Steroid Metabolism In Thermophilic Actinobacteria *Saccharopolyspora Hirsuta* Subsp. *Hirsuta* VKM Ac-666^T

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Abstract

Application of thermophile microorganisms opens new prospects in steroid biotechnology, however little is known on steroid catabolism by the thermophile strains.

The thermophilic *Saccharopolyspora hirsuta* subsp. *hirsuta* strain VKM Ac-666^T is capable of structural modification of different steroids, and fully degrades cholesterol. The intermediates of the cholesterol degradation pathway were identified as cholest-4-en-3-one, cholesta-1,4-dien-3-one, 26-hydroxycholest-4-en-3-one, 3-oxo-cholest-4-en-26-oic acid, 3-oxo-cholesta-1,4-dien-26-oic acid, 26-hydroxycholesterol, 3 β -hydroxy-cholest-5-en-26-oic acid by MS, and H¹- and C¹³-NMR analyses. The data evidence sterol degradation by the strain occurs simultaneously through the aliphatic side chain hydroxylation at C26 and the A-ring modification that are putatively catalyzed by cytochrome P450 monooxygenase CYP125 and cholesterol oxidase, respectively.

The genes orthologous to those related to the sterol side chain degradation, steroid core rings A/B and C/D disruption and the steroid uptake were revealed. Most of the genes related to steroid degradation are grouped in three clusters. The sets of the genes putatively involved in steroid catabolism and peculiarities of their organization in *S. hirsuta* are discussed.

Despite steroids abundance in the environments, the ability to degrade them is not widespread among thermophilic bacteria as follows from the bioinformatic analysis of 52 publicly available genomes. Only seven candidate strains were revealed to possess the key genes related to the only known 9(10)-*seco* pathway of steroid degradation.

The results contribute to the knowledge on diversity of microbial steroid degraders, the features of sterol catabolism by thermophilic actinobacteria and could be useful for application in the pharmaceutical and environmental biotechnology.

Introduction

Sterols (e.g., cholesterol, ergosterol, phytosterols) are steroid 3 β -alcohols with the alkyl side chain consisting of 8–10 carbon atoms. In vertebrates, the bile acids and other bioactive steroids such as corticosteroids, sex hormones and vitamin D originate from cholesterol *via* structural modification of the steroid core and decomposition of the aliphatic side chain. Structurally, bile acids differ from sterols by cis-A/B-ring juncture, α -orientation of hydroxyl at C3, saturated steroid core and a C5 acyl side chain.

Due to the unique lipophilic/amphiphilic properties, steroidal compounds play vital functions in all living organisms. Annually large amounts of sterols, bile acids, natural and synthetic steroids enter into the environment *via* the decay of biomass or excretion by humans and animals. Modern bioinformatics studies of publicly available genomes/metagenomes highlighted the global distribution of actinobacteria capable of sterol and cholate degradation from different ecological niches (soil, aquatic environments, wastes, etc.) (Bergstrand et al. 2016; Holert et al. 2018). Currently, the so-called 9(10)-*seco*-steroid

pathway is the only known for the sterol and cholate degradation by actinobacteria (Philipp 2011; Donova and Egorova 2012; Olivera and Luengo 2019; Giorgi et al. 2019). Moreover, most strains degrade sterols and cholates *via* 3-keto-1,4-diene structures that are conditioned by the consecutive oxidations of 3-hydroxy group and introduction of the corresponding double bond(s) into the steroid core.

Actually, degradation of sterols (such as cholesterol, or phytosterols) by actinobacteria is in the focus due to its exclusive role in pathogenicity of *Mycobacterium tuberculosis*, as well as application of the non-pathogenic actinobacterial species and engineered strains in biotechnology for production of the value-added steroids for the pharmaceutical industry, biomedicine and veterinary.

Sterol catabolism is a multi-step process that included cascade reactions of the side chain degradation and decomposition of the steroid core rings A/B and C/D. This pathway was reported to be controlled by two TetR-type transcriptional repressors, KstR and KstR2 (Kendall et al. 2007, 2010; Uhía et al. 2011, 2012). Cholesterol catabolism has been intensively studied in mycolic acid-containing actinobacteria comprising pathogenic species: *M. tuberculosis* (Griffin et al. 2011), *Rhodococcus* strains (Van der Geize et al. 2007; McLeod et al. 2006), as well as in the non-pathogenic species such as *Mycolicibacterium smegmatis* mc2155 (Uhía et al. 2012), *Gordonia cholesterolivorans* (Drzyzga et al. 2011), and also in the not-containing mycolic acid bacterium *Nocardioides simplex* (Shtratnikova et al. 2020). Aerobic cholate degradation has been mainly studied for *Rhodococcus* strains (e.g. *R. jostii* RHA1) (Mohn et al. 2012) and gammaproteobacteria *Pseudomonas* sp. strain Chol1 (Philipp et al. 2006).

Noteworthy, the molecular mechanisms of steroid catabolism have been studied mainly for mesophilic actinobacteria species, while little is known on the features of thermophilic actinobacteria capable of steroid oxidation. Meanwhile, application of thermophilic strains for steroid bioconversion is of great interest since it may provide economically feasible biotechnologies due to decrease the production costs for the bioreactor cooling, especially in the countries with hot climate. Besides, higher steroid solubility at the elevated temperatures is favorable for bioprocess performance.

Thermophilic microorganisms and their enzymes are widely used in the production of food products, detergents, the pulp and paper, textile and mining industries (Gallo et al. 2021). The impressive example is a Taq-polymerase (named after *Thermus aquaticus*) that is indispensable for PCR techniques in medicine and biology (Ishino and Ishino 2014). However, despite of steroid abundancy in the environments, little is known on structural modifications of steroid compounds by thermophilic bacteria. An example for this is a conversion of progesterone by the moderately thermophilic bacterium *Bacillus thermoglucosidasius* (syn. *Parageobacillus thermoglucosidasius*) resulting in 6 α -/6 β -hydroxy-derivatives, as well as androstenedione and testosterone (Sideso et al. 1998). Another thermophilic bacterium was reported to perform reduction of the progesterone C20 carbonyl group to 20 α - or 20 β -hydroxy group along with the hydroxylation at C6 (Smith et al. 1992). Regio- and stereospecific reduction of the 3-keto group as well as the Δ^4 -double bond in various steroid ketones of the androstane and pregnane series was carried out by the extremely thermophilic bacterium *Calderiella acidophila* (Sodano et al. 1982). The

strain of *Geobacillus kaustophilus* hydroxylated progesterone and testosterone (Al-Tamimi et al. 2010). However, the data on sterol catabolism in thermophilic bacteria are scarce (Holert et al. 2018).

The moderately thermophilic *Saccharopolyspora hirsuta* subsp. *hirsuta* VKM Ac-666^T originated from the sugarcane bagasse (Lacey and Goodfellow 1975) is able to transform lithocholic acid (Kollarov et al. 2013) and steroid compounds (dehydroepiandrosterone, androstenedione, 3 β ,7(α / β)-dihydroxy-5-ene-D-homo-lactones) (Lobastova et al. 2019). Recently, the Ac-666^T whole genome was sequenced and its preliminary bioinformatics analysis has been performed (Lobastova et al. 2020).

In this work, cholesterol degradation by *S. hirsuta* VKM Ac-666^T was studied and the main intermediates were identified. The set of the genes putatively involved in sterol metabolism and cholate catabolism pathways, as well as their organization and clustering were revealed. The presence of the genes coding for the key enzymes accounting for steroid degradation was estimated in the genomes of thermophilic bacteria of different taxa and potent microbial steroid degraders were predicted that might function at the elevated temperatures.

