ISSN 0006-2979, Biochemistry (Moscow), 2023, Vol. 88, No. 9, pp. 1347-1355 © Pleiades Publishing, Ltd., 2023.
Published in Russian in Biokhimiya, 2023, Vol. 88, No. 9, pp. 1631-1641.

Expression of Synthetic *cyp102A1-LG23* Gene and Functional Analysis of Recombinant Cytochrome P450 BM3-LG23 in the Actinobacterium *Mycolicibacterium smegmatis*

Veronika Y. Poshekhontseva[1,a*], Nikolai I. Strizhov[1,b], Mikhail V. Karpov[1,c],
Vera M. Nikolaeva[1,d], Alexey V. Kazantsev[2,e], Olesya I. Sazonova[1,f],
Andrey A. Shutov[1,g], and Marina V. Donova[1,h]

[1]*Skryabin Institute of Biochemistry and Physiology of Microorganisms,*
Pushchino Center for Biological Research, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia
[2]*Faculty of Chemistry, Lomonosov Moscow State University, 119991 Moscow, Russia*
[a]*e-mail: rikahameleon@mail.ru*
[b]*e-mail: nicolai@strizhov.de*
[c]*e-mail: mikhail.v.karpov@mail.ru*
[d]*e-mail: nikolae_vera@rambler.ru*
[e]*e-mail: mak@org.chem.msu.ru*
[f]*e-mail: sazonova_oi@rambler.ru*
[g]*e-mail: w___w@rambler.ru*
[h]*e-mail: mv_donova@rambler.ru*

Received May 16, 2023
Revised June 15, 2023
Accepted June 16, 2023

Abstract—Cytochrome CYP102A1 (P450 BM3) of *Priestia megaterium* (bas. *Bacillus megaterium*) has several unique functional features and thus provides an ideal object for directed evolution and other synthetic applications. Previously, the CYP102A1-LG23 mutant with 14 mutations in the heme part was obtained that hydroxylates several androstanes at C7β with the formation of products with the anti-inflammatory and neuroprotective activities. In this study, synthetic *cyp102A1-LG23* gene encoding the P450 BM3 mutant was expressed as a component of either monocistronic operon or bicistronic operon containing the *gdh* (glucose dehydrogenase, GDH) or *zwf2* (glucose 6-phosphate dehydrogenase, G6PD) gene in *Mycolicibacterium smegmatis* BD cells. The recombinant bacteria were able hydroxylate androst-4-ene-3,17-dione (AD) into 7β-OH-AD. Their biocatalytic activity was increased twice by increasing the solubility of CYP102A1-LG23 protein in the cells and supplementing the cells with the additional cofactor regeneration system by introducing GDH and G6PD. The maximum 7β-OH-AD yield (37.68 mol%) was achieved by co-expression of *cyp102A1-LG23* and *gdh* genes in *M. smegmatis*. These results demonstrate the possibility of using synthetic genes to obtain recombinant enzymes and expand our understanding of the processes involved in steroid hydroxylation by bacterial cytochromes. The data obtained can be used to develop new approaches for microbiological production of 7β-hydroxylated steroids in genetically modified *Mycolicibacterium* species.

DOI: 10.1134/S0006297923090146

Keywords: cytochrome CYP102A1 (P450 BM3), heterologous expression, hydroxylation, 7β-hydroxyandrost-4-ene-3,17-dione, *Mycolicibacterium smegmatis*, bioconversion, steroids

---

Abbreviations: 7β-OH-AD, 7β-hydroxyandrost-4-ene-3,17-dione; AD, androst-4-ene-3,17-dione; G6PD, glucose 6-phosphate dehydrogenase; GDH, glucose dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate.

* To whom correspondence should be addressed.

INTRODUCTION

Steroid compounds exhibit a broad range of biological activities that account for their wide application in medicine. Steroids are used to treat inflammation, endocrine diseases, neurodegenerative disorders, certain cancers, etc. [1].

