (Fig. A.2) [3,52]. Therefore, in these strains the side chain degradation process does not depend on any KshAB activity.

But there are some opposite evidences in other actinobacteria: a relationship between KshAB activity and the degradation of the side chain of cholesterol has been proposed in *R. equi* and *R. erythropolis* [23,54]. Even more, the physiological substrates of KshAB enzymes are described to be CoA thioester derivatives of cholesterol degradation intermediates with a long side chain in *Mycobacterium* [52] although this does not apply to all the *R. rhodochrous* KshA homologues [48]. The dependence or not of the side chain degradation on the KshAB activities could be strain specific.

Table 2Growth of *R. ruber* wild type (WT) and *kshAB* mutants on different substrates after 48 h of culture.

Strain	KshA Remaining	Carbon source									
		CHO	AD	ADD	CHOL	TES	DHEA	PHY	CN	PREG	AC
		1.6 mM	1.6 mM	2.2 mM	2.2 mM	2.2 mM	2.2 mM	0.6 mg/mL	2.2 mM	2.2 mM	2 mM
$\Delta kshA1,2$	KshA3	++	–	–	–	–	–	++	++	++	++
$\Delta kshA1,3$	KshA2	+	++	++	–	++	++	+	+	++	++
$\Delta kshA2,3$	KshA1	++	+	+	++	–	++	++	++	++	++
$\Delta kshA1,2,3$	None	+	–	–	–	–	–	+	+	++	++
$\Delta kshB$	KshA1,2,3	+	–	–	–	–	–	+	+	++	++
WT	KshA1,2,3	++	++	++	++	++	++	++	++	++	++

CHOL: cholic acid; TES: testosterone; DHEA: dehydroepiandrosterone; PHY: phytosterols; CN: cholestenone; PREG: pregnenolone; AC: sodium acetate. +: $0 < A_{600nm} < 0.3$; ++: $A_{600nm} > 0.3$.

3.6. Genetic complementation of *R. ruber* strain *chol-4* mutants

R. ruber $\Delta kshA1,2,3$, devoid of the three KshA activities, is totally unable to use many compounds as the only carbon and energy source (as AD and cholic acid) and is severely impaired to grow using CHO. This strain was successfully complemented with pNV119-derived pNVS recombinant plasmids harbouring a functional copy of *kshA1*, *kshA2* or *kshA3* *R. ruber* genes. Fig. 6 depicts how mutant cells transformed with the three pNVS-derived recombinant plasmids recovered or not the ability to grow in some particular steroid carbon sources, confirming the data of mutant growing experiments.

Complementation data of the $\Delta kshA1,2,3$ strain suggested that KshA2 is only able to act on substrates with a short side chain (AD, ADD, testosterone, DHEA) in *R. ruber*. On the other hand, KshA3 can only grow using cholesterol as carbon source.

KshA1 is essential for *R. ruber* to grow in cholic acid but it can also substitute KshA3 in the cholesterol degradation. KshA1 isoform may have a broader range of substrates as *R. ruber* $\Delta kshA2,3$ strain can also grow in ADD and AD although with a doubling time 10 times higher than when KshA2 is the only KshAB active in the cell. Surprisingly, complementation of the $\Delta kshA1,2,3$ strain with KshA1 perfectly restores growth in CHO or cholic acid but not in AD, ADD or DHEA, whereas the $\Delta kshA2,3$ could do it to certain point (Figs. 5 and 6). It could be possible that the growth on these substrates has some regulation requirements that the above complementation does not fulfil.

Petrusma et al. proposed that each KshAB phylogenetic branch could contain enzymes functionally related with a similar spectrum of substrates [19,24]. This is confirmed by the involvement in the cholate metabolism of *R. ruber* KshA1, *R. rhodochrous* KshA1 (ADY18310) and *R. jostii* RHA1KshA (ABG97588), all of them belonging to the same branch (see Fig. 3) [19,42,43,53].