Materials And Methods

Materials

Androst-4-ene-3,17-dione (AD) and testosterone (T) were obtained from Sigma-Aldrich (USA); androsta-1,4-diene-3,17-dione (ADD) and 1(2)-dehydrotestosterone (DT) – from Steraloids (USA), cholesterol (Serva, Germany), cholestenone – from Maybridge (England), malt extract for microbiology and corn steep solids were obtained from Sigma-Aldrich (USA), randomly methylated β -cyclodextrin (MCD) – from Wacker-Chemie GmbH (Germany), soluble starch and yeast extract – from Difco (USA). Other materials and solvents were of analytical grade and purchased from domestic commercial suppliers.

Microorganism

The strain *Saccharopolyspora hirsuta* subsp. *hirsuta* VKM Ac-666^T was obtained from the All-Russian Collection of Microorganisms (VKM).

Microorganism cultivation and cholesterol conversion

The strain was cultivated on the medium GSMY (Park et al. 2005) containing (g/L): glucose – 7, soluble starch – 10, malt extract – 5, yeast extract – 4.5, CaCO₃ – 0.05 (pH 7.0-7.2) aerobically (200 rpm) at 45°C for 48 h. The seed culture (5 ml) was added into 750-ml shake flasks containing 50 ml GSMY medium. Cultivation was carried out aerobically on a rotary shaker (200 rpm) at 45°C for 24 h. After 24 h of cultivation, aqueous solution of cholesterol (0.5 g/l) with MCD (8.9 g/l) was added aseptically and cultivation continued at the same conditions for 144 h. The experiments were performed in triplicates.

Steroid metabolite isolation and identification

After 48 and 144 h of cholesterol conversion, the biomass was separated from the broth (500 mL) by centrifuge (8000× g, for 30 min). Isolation and purification of the steroid intermediates and products were performed as described earlier (Shtratnikova et al. 2020). HPLC analyses were performed using reversed-phase HPLC on Agilent Infinity 1200 system (Agilent Technologies, Germany SA) with Symmetry column (250 × 4.6 mm, 5 µm) with precolumn Symmetry C18 (5 µm, 3.9 x 20 mm) (Waters, USA) at 50°C and flow rate 1 ml/min. For assays of steroids two mobile phases (acetonitrile:water:acetic acid (60:40:0.01 v/v/v/) and acetonitrile: 2-propanol: water (50:45:5, v/v/v)) with UV-detection at 200 nm (for compounds with 3β-ol-5-ene configuration) and 240 nm (for compounds with 3-oxo-4-ene configuration) were used. MS spectra of II, III and IV were recorded on a tandem mass spectrometer LCQ Advantage MAX (Thermo Finnigan, USA) in positive ions $[M+H]^+$, at evaporator temperature 350°C, capillar – 170°C. MS/MS spectra were obtained using normalized collision energy (Normolized Collision Energy™) ranging from 20 to 40%. Collection and processing of data were performed using the Xcalibur software. HRMS experiments for V, VI, VII, VIII were performed with Orbitrap Elite mass-spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) with an ESI source.

^1H - and ^{13}C -NMR spectra were recorded at 400 and 100.6 MHz respectively with a Bruker Avance 400 spectrometer. Chemical shifts were measured relative to solvent signal. Only characteristic signals in ^1H -NMR of steroids are given.

Cholesterol bioconversion intermediates characteristics:

Cholest-4-en-3-one (II) M.w. 384; Rt (mobile phase acetonitrile:2-propanol:water 50:45:5 v/v/v/, λ 240 nm) 8.9 min; MS (intensity,%) $[M + H]^+$: 385(100), 279(5), 226(9), 149(3), 109(4). ^1H -NMR (CDCl_3) δ: 5.73 (s, 1H, H-4), 1.18 (s, 3H, 19- CH_3), 0.92 (d, J = 6.7 Hz, 3H, 21- CH_3), 0.87 (d, J = 6.6 Hz, 3H, 26(27)- CH_3), 0.86 (d, J = 6.6 Hz, 3H, 26(27)- CH_3), 0.71 (s, 3H, 18- CH_3).

Cholesta-1,4-dien-3-one (III) M.w. 382; Rt (mobile phase acetonitrile:2-propanol:water 50:45:5 v/v/v/, λ 240 nm) 7.2 min; MS (intensity,%) $[M + H]^+$ (collision energy 33 eV): 383(90), 279(5), 365(55), 325(133), 271(15), 247(100), 175(40), 163(48), 135(11), 121(8).

26-Hydroxycholest-4-en-3-one (IV) M.w. 400; Rt (mobile phase acetonitrile:2-propanol:water 50:45:5 v/v/v/, λ 240 nm) 3.9 min; Rt (mobile phase acetonitrile:water:acetic acid (60:40:0.01 v/v/v/, λ 240 nm) 81.8 min; MS (intensity,%) $[M + H]^+$ (collision energy 33 eV): 401(100), 369(33).

3-Oxo-cholest-4-en-26-oic acid (V) M.w. 414; Rt (mobile phase acetonitrile:2-propanol:water 50:45:5 v/v/v/, λ 240 nm) 3.7 min; Rt (mobile phase acetonitrile:water:acetic acid (60:40:0.01 v/v/v/, λ 240 nm) 50.9 min; HRMS-ESI (m/z): $[M-H]^+$ calcd for $\text{C}_{27}\text{H}_{41}\text{O}_3$ 413,3056; found 413,3059. ^1H -NMR (CDCl_3) δ: 5.73 (br. s., 1H, 4-H), 1.18 (s, 3H, 19- CH_3), 1.17 (d, J = 7.0 Hz, 3H, 27- CH_3), 0.91 (d, J = 6.5 Hz, 3H, 21- CH_3), 0.70 (s, 3H, 18- CH_3). ^{13}C -NMR (CDCl_3) δ: 199.9 (C-3), 182.3 (C-26), 171.9 (C-5), 123.7 (C-4), 56.0, 55.8, 53.8, 42.4, 39.6, 39.2, 38.6, 35.63, 35.56, 33.92, 33.86, 32.9, 32.0, 28.1, 24.1, 23.6, 21.0, 18.5, 17.3, 16.7, 11.9.

3-Oxo-cholesta-1,4-dien-26-oic acid (VI) M.w. 412; Rt (mobile phase acetonitrile:2-propanol:water 50:45:5 v/v/v/, λ 240 nm) 3.3 min; Rt (mobile phase acetonitrile:water:acetic acid (60:40:0.01 v/v/v/, λ 240 nm) 32.2 min; HRMS-ESI (m/z): $[M-H]^+$ calcd for $C_{27}H_{39}O_3$ 411,2899; found 411, 2903. 1H -NMR ($CDCl_3$) δ : 7.06 (d, J = 10.1 Hz, 1H, 1-H), 6.24 (dd, J = 1.9, 10.1 Hz, 1H, 2-H), 6.08 (br. s., 1H, 4-H), 1.23 (s, 3H, 19-CH₃), 1.17 (d, J = 7.0 Hz, 3H, 27-CH₃), 0.91 (d, J = 6.5 Hz, 3H, 21-CH₃), 0.73 (s, 3H, 18-CH₃). ^{13}C -NMR ($CDCl_3$) δ : 186.6 (C-3), 182.3 (C-26), 169.8 (C-5), 156.3 (C-1), 127.4 (C-2), 123.7 (C-4), 56.0, 55.4, 52.3, 43.7, 42.6, 39.4, 39.2, 35.6, 35.5, 35.4, 33.9, 33.7, 32.9, 28.1, 24.4, 23.6, 22.8, 18.6, 18.5, 16.7, 12.0.