The biological activity of a steroid depends on the degree of oxidation of its cycloalkane core and the presence of functional oxygen-containing groups in the molecule. Hydroxylated steroids are commonly known to be more active than higher activity compared to their nonhydroxylated analogs. Hydroxyl groups attached to the cycloalkane core increase the polarity and solubility of the molecule and affect its toxicity and sorption properties. Regiospecific and stereospecific positioning of hydroxyl groups is important for steroid binding with the respective cell receptors triggering metabolic reaction cascades in the body [2-5].

Chemical synthesis of hydroxylated steroids often includes multiple steps and is difficult (if possible at all) to perform. Hydroxylation of inactive C–H bonds with the use of microorganisms or enzymes may help to solve the problem [1, 6]. One of the promising approaches involves construction and use of enzymes with regiospecific and stereospecific activities toward steroid compounds [7, 8].

Cytochrome P450 monooxygenases (P450s or CYPs) are of particular interest for gene and protein engineering, as they play a crucial role in oxidative functionalization of inactive carbon atoms. Cytochrome P450 BM3 (CYP102A1) of *Priestia megaterium* (bas. *Bacillus megaterium*) has several unique functional features and thus provides an ideal object for directed evolution and other synthetic applications. In contrast to most P450s, P450 BM3 is a water-soluble protein. Another important feature is bifunctionality ensured by the two-component structure of this protein, i.e., the natural fusion between the P450 monooxygenase domain and reductase domain. P450 BM3 is the fastest catalyst among all currently known P450s. Its turnover number reaches 17,000 m^{-1} (with arachidonic acid as a substrate) [9], which is several orders of magnitude higher than the turnover numbers of the majority of mammalian P450s [10]. The reason for such a high catalytic activity might be the presence of redox partners in the same protein molecule, which ensures rapid electron transfer from the flavin to the heme [11].

Natural P450 BM3 is not involved in steroid metabolism [12]. Several mutant variants of this protein have been obtained by directed evolution, including P450 BM3-LG23, which has 14 mutations in the heme part and hydroxylates several androstanes as steroid substrates [13].

Because actinobacteria of the genus *Mycolicibacterium* are capable of utilizing sterols (cholesterol and phytosterols) commonly used to produce therapeutic steroids and their precursors [14, 15]. The nonpathogenic fast-growing *Mycolicibacterium smegmatis* (syn. *Mycobacterium smegmatis*) strain mc^2 155 provides a convenient host to express heterologous steroidogenic genes because this strain contains an efficient system for the transport of exogenous steroid compounds and has a high transformation rate [16]. We have previously introduced deletions into *kshB* (3-ketosteroid Δ1-dehydrogenase) and *kstD* (reductase subunit of 3-ketosteroid 9α-hydroxylase) to prevent complete destruction of the cycloalkane core in steroids to obtain the mutant *M. smegmatis* BD strain that oxidized phytosterol and cholesterol into androst-4-ene-3,17-dione (AD) [15].

In this work, *M. smegmatis* BD was used to obtain recombinant strains expressing heterologous P450 BM3-LG23 and hydroxylating AD at C7β (Fig. 1). AD hydroxylation, a reaction that is required for oxidative functionalization of steroids, is one of the most difficult to perform artificially. Along with C7β hydroxylation, the hydroxyl group was introduced at C1β. Production of 1β-hydroxysteroids and 1β,7β-dihydroxysteroids with the use of mutant P450 BM3 variants has not been reported before.

Our approach can be used to construct other microbial catalysts based on CYP102A1-LG23; it opens a possibility of a one-step production of hydroxylated androstenes from phytosterol.