R. rhodochrous and *R. jostii* RHA1 contain up to five different KshAs and, consistently, the spectrum of their actions differs from that of *R. ruber* that only contains three KshAs. It has been reported in *R. rhodochrous* that KshA1 is involved in the cholic acid metabolism; KshA2 is involved in the degradation of steroids with none or a short side chain; KshA3 is only involved in the CHO metabolism; KshA4 is involved in the degradation of steroids without any side chain; and lastly, KshA5 is a broad specificity isoform [19].

Surprisingly, while the branches dedicated to cholic acid and CHO contain just one KshA isoform of *R. rhodochrous*, the branch related to AD metabolism (Fig. 3) contains the rest of the isoforms of this bacterium. In general, the AD and cholate branches contain more KshA isoforms than the CHO branch (Fig. 3).

R. ruber KshA2 is an isoform related to the AD, testosterone and DHEA metabolism. This isoform keeps only a 67% aa identity with a KshA from RHA1 (67% aa identity: ABG94295) but the genomic surroundings of both strains are similar (Fig. 2).

Any of *R. ruber* KshA1 or KshA3 was able to restore the growth on cholesterol when expressed in the *R. ruber* $\Delta kshA1,2,3$ strain, while

KshA2 restores the growth on AD. *R. ruber* KshA2 shows a substrate preference for steroids with a short side chain (AD, ADD). In *R. erythropolis* SQ1, KshA2 has been suggested to prevent the accumulation of ADD to reach toxic levels during sterol catabolism [27]. On the other side, KshA2 from *R. rhodochrous* was able to restore the growth on cholesterol in *R. rhodochrous* RG32 mutant (devoid of their five KshAs). The physiological roles of the KshA homologues are clearly strain-dependent.

3.7. Monitoring of degradation intermediaries by TLC

KstD and KshAB are two key enzymes involved in the steroid ring opening and therefore their gene deletions should block this opening to yield 9OHAD or ADD, respectively (Fig. A.2), two C19-steroid intermediaries that have drawn great attention from both the basic and applied (industrial) research [24,55,56].

R. ruber wild type strain and *kshAB* knockout mutants grown on several steroid compounds were used to analyse possible catabolism intermediaries. The harvested resting cells were chloroform extracted and analysed by TLC as described in section 2.5.

Data from cultures in AD are shown in Fig. 7AB. A similar accumulation of ADD was observed in cultures of all *kshAB* mutants, but not in the wild type cells. This result was the same in samples taken from both, cell cultures and resting cell assays. In cells with faint or null KshAB activity, KstD transformed AD into ADD which accumulated and was clearly detected (Fig. 7AB).

This result is consistent with the data of culture growth of those *kshA* and *kshB* mutants that cannot grow on AD ($\Delta kshB$, $\Delta kshA1,2$ and $\Delta kshA1,2,3$) and even in the case of the $\Delta kshA2,3$ strain, able to grow in AD although with a very high doubling time (Fig. 5). However, the ADD accumulation detected in cultures of the $\Delta kshA1,3$ strain, that grows in AD almost as well as the wild type does (Fig. 5) was unexpected. KshA2 is the major activity in the AD/ADD hydroxylation process and seems to be somehow helped in this task by KshA1. The absence of KshA1 may reduce the production of hydroxylated molecules (what would lead to the accumulation of ADD) with no meaningful effect on the growing rate.

Two more conclusions can be drawn from these results: i) the ADD accumulation, at least to the level reached in these assays, is not a serious problem for the cell growth; and ii) the KstD activities work more efficiently than the KshA2 B activity, as in samples of $\Delta kshA1,3$ cells growing in AD no trace of 9OHAD is detected, while the accumulated ADD remained in the cell even after 48 h (Fig. 7A). It should be noted that 9OHAD was however detected in a *R. ruber* *kstD* mutant [16] and also when the chimeric KshAB (KshA from *Mycobacterium smegmatis* mc2 and KshB from *Gordonia neofelifaecis* NRRL B-59395) was heterologously expressed in the AD producer strain *Mycobacterium neoaurum* NRRL B-3805 [44,57].