26-Hydroxycholesterol (cholest-5-ene-3 β ,26-diol) (VII) M.w. 402; Rt (mobile phase acetonitrile:2-propanol:water 50:45:5 v/v/v/, λ 200 nm) 3.9 min; Rt (mobile phase acetonitrile:water:acetic acid (60:40:0.01 v/v/v/, λ 200 nm) 78.9 min; HRMS-ESI (m/z): $[M-H]^+$ calcd for $C_{27}H_{45}O_2$ 401,3420; found 401,3415. 1H -NMR ($CDCl_3$) δ : 5.36 (br. s., 1H, 6-H), 3.53 (m, 1H, 3 α -H), 3.51 (dd, J = 6.0, 10.6 Hz, 1H, CH₂OH), 3.43 (dd, J = 6.4, 10.6 Hz, 1H, CH₂OH), 1.01 (s, 3H, 19-CH₃), 0.92 (d, J = 6.5 Hz, 3H, 21-CH₃), 0.91 (d, J = 6.7 Hz, 3H, 27-CH₃), 0.68 (s, 3H, 18-CH₃). ^{13}C -NMR ($CDCl_3$) δ : 140.8 (C-5), 121.7 (C-6), 71.8 (C-3), 68.5 (C-26), 56.8, 56.1, 50.1, 42.32, 42.28, 39.8, 37.2, 36.5, 36.1, 35.8, 35.7, 33.5, 31.9, 31.7, 28.2, 24.3, 23.4, 21.1, 19.4, 18.7, 16.5, 11.9.

3 β -Hydroxy-cholest-5-en-26-oic acid (VIII) M.w. 416; Rt (mobile phase acetonitrile:2-propanol:water 50:45:5 v/v/v/, λ 200 nm) 3.6 min; Rt (mobile phase acetonitrile:water:acetic acid (60:40:0.01 v/v/v/, λ 200 nm) 45.9 min; HRMS-ESI (m/z): $[M-H]^+$ calcd for $C_{27}H_{43}O_3$ 415,3212; found 415,3217. 1H -NMR (CD_3OD) δ : 5.33 (d, J = 5.2 Hz, 1H, 6-H), 3.39 (m, 1H, 3 α -H), 1.12 (d, J = 7.0 Hz, 3H, 27-CH₃), 1.01 (s, 3H, 19-CH₃), 0.93 (d, J = 6.5 Hz, 3H, 21-CH₃), 0.71 (s, 3H, 18-CH₃). ^{13}C -NMR (CD_3OD) δ : 180.8 (C-26), 142.2 (C-5), 122.5 (C-6), 72.4 (C-3), 58.2, 57.5, 51.7, 43.5, 43.0, 41.2, 40.7, 38.6, 37.7, 37.0, 35.4, 33.3, 33.0, 32.3, 29.3, 25.3, 24.8, 22.2, 19.9, 19.2, 17.6, 12.4.

Genome analysis

Annotation of the genome was carried out using NCBI PGAP (Tatusova et al. 2016), RAST (<http://rast.nmpdr.org/>) (Aziz et al. 2008; Overbeek et al. 2014) and KAAS (<https://www.genome.jp/tools/kaas/>) (Moriya et al. 2007). Orthologous and paralogous relations between the genes of the *S. hirsuta* subsp. *hirsuta* VKM Ac-666^T, *Mycobacterium tuberculosis* H37Rv and *Rhodococcus jostii* RHA1 genomes were found using OrthoFinder 2.5.1 (Emms and Kelly 2015, 2019) with inflation parameter 1.5. Additional analysis was performed using BLAST search (Altschul et al. 1990) against the non-redundant protein database. Reciprocal BLAST was used in several cases for searching genes which corresponded to the known genes one-to-one. Conservative domains in the protein sequences were determined using a CDD database (<https://www.ncbi.nlm.nih.gov/cdd/>) (Lu et al. 2020). The pairwise similarity between the gene and protein sequences was determined using TaxonDC 1.3.1 (Tarlachkov and Starodumova 2017).

BLAST search for steroid catabolism genes

Search for the key genes of the steroid catabolic 9,10-*seco*-pathway: *kstD* and *kshAB* was carried out against several dozen available genomes of thermophilic strains using the BLAST + program (Camacho et al. 2009). The protein sequences of KstD (NP_218054.1) and KshA (NP_218043.1), KshAB (NP_218088.1) of *M. tuberculosis* H37Rv were used as the reference ones. The list of bacteria to be screened (Supplementary Table S1) was compiled on the basis of the literature data (Shivlata and Satyanarayana 2015) on having complete genomes or annotated contigs thermophilic and thermotolerant actinobacteria, and from available sources on other known thermophilic bacteria of diverse phylogenetic positions.

The complete genomes of *Geobacillus kaustophilus* and *Parageobacillus thermoglucosidasius* strains capable of performing some modifications of steroid compounds were screened for the steroid catabolism genes (Supplementary Table S2) using the BLAST + program (Camacho et al. 2009).

Results

Cholesterol bioconversion

As shown in Fig. 1, the *S. hirsuta* strain fully utilized cholesterol for 144 h.

Among the cholesterol bioconversion intermediates, the steroid compounds with both 3-oxo-4-ene and 3 β -hydroxy-5-ene moieties were isolated and their structures were characterized by HPLC, mass spectrometry and ¹H- and ¹³C-NMR-spectroscopy (Supplementary Figs. 1–30). A number of 3-keto-4-ene-steroids were detected in the broth, such as cholest-4-en-3-one (II), cholesta-1,4-dien-3-one (III), 26-hydroxycholest-4-en-3-one (IV), 3-oxo-cholest-4-en-26-oic acid (V), 3-oxo-cholesta-1,4-dien-26-oic acid (VI). Steroids with 3 β -hydroxy-5-ene moiety were identified as 26-hydroxycholest-5-en-3 β -ol (VII), 3 β -hydroxycholest-5-en-26-oic acid (VIII). The compound VIII was accumulated to give about 17 mol% for 144 h. No any steroids without lateral chain (C₁₉-steroids), or partially oxidized side chain (C₂₂- or C₂₄-steroids) were detected among the intermediates by TLC and HPLC.

Cholestenone (II) was observed as a major intermediate to reach a maximum of ~ 20 mol% for 24 h. Its abrupt decrease after 48 h was accompanied with rough accumulation of 3-oxo-cholest-4-en-26-oic acid (V) which reached highest level of ~ 17 mol% to 72 h and then drastically decreased to 2–3 mol% (Fig. 1b). 26-Hydroxycholesterol (cholest-5-ene-3 β ,26-diol) (VII) accumulated to achieve about 10 mol% during 72 h and then decreased. Its decrease after 72 h accompanied with the elevation in the rate of VIII (Fig. 1b). Minor amounts of cholesta-1,4-dien-3-one (III) were observed with maximum level less than 4 mol% for 24 h conversion. Noteworthy, the total steroid content after 144 h incubation was low, thus confirming degradation of the steroid core.

Based on the structures and the time-courses of the steroids detected, the following scheme of cholesterol bioconversion with *S. hirsuta* VKM Ac-666^T was proposed (Fig. 2).

General clustering of steroid catabolic gene homologs

When analyzing the genome of *S. hirsuta* (DDBJ/ENA/GenBank accession no. VWPH000000000) the genes putatively involved in steroid catabolism were mainly grouped into three clusters: cluster 1 (F1721_32550-F1721_33735), cluster 2 (F1721_00675-F1721_00760) and cluster 3 (F1721_28735-F1721_28770) and number of the genes were revealed outside the clusters (Fig. 3, Table S3).