MATERIALS AND METHODS

Reagents. Acetamide (AcA) (Sigma-Aldrich, USA), AD (Steraloids, USA), agarose (Invitrogen, United Kingdom), methyl-β-cyclodextrin (MCD) (Wacker Chemie, Germany), ethidium bromide (Serva, Germany),

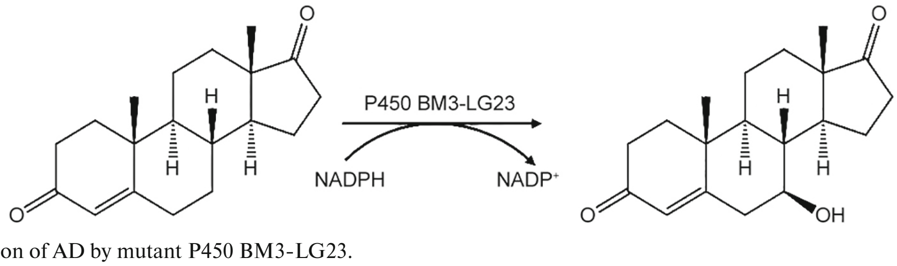


Fig. 1. C7β hydroxylation of AD by mutant P450 BM3-LG23.

Table 1. Primers, strains, and plasmids used in this work

Name	Description	Source
Strains		
Escherichia coli DH 5α	F^-, *endA1*, *glnV44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *deoR*, *nupG*, *purB20*, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ^-	Invitrogen
Mycolicibacterium smegmatis BD	mc^2 155 Δ(*kshB*, *kstD*), AD-producing mutant	[15]
Plasmids		
pMyNT	shuttle expression vector for replication in *E. coli* and mycobacteria; contains *Ami* promoter, sequence for N-terminal 6 × His-tag, TEV cleavage site, *Hyg*^{R}	[17]
pMyNTA	pMyNT derivative; contains *Nde*I restriction site	this work
pNS25	plasmid with optimized synthetic genes for type 2 G6PD of *M. tuberculosis* H37Rv (*zwf2*) and 17β-hydroxysteroid dehydrogenase of *Cohliobolus lunatus*	[18]
pSKLG23	pBluescript II SK(+) vector with optimized synthetic mutant variant (*cyp102A1-LG23*) of *cyp102A1* from *Bacillus megaterium* NBRC 15308	GenScript/ this work
pSKGDH	pBluescript II SK(+) vector with optimized synthetic gene for GDH from *Bacillus megaterium* AS1.223 (*gdh*)	GenScript/ this work
pNS38	pMyNT vector with *cyp102A1-LG23*	this work
pVP1	pMyNTA vector with *cyp102A1-LG23*	this work
pVP2	pMyNTA vector with bicistronic operon of *cyp102A1-LG23* and *gdh*	this work
pVP3	pMyNTA vector with bicistronic operon of *cyp102A1-LG23* and *zwf2*	this work
Primers		
NdeIlF	5'-cgataagagaaagggagtccaCATATGaagcaccaccaccac-caccacc-3'	this work
NdeIlR	5'-ggtggtggtggtggtggtgcttCATATGtggactccctttctctt-atcg-3'	this work
ClaIzwf2F	5'-tagacATCGAT**aggagg**taaaggcgatgaaaccggcccacg-ccgcggc-3'	this work
ClaIzwf2R	5'-gccatATCGATtcatggccgccgccattcccggc-3'	this work

Note. Restriction endonuclease recognition sites are with capital letters in the primers (*Nde*I – CATATG, *Cla*I – ATCGAT). The ribosome-binding site sequence (**aggagg**) is in bold.

kanamycin (BioKhim, Russia), hygromycin, ampicillin, glycerol, yeast extract, peptone, agar-agar (Panreac, Spain), tryptone (Dia-M, Russia), Tween-80 (Serva), DNA-modifying enzymes, DNA extraction kits (Thermo Fisher Scientific, USA), and silica gel (Fluka, USA) were used in the study. Other reagents were from Russian manufacturers and were of analytical grade.

Bacterial strains, plasmids, and cultivation conditions. Primers, bacterial strains, and plasmids used in this work are shown in Table 1.

Escherichia coli strain DH5α was used to clone the plasmid constructs. The bacteria were cultivated in LB medium at 37°C at 200 rpm [19]. Transformed cells were selected using kanamycin (50 µg/ml), hygromycin (50 µg/ml), or ampicillin (100 µg/ml).