TLC intermediary analysis in cells grown in cholesterol provide with other suggestions. In cultures of *R. rhodochrous* RG32 mutant (devoid of

Strain	KshA remaining	CHO 1.6 mM		ADD 2.2 mM		AD 2.2 mM	
		Amax 600 nm	DT (hours)	Amax 600 nm	DT (hours)	Amax 600 nm	DT (hours)
WT	KshA1,2,3	2.0 ± 0.1	4.7 ± 0.4	3.00 ± 0.10	2.2 ± 0.0	2.2 ± 0.0	2.1 ± 0.1
$\Delta kshA1,3$	KshA2	0.3 ± 0.0	15.1 ± 0.7	3.3 ± 0.1	1.8 ± 0.1	2.1 ± 0.1	3.5 ± 0.4
$\Delta kshA2,3$	KshA1	1.9 ± 0.2	3.9 ± 0.6	2.0 ± 0.5	21.9 ± 3.8	2.2 ± 0.0	19.8 ± 0.6
$\Delta kshA1,2$	KshA3	2.1 ± 0.1	4.1 ± 0.6	ng	ng	ng	ng
$\Delta kshA1,2,3$	none	0.3 ± 0.0	16.1 ± 1.2	ng	ng	ng	ng
$\Delta kshB$	KshA1,2,3	0.3 ± 0.0	18.9 ± 1.3	ng	ng	ng	ng

Fig. 5. Growth curves of *R. ruber* wild type (WT) and mutant strains in minimal medium supplemented with different steroids as only carbon source. A representative experiment of 3–4 replicas is shown. The standard error of the mean was calculated by GraphPad Prism 5.0. The $\Delta kshA1,2,3$ strain growth curve on cholesterol follows the same pattern as those of $\Delta kshA1,3$ or $\Delta kshB$ strains in panel B. $\Delta kshA1,2$ strain did not grow in AD or ADD.

its five *kshA* genes) ADD and $\Delta^{1,4}$ -BNC have been identified as intermediaries of the process [19,53]. A similar result has been reported from *R. equi* *kshB* mutant [23]. Our results with *R. ruber* Chol-4 $\Delta kshA1,2,3$ and $\Delta kshA1,3$ strains show that they can hardly grow on cholesterol (Fig. 5), what we firstly explained by the low efficient degradation of the carbon side chain of the molecule, a process that therefore would be independent of the KshAB action. However, bio-transformation assays with both the wild type and these *R. ruber* mutant cells growing in cholesterol yielded no significant traces of ADD or 9OHAD spots in TLC (Fig. 7C). The absence of intermediaries of the cholate catabolism was reported after the GC–MS analysis of cultures of a *R. jostii* Rha1 *kshA* mutant [43].

These data lead to think that, owing to the different alternative combinations of isoforms, particular diversifications of the catabolic pathway may be possible among different strains. It has been recently proposed that sterols can be catabolized in *R. equi* USA-18 through two different routes (via AD or via $\Delta^{1,4}$ -BNC) that converge in 9OHADD [23]. Alternative pathways that do not pass through AD have been also suggested in the testosterone catabolism of *Steroidobacter denitrificans* [58]. In all those cases the degradation pathways would converge into ADD or 9OHAD intermediaries, although a preference for steroid catabolism via 9OHAD has been suggested in *Rhodococcus* [27] and *Mycobacterium* [5].

In the light of the above opinions, the AD/ADD branch mediated by

Fig. 6. Complementation of the $\Delta kshA1,2,3$ strain with the pNVS vectors (pNV119-derived recombinant plasmids harbouring a functional copy of each *R. ruber kshA* ORFs). Complementation with KshA1 restore the growth on cholesterol and cholic acid; with KshA2, growth on AD, ADD, cholesterol and DHEA was restored; complementation with KshA3 only restore the growth on cholesterol.