Cluster 1 (Fig. 3) contains candidate genes related to the Mce4 system, namely orthologs of the transporter operon *mceABCDEF* and the genes coding for two permease subunits YrbEa and YrbEb; candidate genes related to a sterol side chain degradation pathway (*cyp125*, *fadD19*, *fadE26*, *fadEE27*, *echA19*, *hsd4A*, *fadA5*, *ltp3*, *ltp4*, *fadD17*, *fadE28*, *fadE29*, *hsd4A* and *ltp2*); and ring A/B oxidation (*kstD3*, *kshAB* and operon *hsaBCDAFGE*); and ring C/D degradation (*ipdAB*, *ipdC*, *fadE30*, *fadE31*, *fadE32*, *fadE33*), steroid delta-isomerase *ksdI* (Table S3).

A candidate gene encoding FadD3 lies alone in the Ac-666^T genome out of clusters. The genes *choD* and *choE* presumably encoding cholesterol oxidases were found out of clusters in Ac-666^T (Fig. 3, Table S3).

In the Ac-666^T genome, clusters 2 and 3 (Fig. 3) contain candidate genes related to the cholate degradation pathway, namely orthologs of *kshAB* subunits; two orthologs of *kstDs*: *kstD2* and *kstD1*; the A/B-rings opening operon *hsaEGF* and orthologs of *hsaD3* and *hsaB3*; steroid delta-isomerase *ksdI*; a predicted transcriptional regulator *kstR3*; an operon that contain orthologs of genes encoding degradation of the cholate side chain *casACEHI* (Table S3).

Figure 4 shows the proposed scheme of cholesterol bioconversion with the participation of the candidate genes of *S. hirsuta* VKM Ac-666^T.

BLAST search for the key enzymes of steroid catabolism in 52 thermophilic/thermotolerant strains

The key enzymes of steroid catabolism: KstD, KshA and KshB of *M. tuberculosis* H37Rv were used as reference enzymes in the BLAST search carried out against several dozens of publicly available genomes of the thermophilic bacteria of different phylogenetic positions, including the genomes of the thermophiles for which it has been reported that they catalyze some modifications of steroid compounds.

Seven actinobacterial strains, namely, *Thermomonospora curvata* DSM 43183 *Amycolatopsis granulosa* DSM 45669, *Amycolatopsis methanolica* strain 239T, *Amycolatopsis thermalba* strain 50.9b, *Thermocatellispora tengchongensis* DSM 45615, *Amycolatopsis ruanii* strain 49.3e, *Microbispora siamensis* NBRC 104113 of the 52 tested thermophilic and thermotolerant species were found to possess the proteins that are from 41.6 to 64.2% similar to the reference KstD and KshAB of *M. tuberculosis* H37Rv (Supplementary Table S4).

The complete genomes of two strains capable of performing some modifications of steroid compounds, - *Geobacillus kaustophilus*, *Parageobacillus thermoglucosidasius* were screened for the enzymes involved in steroid catabolism, namely, ChoD, ChoL, Ltp3-4, Hsd4A, FadE26-30, ChsH1-2, FadD17, FadD19, EchA19, HsaA-E, KstD, KshAB, IpdAB, FadD3, EchA20 (Supplementary Table S2). Most of the proteins were absent in these two strains (Supplementary Table S5). On the other hand, the products of separate genes showed similarity with the reference enzymes. The enzymes that are 47% and 45% similar to the reference FadA5 were revealed in *G. kaustophilus* and *P. thermoglucosidasius*, respectively; and the enzymes with 48% and 41% identity to HsaF and HsaE, respectively, were identified in *P. thermoglucosidasius* (Supplementary Table S5).

Discussion

Several thermophilic bacteria species have been reported to catalyze distinct reactions of steroid structural modifications, while sterol degradation by thermophilic microorganisms has not been studied so far. As shown in this research, the strain of *S. hirsuta* fully utilized cholesterol (Fig. 1), and the degradation pathway was predicted (Fig. 2) based on the time-courses of the intermediates (Fig. 1) and genome-wide bioinformatics analysis (Fig. 3).

The set and the order of the genes putatively involved in steroid catabolism in the clusters of *S. hirsuta* genome are similar to the clusters described for the reference actinobacteria: *M. tuberculosis* H37Rv and *R. jostii* RHA1 (Olivera and Luengo 2019) (Fig. 3). In general, cluster 1 in *S. hirsuta* is similar to the cluster of the sterol catabolic pathway in *M. tuberculosis* H37Rv (Fig. 3). The differences are that *hsa* genes in *S. hirsuta* are represented as a “complete” operon *hsaBCDAFGE*, while in *M. tuberculosis* H37Rv (as well as in *R. jostii* RHA1) the genes of this block are divided into two parts *hsaFGE* and *hsaBCDA* within the cluster genes (Fig. 3). The orthologs of *kshAB* in cluster 1 in the *S. hirsuta* genome locate close to each other (Fig. 3, Table S3); there is only one gene of an undefined function between them, in contrast to *kshA* and *kshB* from *M. tuberculosis* H37Rv, which are very far from each other in mycobacterial cluster of cholesterol catabolism (Fig. 3).

Together, clusters 2 and 3 of the *S. hirsuta* contain the vast majority of orthologs of the cholate cluster genes from *R. jostii* RHA1 participating in the bile acids rings A/B degradation, but not all orthologs of the genes from *R. jostii* RHA1 coding for the enzymes of the bile acids side chain degradation (Fig. 3, Table S3).

ChOs and 3-HSDs

In many actinobacteria, the sterol degradation pathway is known to begin with the modification of 3 β -hydroxy-5-ene into the 3-keto-4-ene structure of the ring A by the action of cholesterol oxidases (ChOs) or 3 β -hydroxydehydrogenases (3-HSDs) (Donova 2007; Yam et al. 2009), while cytochrome P450-mediated hydroxylation at C26(27) of the cholesterol side chain was reported to be initial reaction in the sterol degradation in *Rhodococcus* strains (Rostłoniec et al. 2009; Kreit 2017). As evidenced from the time course of cholesterol conversion by *S. hirsuta* the initial reactions of cholesterol degradation, i.e.

modification of 3 β -ol-5-ene-moiety of the steroid A-ring and hydroxylation of the sterol side chain at C26(27), occurred independently (Fig. 2). During first 24 h cholesterol (I) mainly transformed to cholestenone (II) and in a lesser extent – to 26-hydroxycholesterol (VII). Cholestenone (II) further subjected to the side chain hydroxylation at C26(27) to form the corresponding 3-keto-4-en-26-alcohol (IV). In turn, the 3 β -ol-5-ene moiety in 26-hydroxycholesterol (VII) modified to the corresponding 3-keto-4-ene also resulting in the compound (IV).

Cholesterol oxidases are most likely involved in the 3 β -hydroxyl group dehydrogenation and $\Delta^5 \rightarrow \Delta^4$ -isomerization (I $\rightarrow$ II, VII $\rightarrow$ IV) in *S. hirsuta* since no candidate genes responsible for the 3 β -hydroxysteroid dehydrogenase (3-HSD) were found in the Ac-666^T genome (Lobastova et al. 2020). Two candidate *cho* genes: *choD* F1721_14655 and *choE* F1721_09795 encoding the cholesterol oxidases were revealed in the genome and both are situated out of the clusters related to cholesterol catabolism in Ac-666^T. It corresponds to the literature data evidencing that in most cases the genes coding for cholesterol oxidases in actinobacteria located out of the clusters of the genes that involved in cholesterol catabolism (Kreit 2017).