Mycolicibacterium smegmatis BD cells were grown in M3 medium at 37°C at 200 rpm [15]. Transformed cells were selected using hygromycin (50-75 µg/ml).

Synthetic sequences and site-directed mutagenesis. Nucleotide sequences of the wild-type *cyp102A1* were

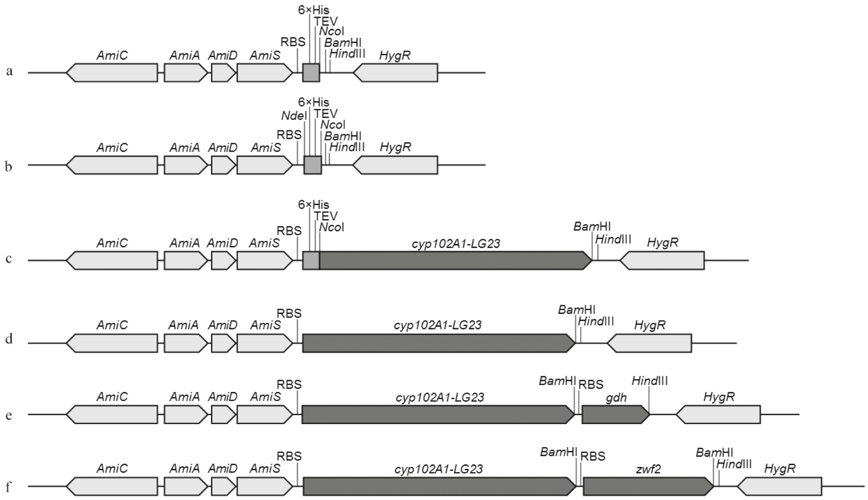


Fig. 2. Genetic elements of monocistronic and bicistronic constructs. a) Map of the pMyNT plasmid expression vector. b) Map of the pMyNTA plasmid expression vector. c) Plasmid pNS38 with the *cyp102A1-LG23* gene. d) Plasmid pVP1 with the *cyp102A1-LG23* gene. e) Plasmid pVP2 with the *cyp102A1-LG23* and *gdh* bicistronic expression cassette. f) Plasmid pVP3, with the *cyp102A1-LG23* and *zwf2* bicistronic expression cassette. *AmiC*, *AmiA*, *AmiD*, and *AmiS*, components of the acetamidase promoter; 6 × His, hexahistidine tag; *HygR*, hygromycin resistance gene; RBS, ribosome-binding site.

retrieved from KEGG (BG04_163; *Bacillus megaterium* NBRC 15308) and GenBank (ACCESSION J04832, VERSION J04832.1); the amino acid sequence of the heme fragment of CYP102A1-LG23 was retrieved from RCSB PDB (PDB ID: 6LY4). The original nucleotide sequence of *gdh* (glucose dehydrogenase, GDH) was from GenBank (ACCESSION AY930464, VERSION AY930464.1; *Bacillus megaterium* AS1.223).

Synthetic sequences of mutant *cyp102A1-LG23* [13] and *gdh* genes were optimized for efficient heterologous expression in *M. smegmatis* cells using the OptimumGeneTM algorithm. The optimized sequences were synthesized by GenScript (USA) and cloned into the pBluescript II SK(+) vector to produce pSKLG23 and pSKGDH plasmids, respectively (Table 1). The optimized synthetic sequence of *zwf2* gene coding for type 2 glucose 6-phosphate dehydrogenase (G6PD) of *Mycobacterium tuberculosis* H37Rv was obtained from pNS25 [18].

The pMyNTA vector was constructed using the pMyNT shuttle expression vector [17] by introducing the *Nde*I site at the position of the ATG start codon via site directed mutagenesis carried out according to the QuickChange™ Site-Directed Mutagenesis System (QCM) protocol (Stratagene, USA). The oligonucleotide primers NdeIlF and NdeIlR (Table 1) were used to amplify pMyNT. The resulting mixture was digested with the *Dpn*I restriction endonuclease and cloned in *E. coli* DH5α cells.