Fig. 7. Thin-layer chromatography-based monitoring of steroid degradation intermediaries. A. TLC of samples from Chol-4 cultures growing in AD. B, C. Biotransformation studies done on *R. ruber* strain Chol-4 WT and mutants on AD (panel B) and cholesterol (panel C).

KshA2 in *R. ruber* Chol-4 may not be the main pathway of the cholesterol degradation in this strain. In this point attention must be focused on the KshA3 activity, whose only presence allows a good growing of *R. ruber* in cholesterol (Fig. 5). KshA3-based pathway could be an alternative to the AD/ADD branch in the cholesterol catabolism of *R. ruber*, that would by-pass AD and ADD intermediaries. TLC spots are hardly visualised in cholesterol-grown resting cells only in the case of the $\Delta ksh1,3$ strain, where KshA3 is lacking and KshA2 is present (Fig. 7C). The absence of TLC spots when analysing cultures of wild type *R. ruber* grown in cholesterol suggests that the degradation of this molecule occurs mainly through the alternative KshA3 pathway. By contrast, AD/9OHAD (both easily detectable by TLC) have been proved to be intermediaries from cholesterol degradation in a *kstD* mutant of *M. smegmatis* [59], supporting the previous suggestion about different and particular alternative ways to catabolize cholesterol in bacteria.

3.8. Conclusions

In conclusion, in this study a collection of *kshA* and *kshB* mutants have been constructed in *R. ruber* and the role of these activities in the growth behaviour in different substrates have been discussed. KshA2 subunit is the main responsible of steroid substrates with short side chain while KshA3 subunit is specific for steroids with longer side chains. KshA1 activity, being necessary for cholic acid degradation, also helps to the other KshA3 and KshA2 isoforms in their action. These studies have also suggested that the side chain degradation during the steroid catabolism is independent of the KshAB activities in *R. ruber* in the same way that has been described for other *Rhodococcus* strains [23,53]. A cholesterol degradation pathway alternative to the AD/ADD branch in *R. ruber* and mainly based on the KshA3 and KshA1 activities is being subject of further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jsmb.2017.06.011>.