No orthologs of 3 β -HSD were identified also in the genome of *N. simplex* VKM Ac-2033D, and highly efficient oxidation of different sterols (cholesterol, sitosterol, stigmasterol, campesterol) to the corresponding stenones (cholestenone, sitostenone, stigmastenone, campestenone) is attributed to the activity of cholesterol oxidase in this strain (Shtratnikova et al. 2021).

On the other hand, the presence of both cholesterol oxidases and 3 β -HSDs have been identified in the genomes of mycobacterial strains (Uhia et al. 2012; Bragin et al. 2013). The functionality and role of the enzymes can differ in different actinobacteria. As shown for *Mycolicibacterium neoaurum* ATCC 25795 (syn. *Mycobacterium neoaurum*), two cholesterol oxidases (ChoM1 and ChoM2) are accounting for the 3 β -ol-5-ene to 3-keto-4-ene modification and essential for the cell growth on cholesterol (Yao et al. 2013). In contrast, the knock-outs of the cholesterol oxidases ChoD (orthologous to ChoM1) in other relative mycobacteria (*M. neoaurum* VKM Ac-1815D, *M. smegmatis* mc² 155) did not block cholesterol to cholestenone oxidation (Uhia et al. 2011; Ivashina et al. 2012) thus evidencing the presence of other enzymes involved in 3 β -ol-5-ene to 3-keto-4-ene modification such as 3 β -HSDs.

Most of the actinobacterial ChOs are of the dual functions: catalyze both oxidation of the 3 β -hydroxy group and $\Delta^5 \rightarrow \Delta^4$ isomerization of 3 β -hydroxy-5-ene steroids. A separate Δ^5 -3-ketosteroid isomerase, encoded by the gene *ksi* in *Comamonas testosteroni* was shown to be responsible for the $\Delta^5 \rightarrow \Delta^4$ isomerization (Horinouchi et al. 2012). The genome of *S. hirsuta* contains two candidate genes *ksi* (*ksdI*). *KsdI* F1721_32675 is located between *cyp125* F1721_32680 and *ltp3-ltp4* F1721_32665-F1721_32660 in cluster 1, and another *ksdI* F1721_00740 is situated among the genes putatively involved in the A/B-rings oxidation: *kshB3* F1721_00735 and *hsaB3* F1721_00745 in cluster 2 (Fig. 3, Table S3). In the genome of *N. simplex*, only one of two *ksdI* genes, - KR76_23530 with an unclear function was up-regulated in the presence of phytosterol (Shtratnikova et al. 2021).

CYP 125

The cleavage of the cholesterol/cholestenone side chain by actinobacteria begins with hydroxylation of the terminal methyl group catalyzed by steroid 26(27)-monooxygenase to form the corresponding 26(27)-alcohols (Kreit 2017). As shown for *R. jostii* RHA1, the same enzyme is accounting for further oxidation to the corresponding carboxylic C26-oic acids (Rostöniec et al. 2009). Cytochrome P450 monooxygenases encoded by *cyp125* had been isolated and characterized from *M. tuberculosis* (Capyk et al. 2009b) and *R. jostii* RHA1 (Rostöniec et al. 2009). Cyp125 from the clinical strain *M. tuberculosis* CDC1551 was shown to be involved in the oxidation of 26-hydroxycholest-4-en-3-one to cholest-4-en-3-one-26-oic acid (Ouellet et al. 2010). The genes *cyp125*, *cyp142*, *cyp124* were reported to encode the enzymes performing terminal hydroxylation at C(26)27 (Kreit 2017).

The candidate gene *cyp125 F1721_32680* was identified in the *S. hirsuta* genome (Figs. 3, 4). The gene product could be responsible for the modifications to 26-hydroxycholestenone, 3-oxocholest-4-ene-26-oic acid, 3-oxocholesta-1,4-diene-26-oic acid, 26-hydroxycholesterol, and 3 β -hydroxycholest-5-ene-26-oic acid (Fig. 2) that have been identified among the intermediates/metabolites from cholesterol. Probably, this strain possesses a specific steroid 26(27)-monooxygenase capable of oxidizing the C27-sterol side chain regardless 3 β -hydroxy-5-ene- or 3-oxo-4-ene- (3-oxo-1,4-diene-) structure of the ring A. No orthologs of *cyp124*, or *cyp142* were identified in *S. hirsuta* genome.

Side chain degradation

As well-established for many actinobacteria, the aliphatic side chain of sterols is degraded through a cascade of reactions similar to the β -oxidation of fatty acids. The C26-oic acid is activated by the coenzyme A (CoA) and then the side chain is shortened with release of two propionyl-CoAs and one acetyl-CoA (Fig. 4).

The genes *chsE4 (fadE26)* and *chsE5 (fadE27)* were shown to encode acyl-CoA dehydrogenases forming a complex that oxidizes 3-oxo-cholest-4-ene-26-oyl-CoA (Yang et al. 2015). ChsE4-ChsE5 complex of *M. tuberculosis* H37Rv was shown to take on the function of the blocked ChsE1-ChsE2 enzymes. The strain also synthesized ChsE3 specifically catalyzing the oxidation of 3-oxochole-4-ene-24-oyl-CoA in the second round of β -oxidation of the cholesterol side chain (Yang et al. 2015). The orthologous genes, namely, *chsE1 (F1721_33645)*, *chsE2 (F1721_32785)*, *chsE3 (F1721_28750)*, *chsE4 (F1721_32605)* and *chsE5 (F1721_32610)* were found in the genome of *S. hirsuta* (Figs. 3, 4).

The steroid metabolite with the C5 carbon side chain is ligated further by the steroid-24-oyl-coenzyme A synthetase. The phylogenetic analysis of more than 70 acyl-CoA synthetases aimed on the elucidation of their physiological role revealed four different types of the acyl-CoA synthetases from *R. jostii* RHA1 and *M. tuberculosis* H37Rv which were specific to the chain length of steroids (Casabon et al. 2014). FadD19 from *M. tuberculosis* H37Rv activated cholesterol metabolites with the C8 steroid side chain, whilst FadD17 from *M. tuberculosis* H37Rv $\rightarrow$ the C5- or longer side chain, and CasG from *R. jostii* RHA1 $\rightarrow$ the C5 cholate side chain. The metabolites with the C3 side chain accumulated during the cholate

oxidation by *R. jostii* RHA1 were activated by the steroid-22-oyl-CoA synthetase *CasI* (Casabon et al. 2014). The orthologs of *fadD19* (F1721_32635), *fadD17* (F1721_32615), *casG* (F1721_02405) and *casI* (F1721_28770) encoding isofunctional acyl-coenzyme A synthases were revealed in the genome of *S. hirsuta* (Fig. 3). Probably, the presence of the homologous genes encoding various acyl-coenzyme A synthases of cholesterol and cholate catabolic pathways in *S. hirsuta* contributes to the adaptation of the thermophile microorganism in nature.

A special function of acyl-CoA synthetase *FadD19* was reported to consist in its participation in the degradation of C₂₄-branched sterols (sitosterol, stigmasterol, and others) as it was shown for *R. rhodochrous* (Wilbrink et al. 2011). The same function can be assumed for the *fadD19* ortholog in the *S. hirsuta* (Fig. 4).

As shown for *R. rhodochrous* RG32, the oxidative decomposition of the phytosterols with the branched β -sitosterol-like side chain is mediated by the aldol lyases encoded by *ltp3* and *ltp4* (Wilbrink et al. 2012). The candidate genes *ltp3* (F1721_32665) and *ltp4* (F1721_32660) putatively involved in the degradation of sterols with the C₂₄-branched side chain have been identified also in *S. hirsuta* (Figs. 3, 4).