Monocistronic plasmid constructs for *cyp102A1-LG23* expression in *Mycolicibacterium* cells. DNA cloning was performed according to a standard protocol [20] using pMyNT shuttle expression vector (Fig. 2a) and its derivative pMyNTA (Fig. 2b).

The pSKLG23 plasmid was digested with the *Nco*I and *Bam*HI restriction enzymes. The *cyp102A1-LG23* gene was ligated with pMyNT linearized with the same enzymes. The resulting recombinant plasmid carrying the *cyp102A1-LG23* gene with the nucleotide sequence coding for the N-terminal hexahistidine tag (6 × His-tag) was designated pNS38 (Table 1, Fig. 2c).

The pNS38 plasmid was consecutively digested with *Nco*I, treated with the Klenow fragment of *E. coli* DNA polymerase I, and hydrolyzed with *Bam*HI. The pMyNTA vector was treated consecutively with *Nde*I, Klenow fragment, and *Bam*HI. The *cyp102A1-LG23* gene was cloned in linearized pMyNTA vector to produce recombinant pVP1 plasmid (Table 1, Fig. 2d).

Bicistronic plasmid constructs for *cyp102A1-LG23/gdh* and *cyp102A1-LG23/zwf2* coexpression in *Mycolicibacterium* cells. The pSKGDH plasmid was digested with *Bam*HI and *Hind*III restriction endonucleases, and the *gdh* gene was ligated into pVP1 vector linearized with the same restriction enzymes; the resulting plasmid was designated pVP2 (Table 1, Fig. 2e).

The *zwf2* gene was amplified by PCR with the ClaIzwf2F and ClaIzwf2R primers (Table 1) using the pNS25 plasmid as a template [18]. The amplified *zwf2* gene was digested with *Cla*I, treated with the Klenow fragment, and ligated into the pVP1 vector treated consecutively with *Bam*HI and Klenow fragment to produce the pVP3 plasmid (Table 1, Fig. 2f).

Gene expression in recombinant *Mycolicibacterium* strains. Electrocompetent cells of the mutant *M. smegmatis* BD strain were transformed with the pMyNT vector or pNS38, pVP1, pVP2, and pVP3 genetic constructs by electroporation [21] and plated onto M3 agar supplemented with 75 μg/ml hygromycin. Suspension cultures of *M. smegmatis* BD cells transformed with the plasmids were grown in 5 ml of liquid M3 medium containing 50 μg/ml hygromycin at 37°C at 200 rpm for 12-15 h and used for inoculation of 50 ml of the same medium in 750-ml flasks. The cells were grown with shaking in an incubator at 37°C at 200 rpm to OD$_{600}$ 0.8-1.0. Heterologous gene expression was induced by adding AcA to a final concentration of 2 g/liter. Induced cultures were grown at 25°C at 200 rpm for 48 h and centrifuged at 5020*g* at 4°C for 15 min. The cells were resuspended in 20 ml of buffer (50 mM KH$_2$PO$_4$, 50 mM NaCl, pH 7.4) and disintegrated by sonication with a Q500 ultrasonic homogenizer (USA) according to the manufacturer's protocol. Cell debris was removed by centrifugation at 27,300*g* at 4°C for 2.5 h. Proteins were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) in 10% gel [22].

AD bioconversion by recombinant *Mycolicibacterium* strains. Suspension cultures recombinant *Mycolicibacterium* strains were grown and induced as described above. AD (aqueous suspension with MCD, 1 : 1.5, mol/mol) was added simultaneously with AcA to a final concentration of 200 mg/liter. Glucose solution was added to the fermentation medium (to 10 g/liter) together with the inducer and then daily. The cultures were incubated at 25°C at 200 rpm for 4 days. The samples of culture liquid (0.5 ml) were collected every 24 h. Steroids were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

Biomass production assessment. The growth of bacterial cultures was assessed by measuring OD$_{600}$ in an Eppendorf BioSpectrometer Basic spectrophotometer (Germany). The strains were grown in liquid M3 medium (supplemented with 50 µg/ml hygromycin in the case of transformants) at 37°C at 200 rpm for 48 h.