References

- [1] L.H. Bergstrand, E. Cardenas, J. Holert, J.D. Van Hamme, W.W. Mohn, Delineation of steroid-Degrading microorganisms through comparative genomic analysis, *mBio* 7 (2) (2016) e00166–16.
- [2] H.N. Bhatti, R.A. Khera, Biological transformations of steroidal compounds: a review, *Steroids* 77 (12) (2012) 1267–1290.
- [3] M.V. Donova, O.V. Egorova, Microbial steroid transformations: current state and prospects, *Appl. Microbiol. Biotechnol.* 94 (6) (2012) 1423–1447.
- [4] K.C. Yam, S. Okamoto, Adventures in *Rhodococcus* – from steroids to explosives, *Can. J. Microbiol.* 57 (3) (2011) 155–168.
- [5] K. Yao, L.Q. Xu, F.Q. Wang, D.Z. Wei, Characterization and engineering of 3-ketosteroid-9 α -hydroxylase and 3-ketosteroid-9 α -hydroxylase in *Mycobacterium neoaurum* ATCC 25795 to produce 9 α -hydroxy-4-androstene-3,17-dione through the catabolism of sterols, *Metab. Eng.* 24 (2014) 181–191.
- [6] J.L. García, I. Uhía, B. Galán, Catabolism and biotechnological applications of cholesterol degrading bacteria, *Microb. Biotechnol.* 5 (6) (2012) 679–699.
- [7] R. van der Geize, K. Yam, T. Heuser, M.H. Wilbrink, H. Hara, M.C. Anderton, E. Sim, L. Dijkhuizen, J.E. Davies, W.W. Mohn, L.D. Eltis, A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages, *Proc. Natl. Acad. Sci. U.S.A.* 104 (6) (2007) 1947–1952.
- [8] E. García-Fernández, D.J. Frank, B. Galán, P.M. Kells, L.M. Podust, J.L. García, P.R. Ortiz de Montellano, A highly conserved mycobacterial cholesterol catabolic pathway, *Environ. Microbiol.* 15 (8) (2013) 2342–2359.
- [9] N.M. Nesbitt, X. Yang, P. Fontan, I. Kolesnikova, I. Smith, N.S. Sampson, E. Dubnau, A thiolase of *Mycobacterium tuberculosis* is required for virulence and production of androstenedione and androstadienedione from cholesterol, *Infect. Immun.* 78 (1) (2010) 275–282.
- [10] K.J. McLean, M. Hans, A.W. Munro, Cholesterol, an essential molecule: diverse roles involving cytochrome P450 enzymes, *Biochem. Soc. Trans.* 40 (3) (2012) 587–593.
- [11] N. Doukyu, Characteristics and biotechnological applications of microbial cholesterol oxidases, *Appl. Microbiol. Biotechnol.* 83 (5) (2009) 825–837.
- [12] A. Vrielink, S. Ghisla, Cholesterol oxidase: biochemistry and structural features, *FEBS J.* 276 (23) (2009) 6826–6843.
- [13] L. Fernández de las Heras, J. Perera, J.M. Navarro Llorens, Cholesterol to cholesterol oxidation by ChoG, the main extracellular cholesterol oxidase of *Rhodococcus ruber* strain Chol-4, *J. Steroid Biochem. Mol. Biol.* 139 (2014) 33–44.
- [14] R. van der Geize, G.I. Hessels, R. van Gerwen, J.W. Vrijbloed, P. van der Meijden, L. Dijkhuizen, Targeted disruption of the *kstD* gene encoding a 3-ketosteroid- Δ^1 -dehydrogenase isoenzyme of *Rhodococcus erythropolis* strain SQ1, *Appl. Environ. Microbiol.* 66 (5) (2000) 2029–2036.
- [15] R. van der Geize, G.I. Hessels, L. Dijkhuizen, Molecular and functional characterization of the *kstD2* gene of *Rhodococcus erythropolis* SQ1 encoding a second 3-ketosteroid Δ^1 -dehydrogenase isoenzyme, *Microbiology* 148 (10) (2002) 3285–3292.
- [16] L. Fernández de las Heras, R. van der Geize, O. Drzyzga, J. Perera, J. Maria Navarro Llorens, Molecular characterization of three 3-ketosteroid- Δ^1 -dehydrogenase isoenzymes of *Rhodococcus ruber* strain chol-4, *J. Steroid Biochem. Mol. Biol.* 132 (3–5) (2012) 271–281.
- [17] T.V. Ivashina, V.M. Nikolayeva, D.V. Dovbnya, M.V. Donova, Cholesterol oxidase ChoD is not a critical enzyme accounting for oxidation of sterols to 3-keto-4-ene steroids in fast-growing *Mycobacterium* sp. VKM Ac-1815D, *J. Steroid Biochem. Mol. Biol.* 129 (1–2) (2012) 47–53.
- [18] I. Uhía, B. Galán, S.L. Kendall, N.G. Stoker, J.L. García, Cholesterol metabolism in *Mycobacterium smegmatis*, *Environ. Microbiol. Rep.* 4 (2) (2012) 168–182.