The enoyl-coenzyme A hydratase encoded by *echA19* gene acts at the early stage of sterol side chain degradation (Van der Geize et al. 2007). The *Hsd4A* might act as a dehydrogenase at the early stage of the degradation of the unsubstituted sterol side chain, and as a 17 β -hydroxysteroid dehydrogenase at the last step of the side chain cleavage, or as a D-3-hydroxyacyl coenzyme A dehydrogenase at the branched fatty acids degradation (Van der Geize et al. 2007). The candidate gene *echA19* (F1721_32640) and two distant homologs of *hsd4A*: F1721_32600 and F1721_33680 were revealed in the *S. hirsuta* genome (Fig. 3). Interestingly, both *hsd4A* genes were detected in cluster 1; and each *hsd4A* gene is located at the beginning or at the end of a group of genes encoding the sterol aliphatic side chain cleavage in the cluster. Due to distant homology of two *Hsd4As* (coding by F1721_32600 and F1721_33680) with a.a. identity of the proteins about 49%, one can assume different substrate specificity for these *S. hirsuta* isoenzymes towards the steroids with distinct side chain length.

The role of thiolase *FadA5* at the last cycle of the cholesterol side chain β -oxidation was demonstrated for *M. tuberculosis* H37Rv (Schaefer et al. 2015). Orthologous *fadA5* (F1721_32685) is present in the genome of *S. hirsuta* (Fig. 3).

Steroid/lipid transport system

In *M. tuberculosis* strains the operon that contained the genes for a putative lipid transfer protein (*ltp2/Rv3540c*), 2 MaoC-like hydratases (*chsH1/Rv3541c* and *chsH2/Rv3542c*), 2 acyl-CoA dehydrogenases (*fadE29/chsE2/Rv3543c* and *fadE28/chsE1/Rv3544c*), and cytochrome P450 (*cyp125/Rv3545c*) had been reported to be essential for virulence (Thomas et al. 2011). The orthologous genes *ltp2* (F1721_32770), *fadE28/chsE1* (F1721_33645), *fadE29/chsE2* (F1721_32785), *chsH1* (F1721_32775) and *chsH2* (F1721_32780) were also found in the genome of *S. hirsuta* (Fig. 3). Unlike other actinobacteria, a transposon element (contig VVPH01000033.1 with length 1457 bp) is most likely

located between the *fadE28/chsE1* (F1721_33645) and *fadE29/chsE2* (F1721_32785) in the thermophile Ac-666^T.

Steroid nucleus degradation

The key reactions in the only known 9(10)-*seco* pathway of steroid core degradation are 1(2)-dehydrogenation and 9 α -hydroxylation (e.g. Donova and Egorova 2012). 1(2)-Dehydrogenation is known to be carried out by 3-ketosteroid Δ 1-dehydrogenases (KstDs) (Itagaki et al. 1990). The presence of several KstDs with distinct activities have been reported for actinobacteria species (Bragin et al. 2013; Zhang et al. 2015; Shtratnikova et al. 2016; Guevara et.al 2017; Zhang et al. 2018). In the genome of the Ac-666T strain three putative KstDs were identified (Fig. 3, Table S3). The candidate *kstD3* gene is situated in the cluster 1 (Fig. 3). Two other candidate *kstDs*, namely, *kstD2* and *kstD1* are located side by side in the cluster 2 (Fig. 3). As reported earlier, *S. hirsuta* effectively transformed androst-4-ene-3,17-dione (AD), 3 β -hydroxy-5-en-17-one (DHEA) and 3 β ,7(α / β)-dihydroxy-5-ene-D-homo-lactones into the corresponding 1(2)-dehydrogenated derivatives thus evidencing high KstD activity (Lobastova et al. 2019).

In the present study, detection of the intermediates with a 3-keto-1,4-diene structure such as cholesta-1,4-dien-3-one (III) and 3-oxo-cholesta-1,4-diene-26-oic acid (VI) evidenced that the 1(2)-dehydrogenation can take place at the early stages of sterol catabolism in *S. hirsuta* (Fig. 2). As shown for *M. neoaurum* DSM 1381, KstD1, KstD2, KstD3 catalyze 1(2)-dehydrogenation of various steroid substrates at different stages of sterol degradation. The *kstD1* was highly up-regulated in response to phytosterol, while recombinant KstD2 exhibited a higher enzymatic activity towards the substrates without, or with a short side chain such as AD, or 22-hydroxy-23,24-bisnorchol-4-en-3-one (Zhang et al. 2018). Probably, the presence of several KstDs might provide 1(2)-dehydrogenation of various steroids in *S. hirsuta*.

The *S. hirsuta* KstDs showed higher identity with the KstDs in *N. simplex* (syn. *Pimelobacter simplex*) as compared with the KstDs from mycolic-acid rich actinobacteria, - *M. tuberculosis* and *R. jostii* (Fig. 5). Highest level of the activity towards C₂₁-3-keto-steroids has been demonstrated earlier for *N. simplex* KstD2 encoded by *KR76_27125* (Shtratnikova et al. 2021) that is in close identity with *S. hirsuta* KstD2.

The 1(2)-dehydrogenase of *M. tuberculosis*, which is related to the pathway of cholesterol catabolism, is in the same clade with KstD3 from *S. hirsuta*, while *S. hirsuta* KstD1 is more similar to the corresponding enzymes in *N. simplex*.

The KstD4 from *S. hirsuta* locates in the same clade with KstD4 (TesI) from *N. simplex*.

It is well-known that the 9 α -hydroxylation is carried out by 3-ketosteroid 9 α -hydroxylase KshAB consisting of the oxygenase component (KshA), and the reductase component (KshB) (Capyk et al. 2009a). Five different paralogous genes were reported to encode the KshA subunits in *Mycolicibacterium fortuitum* VKM Ac-1817D (syn. *Mycobacterium* sp. VKM Ac-1817D) (Bragin et al. 2013), thus providing 9 α -hydroxylation of steroid metabolites at the different stages of sitosterol catabolism (Bragin et al. 2019).

Several KshAs with different substrate specificity have also been found in *R. rhodochrous* DSM 43269: KshA1 was shown to participate only in the cholic acid catabolism while KshA5 could hydroxylate several substrates (Petrusma et al. 2011). The number of the genes coding for the kshA or kshB subunits depends on the strain species (Bragin et al. 2013; Shtratnikova et al. 2016). Herein, two orthologs of *kshA* and two orthologs of *kshB* were revealed in the genome of *S. hirsuta* (Fig. 3). One pair of the genes (*kshA* F1721_32745, *kshB* F1721_32755) is located far from the second pair (*kshA* F1721_00725, *kshB* F1721_00735). Most likely, the two KshABs might differ on their substrate specificity in *S. hirsuta*.

It should be noted that no any C19-steroid intermediates such as AD, ADD, testosterone, or 1(2)-dehydrotestosterone were detected during the cholesterol transformation by *S. hirsuta*. It could be explained either by their rapid degradation when their concentrations are below the level of detection, or ring A/B disruption of the “earlier” intermediate steroids (with preserved side chain at C17). For instance, at the bile acid transformation with *Rhodococcus* strains, the 9,10-*seco*-steroid intermediates with the partially degraded side chains were formed, evidencing that side chain degradation and opening of the ring B occurred simultaneously (Costa et al. 2013a, b). The order of 9 α -hydroxylation and 1(2)-dehydrogenation of 3-oxo-4-ene-steroids resulting in the formation of the unstable 9 α -hydroxy-3-oxo-1,4-diene-intermediates depends on the substrate specificity of the corresponding enzymes.