Thin-layer chromatography. Steroids were extracted with a double volume of ethyl acetate. The extracts were applied onto ALUGRAM SIL G/UV254 chromatographic plates (Macherey-Nagel, Germany) and fractionated using benzene : acetone (2 : 1, v/v) as a mobile phase. Steroids were detected in UV light (254 nm) in a CN-15. LC UV Darkroom viewing chamber (Vilber Lourmat, France).

High-performance liquid chromatography. An aliquot of the culture liquid was combined with an equal volume of acetonitrile; the mixture was incubated for 12 h and centrifuged at 2100*g* for 8 min. The supernatant was analyzed using an Agilent Infinity 1260 chromatography system (Agilent Technologies, USA) with a Symmetry RP-18 column (5 µm, 4.6 × 250 mm) and a Symmetry RP-18 precolumn (5 µm, 3.9 × 20 mm) (Waters, USA). Mobile phase: solution A, acetonitrile : THF (tetrahydrofuran) : water (10 : 10 : 80, v/v); solution B, 100% acetonitrile. Flow rate, 1 ml/min; gradient elution (1st stage: eluent A 100%; 2nd stage: eluent A 40% – eluent B 60%); column temperature, 50°C. Steroids were detected at 254 nm.

Isolation and structural analysis of hydroxylated AD derivatives. The culture liquid (~200 ml) collected after 48-h incubation was centrifuged at 27,300*g* at 4°C for 1 h. The supernatant was extracted with ethyl acetate (70 ml) three times, and pooled ethyl acetate extract was evaporated under reduced pressure. Steroids were separated by column chromatography on silica gel 90 (0.2-0.5 mm), and AD hydroxylation products were isolated by a stepwise elution with hexane : ethyl acetate : ethanol:

1st stage: hexane 70% – ethyl acetate 30%;
2nd stage: hexane 50% – ethyl acetate 50%;
3rd stage: hexane 30% – ethyl acetate 70%;
4th stage: ethyl acetate 100%;
5th stage: ethyl acetate 70% – ethanol 30%;
6th stage: ethyl acetate 50% – ethanol 50%;
7th stage: ethyl acetate 30% – ethanol 70%;
8th stage: ethanol 100%.

The resulting fractions were evaporated until dry, dissolved in 500 µl of ethyl acetate, and purified by TCL on ALUGRAM SIL G/UV254 plates (Macherey-Nagel, Germany) in benzene : acetone (3 : 1, v/v). Steroid compounds were detected in UV light in a CN-15. LC UV Darkroom viewing chamber (Vilber Lourmat) at 254 nm.

The structure of steroid components was verified by ^{1}H-NMR spectroscopy. The spectra were recorded with a Bruker Avance 400 NMR spectrometer (Bruker, Germany) at 400 and 100.6 MHz. Chemical shifts were measured relative to tetramethylsilane (^{1}H-NMR).

Statistical data processing. Experimental data were obtained in three biological replicates. Results are presented as mean +/– standard deviation.

RESULTS

Recombinant bacterial producers were obtained using the mutant *M. smegmatis* BD strain (Table 1) [15].