- [19] M. Petrusma, G. Hessels, L. Dijkhuizen, R. van der Geize, Multiplicity of 3-Ketosteroid-9 α -hydroxylase enzymes in *Rhodococcus rhodochrous* DSM43269 for specific degradation of different classes of steroids, *J. Bacteriol.* 193 (15) (2011) 3931–3940.
- [20] M. Petrusma, L. Dijkhuizen, R. van der Geize, Structural features in the KshA terminal oxygenase protein that determine substrate preference of 3-ketosteroid 9 α -hydroxylase enzymes, *J. Bacteriol.* 194 (1) (2012) 115–121.
- [21] C. Dresen, L.Y. Lin, I. D'Angelo, E.I. Tocheva, N. Strynadka, L.D. Eltis, A flavin-dependent monooxygenase from *Mycobacterium tuberculosis* involved in cholesterol catabolism, *J. Biol. Chem.* 285 (29) (2010) 22264–22275.
- [22] P.H. Wang, T.H. Lee, W. Ismail, C.Y. Tsai, C.W. Lin, Y.W. Tsai, Y.R. Chiang, An oxygenase-independent cholesterol catabolic pathway operates under oxic conditions, *PLoS One* 8 (6) (2013) e66675.
- [23] C.H. Yeh, Y.S. Kuo, C.M. Chang, W.H. Liu, M.L. Sheu, M. Meng, Deletion of the gene encoding the reductase component of 3-ketosteroid 9 α -hydroxylase in *Rhodococcus equi* USA-18 disrupts sterol catabolism, leading to the accumulation of 3-oxo-23, 24-bisnorchola-1, 4-dien-22-oic acid and 1, 4-androstadiene-3, 17-dione, *Microb. Cell Fact.* 13 (2014) 130.
- [24] M. Petrusma, R. van der Geize, L. Dijkhuizen, 3-Ketosteroid 9 α -hydroxylase enzymes: rieske non-heme monooxygenases essential for bacterial steroid degradation, *Antonie Van Leeuwenhoek* 106 (1) (2014) 157–172.
- [25] S.M. Barry, G.L. Challis, Mechanism and catalytic diversity of rieske non-heme iron-Dependent oxygenases, *ACS Catal.* 3 (10) (2013).
- [26] A.K. Pandey, C.M. Sasseti, Mycobacterial persistence requires the utilization of host cholesterol, *Proc. Natl. Acad. Sci. U. S. A.* 105 (11) (2008) 4376–4380.
- [27] R. van der Geize, G.I. Hessels, M. Nienhuis-Kuiper, L. Dijkhuizen, Characterization of a second *Rhodococcus erythropolis* SQ1 3-ketosteroid 9 α -hydroxylase activity comprising a terminal oxygenase homologue, KshA2, active with oxygenase-reductase component KshB, *Appl. Environ. Microbiol.* 74 (23) (2008) 7197–7203.
- [28] M. Horinouchi, T. Kurita, T. Hayashi, T. Kudo, Steroid degradation genes in *Comamonas testosteroni* TA441: Isolation of genes encoding a $\Delta^4(5)$ -isomerase and 3 α - and 3 β -dehydrogenases and evidence for a 100 kb steroid degradation gene hot spot, *J. Steroid Biochem. Mol. Biol.* 122 (4) (2010) 253–263.
- [29] W.W. Mohn, R. van der Geize, G.R. Stewart, S. Okamoto, J. Liu, L. Dijkhuizen, L.D. Eltis, The actinobacterial *mecA* locus encodes a steroid transporter, *J. Biol. Chem.* 283 (51) (2008) 35368–35374.
- [30] H. Venkataraman, E.M. Te Poele, K.Z. Rosloniec, N. Vermeulen, J.N. Commandeur, R. van der Geize, L. Dijkhuizen, Biosynthesis of a steroid metabolite by an engineered *Rhodococcus erythropolis* strain expressing a mutant cytochrome P450 BM3 enzyme, *Appl. Microbiol. Biotechnol.* 99 (11) (2015) 4713–4721.
- [31] L. Fernández de las Heras, E. García Fernández, J. María Navarro Llorens, J. Perera, O. Drzyzga, Morphological, physiological, and molecular characterization of a newly isolated steroid-degrading actinomycete, identified as *Rhodococcus ruber* strain Chol-4, *Curr. Microbiol.* 59 (5) (2009) 548–553.
- [32] L. Fernández de las Heras, V. Mascaraque, E. García Fernández, J.M. Navarro-Llorens, J. Perera, O. Drzyzga, ChoG is the main inducible extracellular cholesterol oxidase of *Rhodococcus* sp. strain CECT3014, *Microbiol. Res.* 166 (5) (2011) 403–418.
- [33] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, N.Y., 1989.
- [34] T.A. Hall, BioEdit a user-friendly biological sequence alignment editor and analysis