Steroid core degradation

The next step of steroid core destruction is hydroxylation at C4 in the A ring of 3-hydroxy-9,10-*seco*-androst-1,3,5(10)-triene-9,17-dione (3 β HSA) by the flavin-dependent monooxygenase (HsaAB) resulting in the 3,4-dihydroxy-derivative (3,4-DHSA) (García et al. 2012). The detailed characterization of HsaAB was performed for the monooxygenase from *M. tuberculosis* (Dresen et al. 2010). The catabolic operon *hsaBCDAFGE* F1721_32700-F1721_32730 presumably involved in further degradation of the fragments of the ring A was identified in the genome of *S. hirsuta*. In addition, the candidate genes, namely, *hsaA3* (F1721_00755), *hsaB3* (F1721_00745), *hsaC3* (F1721_00760) and *hsaD3* (F1721_00695) orthologous to the *R. jostii* RHA1 *hsaA3B3C3D3* genes were found in the strain Ac-666^T (Fig. 3, Table S3). The candidate genes *hsaF* and *hsaG* have been revealed encoding HsaF and HsaG that putatively participate in the final stages of the ring A remnants degradation with formation of pyruvate and propionate (Figs. 3, 4).

Degradation of the C/D rings begins with the action of FadD3 which physiological role has been studied in *M. tuberculosis* (Casabon et al. 2013). Unlike mycolic acid rich *M. tuberculosis* H37Rv and *R. jostii* RHA1, as well as not-containing mycolic acids *N. simplex*, in which genomes *fadD3* encoding the HIP-CoA synthetase lies in the corresponding cluster, ortholog of *fadD3* in the *S. hirsuta* genome is located out of clusters (Fig. 3).

The *fadE30* gene encoding the acyl-CoA dehydrogenase was shown to be involved in dehydrogenation at C4 of 5-OH-HIP (Van der Geize et al. 2011). The intermediate with the intact rings C and D: 5-OH-HIC-CoA is produced by two reactions catalyzed by IpdC which introduces a double bond in the C ring and IpdF that oxidizes the 5-OH group in *M. tuberculosis*. Crotonase Ech20 is responsible for the hydrolytic

cleavage of the ring C to give HIEC-CoA. IpdAB encodes an enzyme that hydrolytically cleaves the C ring in the substrate COCHEA-CoA (Crowe et al. 2017). The candidate genes: *ipdAB* F1721_33690-F1721_33695, *ipdC* F1721_33700, *fadE30* F1721_33715, and *echA20* F1721_33685 found in the cluster 1 of the *S. hirsuta* genome represent the genes presumably involved in the degradation of the rings C and D (Fig. 3).

The product of the opening of both the C and D rings is subjected to the action of a putative thiolase FadA6 resulting in acetyl-CoA and 4-methyl-5-oxo-octanedioyl-CoA (Fig. 3) (Olivera and Luengo 2019). The last intermediate undergoes a β -oxidation with an acyl-CoA dehydrogenase FadE32, or by the Fad31-FadE32 complex in *Mycobacterium* (Crowe et al. 2017). Finally, the products of β -oxidation, an acetyl-CoA and 2-methyl- β -keto adipyl-CoA would be released, followed by the formation of propionyl-CoA and succinyl-CoA (Fig. 4). The orthologs of *fadE31* (F1721_33725), *fadE32* (F1721_33730), *fadE33* (F1721_33735) were detected in *S. hirsuta* genome (Fig. 3, Table S3).

Search for the key genes of steroid catabolism in the genomes of thermophilic/thermotolerant bacteria

In order to find out whether steroid degraders are widespread among the thermophile bacteria, the BLAST search for the key genes of the steroid catabolic 9,10-seco-pathway, – *kstD* and *kshAB* has been performed using 52 publicly available genomes of the thermophilic/thermotolerant strains. Only seven actinobacterial strains were proposed to be steroid degraders (Supplementary Table S4). The rest thermophilic/thermotolerant strains do not contain enzymes similar to the reference ones (*KstD* and *KshAB* of *M. tuberculosis* H37Rv) by more than 35% and, most likely do not degrade steroids.

The thermophilic strains of *G. kaustophilus* and *P. thermoglucosidasius* were reported to provide separate reactions of steroid modifications (Sideso et al. 1998; Al-Tamimi et al. 2010). The BLAST search of more than 20 enzymes related to steroid catabolism in these bacteria discovered putative proteins that are 47% and 45% similar to the reference FadA5, respectively, as well as enzymes of *P. thermoglucosidasius* that are similar to HsaF and HsaE of *M. tuberculosis* H37Rv by 48% and 41%, respectively (Supplementary Table S5). Taking into consideration that FadA5 is known to be involved also in fatty acid β -oxidation, the corresponding proteins in *G. kaustophilus* and *P. thermoglucosidasius* may not be intended for steroid catabolism. Products of *hsaE* and *hsaF* participate in the oxidation of a fragment of the steroid nucleus, which is a hydroxydiene-derivative of hexanoic acid, that means that the similar genes do not necessarily participate in the catabolism of steroid compounds. Taking into account the absence of other genes coding for steroid oxidation enzymes, most likely, these enzymes of *G. kaustophilus* and *P. thermoglucosidasius* are not associated with steroid catabolism, and the oxidation/reduction and hydroxylation reactions performed by the strains (Sideso et al. 1998; Al-Tamimi et al. 2010) are catalyzing by the enzymes that are non-specific towards steroids.

Conclusions

The thermophilic strain *Saccharopolyspora hirsuta* subsp. *hirsuta* VKM Ac-666^T is capable of effective metabolizing cholesterol and lithocholic acid, as well as transforming different exogenous steroids (Kollarov et al. 2013; Lobastova et al. 2019). As confirmed in this study, the strain completely degrades cholesterol, and the 26-alcohols both with 3 β -ol-5-ene and 3-keto-4-ene structures of the ring A are the key intermediates. The genes related to sterol metabolism and cholic acid catabolism were first identified in the genome of this thermophilic strain. The organization of the steroid catabolism genes is generally similar to that in other actinobacteria, with some differences related to the individual genes and their grouping. Future transcriptomic and proteomic studies are of significance for clearer understanding of the peculiarities of steroid catabolism in the thermophilic actinobacteria.

The presence of key enzymes accounting for steroid core disruption was identified only in seven of 52 thermophilic bacteria of various phylogenetic positions thus suggesting that steroid degrading activity is not common in the thermophilic species. The potent microbial degraders capable of steroid degradation under the elevated temperatures were determined.

The results contribute to the knowledge on the diversity of microbial steroid degraders, the features of steroid catabolism by the thermophilic actinobacteria and could be useful for application in the steroid pharmaceutical and environmental biotechnology.

Declarations

Conflicts of interest/Competing interests

The authors declare no conflict of interest in this work.

Availability of data and material

Not applicable.

Code availability

Not applicable.

Authors' contributions

T.G.L., V.V.F. and A.A.S. designed research and analyzed data. S.V.T. and E.Y.B. did bioinformatics analysis. V.V.F. and T.G.L. isolated steroid intermediates. A.V.K. performed the NMR measurements. M.V.D. administrated and coordinated the project. T.G.L., V.V.F. and M.V.D. wrote the manuscript. T.G.L., V.V.F., S.V.T. and A.A.S. drew figures. All authors have read and agreed to the published version of the manuscript.

Ethical approval

This study does not involve any human or animal subjects and followed all ethical standards of research.