- program for windows 95/98/NT, Nucl. Acids Symp. Ser. 41 (1999) 95–98.
- [35] L. Fernández de las Heras, S. Alonso, A. de la Vega de Leon, D. Xavier, J. Perera, J.M. Navarro Ilorens, Draft genome sequence of the steroid degrader *Rhodococcus ruber* strain chol-4, Genome Announc. 1 (3) (2013).
- [36] R.K. Aziz, D. Bartels, A.A. Best, M. DeJongh, T. Disz, R.A. Edwards, K. Formsma, S. Gerdes, E.M. Glass, M. Kubal, et al. The RAST Server: rapid annotations using subsystems technology, BMC Genomics 9 (2008) 75.
- [37] J. Yang, R. Yan, A. Roy, D. Xu, J. Poisson, Y. Zhang, The I-TASSER Suite: protein structure and function prediction, Nat. Methods 12 (1) (2015) 7–8.
- [38] G. Yachdav, E. Kloppmann, L. Kajan, M. Hecht, T. Goldberg, T. Hamp, P. Honigschmid, A. Schafferhans, M. Roos, M. Bernhofer, et al., PredictProtein—an open resource for online prediction of protein structural and functional features, Nucleic Acids Res. 42 (Web Server issue) (2014) W337–W343.
- [39] J.S. Papadopoulos, R. Agarwala, COBALT: constraint-based alignment tool for multiple protein sequences, Bioinformatics 23 (9) (2007) 1073–1079.
- [40] R. van der Geize, G.I. Hessels, R. van Gerwen, P. van der Meijden, L. Dijkhuizen, Unmarked gene deletion mutagenesis of kstD, encoding 3-ketosteroid Delta1-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using sacB as counter-selectable marker, FEMS Microbiol. Lett. 205 (2) (2001) 197–202.
- [41] A. Schafer, A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, A. Puhler, Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*, Gene 145 (1) (1994) 69–73.
- [42] J.M. Mathieu, W.W. Mohn, L.D. Eltis, J.C. LeBlanc, G.R. Stewart, C. Dresen, K. Okamoto, P.J. Alvarez, 7-ketocholesterol catabolism by *Rhodococcus jostii* RHA1, Appl. Environ. Microbiol. 76 (1) (2010) 352–355.
- [43] W.W. Mohn, M.H. Wilbrink, I. Casabon, G.R. Stewart, J. Liu, R. van der Geize, L.D. Eltis, Gene cluster encoding cholate catabolism in *Rhodococcus* spp, J. Bacteriol. 194 (24) (2012) 6712–6719.
- [44] J. Yuan, G. Chen, S. Cheng, F. Ge, W. Qiong, W. Li, J. Li, Sheng Wu, Gong Cheng, Xue Bao, [Accumulation of 9alpha-hydroxy-4-androstene-3 17-dione by co-expressing kshA and kshB encoding component of 3-ketosteroid-9alpha-hydroxylase in *Mycobacterium* sp. NRRL B-3805], Chin. J. Biotechnol. 31 (4) (2015) 523–533.
- [45] Y. Hu, R. van der Geize, G.S. Besra, S.S. Gurcha, A. Liu, M. Rohde, M. Singh, A. Coates, 3-Ketosteroid 9alpha-hydroxylase is an essential factor in the pathogenesis of *Mycobacterium tuberculosis*, Mol. Microbiol. 75 (1) (2010) 107–121.
- [46] V.Y. Shtratnikova, M.I. Schelkunov, V.V. Fokina, Y.A. Pekov, T. Ivashina, M.V. Donova, Genome-wide bioinformatics analysis of steroid metabolism-associated genes in *Nocardioideis simplex* VKM Ac-2033D, Curr. Genet. 62 (2016) 643–656.
- [47] J.K. Capyk, I. D'Angelo, N.C. Strynadka, L.D. Eltis, Characterization of 3-ketosteroid 9(alpha)-hydroxylase, a rieske oxygenase in the cholesterol degradation pathway of *Mycobacterium tuberculosis*, J. Biol. Chem. 284 (15) (2009) 9937–9946.
- [48] J.S. Penfield, L.J. Worrall, N.C. Strynadka, L.D. Eltis, Substrate specificities and conformational flexibility of 3-ketosteroid 9alpha-hydroxylases, J. Biol. Chem. 289 (37) (2014) 25523–25536.
- [49] A. Pinto, M. Tarasev, D.P. Ballou, Substitutions of the bridging aspartate 178 result in profound changes in the reactivity of the Rieske center of phthalate dioxygenase, Biochemistry 45 (30) (2006) 9032–9041.
- [50] M. Tarasev, A. Pinto, D. Kim, S.J. Elliott, D.P. Ballou, The bridging aspartate 178 in phthalate dioxygenase facilitates interactions between the Rieske center and the iron(II)–mononuclear center, Biochemistry 45 (34) (2006) 10208–10216.
- [51] R.E. Parales, J.V. Parales, D.T. Gibson, Aspartate 205 in the catalytic domain of naphthalene dioxygenase is essential for activity, J. Bacteriol. 181 (6) (1999) 1831–1837.
- [52] J.K. Capyk, I. Casabon, R. Gruninger, N.C. Strynadka, L.D. Eltis, Activity of 3-ketosteroid 9alpha-hydroxylase (KshAB) indicates cholesterol side chain and ring degradation occur simultaneously in *Mycobacterium tuberculosis*, J. Biol. Chem. 286 (47) (2011) 40717–40724.
- [53] M.H. Wilbrink, M. Petrusma, L. Dijkhuizen, R. van der Geize, FadD19 of *Rhodococcus rhodochrous* DSM43269, a steroid-coenzyme A ligase essential for degradation of C-24 branched sterol side chains, Appl. Environ. Microbiol. 77 (13) (2011) 4455–4464.
- [54] R. van der Geize, G.I. Hessels, R. van Gerwen, P. van der Meijden, L. Dijkhuizen, Molecular and functional characterization of kshA and kshB, encoding two components of 3-ketosteroid 9alpha-hydroxylase, a class IA monooxygenase, in *Rhodococcus erythropolis* strain SQ1, Mol. Microbiol. 45 (4) (2002) 1007–1018.
- [55] P. Fernandes, A. Cruz, B. Angelova, H.M. Pinheiro, J.M.S. Cabral, Microbial conversion of steroid compounds: recent developments, Enzyme Microb. Technol. 32 (2003) 688–705.
- [56] F.Q. Wang, K. Yao, D.Z. Wei, From soybean phytosterols to steroid hormones, in: Hany El-Shemy (Ed.), Soybean Health, InTech—Open Access Publisher, Rijeka, 2011, pp. 232–252, <http://dx.doi.org/10.5772/18808>.
- [57] A. Rodríguez-García, E. Fernández-Alegre, A. Morales, A. Sola-Landa, J. Lorraine, S. Macdonald, D. Dovbnya, M.C. Smith, M. Donova, C. Barreiro, Complete genome sequence of '*Mycobacterium neoaurum*' NRRL B-3805, an androstenedione (AD) producer for industrial biotransformation of sterols, J. Biotechnol. 224 (2016) 64–65.
- [58] Y.R. Chiang, J.Y. Fang, W. Ismail, P.H. Wang, Initial steps in anoxic testosterone degradation by *Steroidobacter denitrificans*, Microbiology 156 (Pt 7) (2010) 2253–2259.
- [59] A. Brzostek, T. Sliwinski, A. Rumijowska-Galewicz, M. Korycka-Machala, J. Dziadek, Identification and targeted disruption of the gene encoding the main 3-ketosteroid dehydrogenase in *Mycobacterium smegmatis*, Microbiology 151 (Pt 7) (2005) 2393–2402.