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Figures

Figure 1

Time-courses of cholesterol (a) and the intermediates/metabolites (b) at the *S. hirsuta* VKM Ac-666T cholesterol bioconversion. I – Cholesterol (cholest-5-ene-3 β -ol), II - Cholest-4-en-3-one, III Cholesta-1,4-dien-3-one, IV – 26-Hydroxycholest-4-en-3-one, V - 3-Oxo-cholest-4-en-26-oic acid, VI - 3-Oxo-cholesta-1,4-dien-26-oic acid, VII - 26-Hydroxycholesterol (cholest-5-ene-3 β ,26-diol), VIII - 3 β -hydroxy-cholest-5-en-26-oic acid. The data are the average of triplicates

Figure 2

Scheme of the cholesterol bioconversion by *S. hirsuta* VKM Ac-666T. Compounds: I - Cholesterol, II - Cholest-4-en-3-one, III - Cholesta-1,4-dien-3-one, IV - 26-Hydroxycholest-4-en-3-one, V - 3-Oxo-cholest-4-en-26-oic acid, VI - 3-Oxo-cholesta-1,4-dien-26-oic acid, VII - 26-Hydroxycholesterol, VIII - 3β-Hydroxycholest-5-en-26-oic acid. Biochemical reactions: 1 - 3β-Hydroxyl group dehydrogenation and $\Delta^5 \rightarrow \Delta^4$ -isomerization; 2 - 3-Oxo-4-ene-steroids 1(2)-dehydrogenation; 3 - C26(27)-Hydroxylation; 4 - C26-Alcohol hydroxylation; 5 - Oxidative side-chain degradation

Mycobacterium tuberculosis H37Rv

Rhodococcus jostii RHA1

Saccharopolyspora hirsuta VKM Ac-666^T

Figure 3

Organization of the *S. hirsuta* genes encoding the 9,10-seco pathway involved in cholesterol and cholic acid catabolism. For comparison, the organization of the corresponding genes of *Mycobacterium tuberculosis* H37Rv and *Rhodococcus jostii* RHA1 is shown (Olivera and Luengo 2019). Genes encoding enzymes involved in cholesterol or bile acids side chain degradation are shown in green; genes participating in ring A/B degradation are in red for the enzymes from cholesterol metabolism and orange for cholic acid catabolism; in purple genes coding for ring C/D degradation are shown; blue color indicates genes coding for transport systems; regulatory elements are indicated in yellow

Figure 4

Proposed biochemical scheme of cholesterol catabolism in *S. hirsuta* VKM Ac-666T. Genes encoding respective proteins are denoted. (a) Modification of 3 β -ol-5-ene to 3-keto-4-ene moiety in A-ring of steroid core and degradation of the sterol side chain to C19-steroids; (b) Steroid core modifications; (c) Steroid core degradation via 9(10)-seco pathway. I - Cholesterol; II - Cholest-4-en-3-one; IV - 26-Hydroxy-cholest-4-en-3-one; V - 3-Oxo-cholest-4-en-26-oic acid; VII - Cholest-5-ene-3 β ,26-diol; VIII - 3 β -Hydroxy-cholest-5-en-26-

oic acid; IX - 3-Oxo-cholest-4-en-26-oyl-CoA; X - 3-Oxo-cholesta-4,24-dien-26-oyl-CoA; XI - 24-Hydroxy-3-oxo-cholest-4-en-26-oyl-CoA; XII - 3,24-Dioxo-cholest-4-en-26-oyl-CoA; XIII - 3-Oxo-chol-4-en-24-oyl-CoA; XIV - 3-Oxo-chola-4,22-dien-24-oyl-CoA; XV - 22-Hydroxy-3-oxo-chol-4-en-24-oyl-CoA; XVI - 3,22-Dioxo-chol-4-en-24-oyl-CoA; XVII - 3-Oxo-4-pregnene-20-carboxyl-CoA; XVIII - Androst-4-ene-3,17-dione (AD); XIX - Androsta-1,4-diene-3,17-dione (ADD); XX - 9 α -Hydroxy-AD; XXI - Unstable 9 α -hydroxy-ADD; XXII - 3 β -Hydroxy-9,10-seco-androsta-1,3,5(10)-triene-9,17-dione (3 β HSA); XXIII - 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA); XXIV - 4,5-9,10-Diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA); XXV - 2-Hydroxyhexa-2,4-dienoic acid (2-HHD); XXVI - 4-Hydroxy-2-oxohexanoic acid; XXVII - 9,17-Dioxo-1,2,3,4,10,19-hexanorandrost-5-oic acid (DOHNAA) or 3 α -H-4 α -(3'-propanoate)-7 β -methylhexahydro-1,5-indadione (HIP); XXVIII - 9,17-Dioxo-1,2,3,4,10,19-hexanorandrost-5-oyl-CoA (HIP-CoA); XXIX - 9-Hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-5-oic acid or 3 α -H-4 α -(3'-propanoate)-5 α -hydroxy-7 β -methylhexahydro-1-indanone (5-OH-HIP); XXX - 9-Hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-5-oyl-CoA (5-OH-HIP-CoA); XXXI - 9-Hydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-6-ene-5-oyl-CoA (HIPE-CoA); XXXII - 7,9-Dihydroxy-17-oxo-1,2,3,4,10,19-hexanorandrost-5-oyl-CoA; XXXIII - 9-Hydroxy-7,17-dioxo-1,2,3,4,10,19-hexanorandrost-5-oyl-CoA; XXXIV - 9-Hydroxy-17-oxo-1,2,3,4,5,6,10,19-octa-norandrost-7-oyl-CoA or 3 α -H-4 α -(carboxylCoA)-5 α -hydroxy-7 β -methylhexahydro-1-indanone (5-OH-HIC-CoA); XXXV - 9,17-dioxo-1,2,3,4,5,6,10,19-octa-norandrost-7-oyl-CoA; XXXVI - 9-hydroxy-17-oxo-1,2,3,4,5,6,10,19-octa-norandrost-8(14)-en-7-oyl-CoA; XXXVII - 9,17-Dioxo-1,2,3,4,5,6,10,19-octa-norandrost-8(14)-en-7-oyl-CoA or 7 α -Methyl-1,5-dioxo-2,3,5,6,7,7a-hexahydro-1H-indene-4-carboxylic acid (HIEC-CoA); XXXVIII - 9-Oxo-1,2,3,4,5,6,10,19-octanor-13,17-secoandrost-8(14)-ene-7,17-dioic acid-CoA-ester or (R)-2-(2-carboxyethyl)-3-methyl-6-oxocyclohex-1-ene-1-carboxyl-CoA (COCHEA-CoA); XXXIX - 6-Methyl-3,7-dioxo-decane-1,10-dioic acid-CoA ester; XL - 4-Methyl-5-oxo-octane-1,8-dioic acid-CoA ester; XLI - 4-Methyl-5-oxo-oct-2-ene-1,8-dioic acid-CoA ester (MOODA-CoA); XLII - 3-Hydroxy-4-methyl-5-oxo-octane-1,8-dioic acid-CoA ester; XLIII - 4-Methyl-3,5-dioxo-octane-1,8-dioic acid-CoA ester; XLIV - 2-Methyl-3-oxo-hexane-1,6-dioic acid-CoA ester; XLV - Succinyl-CoA; XLVI - Propionyl-CoA. Adopted from: (Van der Geize et al. 2007; Nesbitt et al. 2010; Thomas et al. 2011; Carere et al. 2013; Casabon et al. 2013; Yang et al. 2015; Crowe et al. 2017; Horinouchi et al. 2019; Stratnikova et al. 2021)

Figure 5

Dendrogram showing the phylogeny of KstD homologs

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