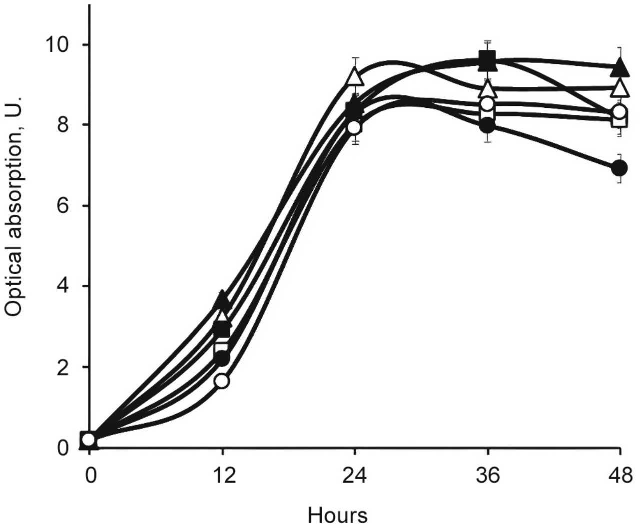


Fig. 3. Growth of parental *M. smegmatis* BD strain (–Δ–) and its recombinant derivatives carrying pMyNT (–▲–), pNS38 (–■–), pVP1 (–□–), pVP2 (–●–), or pVP3 (–○–) plasmids.

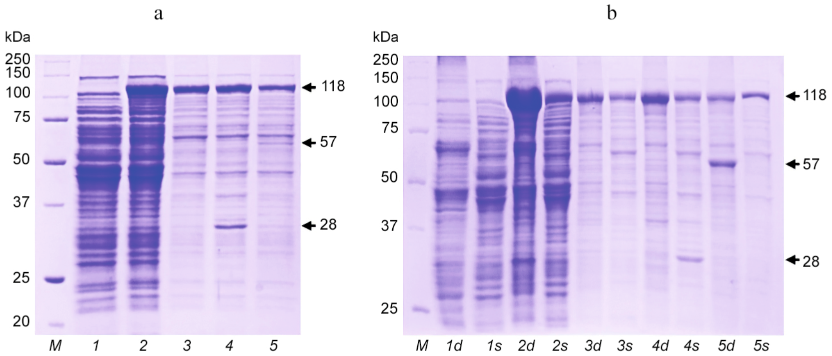


Fig. 4. SDS-PAGE analysis of (a) heterologous gene expression and (b) solubility of recombinant proteins in the AcA-induced cultures of recombinant *M. smegmatis* BD strains. M, molecular weight markers (Bio-Rad, USA); s, supernatant; d, cell debris. Plasmids: *1*) pMyNT (control); *2*) pNS38; *3*) pVP1; *4*) pVP2; *5*) pVP3. Arrows indicate CYP102A1-LG23 (118 kDa), GDH (28 kDa), and G6PD (57 kDa).

A set of plasmids was constructed to express synthetic *cyp102A1-LG23* as a single gene and in the content of bicistronic operons with the *gdh* or *zwf2* genes (Table 1) under control of the inducible acetamidase (*Ami*) promoter.

The obtained recombinant strains did not differ in the cultivation and morphological properties when grown on M3 agar but showed a slightly lower growth rate in the liquid medium compared with the parental *M. smegmatis* BD strain (Fig. 3).

The highest level of CYP102A1-LG23 expression was observed in *Mycolicibacterium* cells carrying the pNS38 plasmid (Fig. 4a, lane *2*). Fractionation of cell homogenate showed that the recombinant protein accumulated almost fully in inclusion bodies (Fig. 4b, lane *2d*). When the hexahistidine tag was removed from the N-terminus of the mutant cytochrome, up to 40-50% of the protein occurred in a soluble form (Fig. 4b; lanes *3d*, *3s*). GDH was detected in the soluble cell fraction (Fig. 4b, lane *4s*), while G6PD was found in the membrane fraction (Fig. 4b, lane *5d*). The recombinant proteins synthesized upon induction with AcA showed expected molecular weights: CYP102A1-LG23, 118 kDa; G6PD, 57 kDa; and GDH, 28 kDa. In all recombinant strains, production of the second protein (GDH or G6PD) encoded in the bicistronic construct was much lower than that of the first protein (Fig. 4a; lanes *4*, *5*).

In vivo production of 7β-hydroxyandrost-4-ene-3,17-dione (7β-OH-AD) by the strain carrying the pNS38 plasmid (Fig. 5) was low (15.56 mol%) indicating low enzymatic activity of the insoluble protein. Decreasing the *cyp102A1-LG23* expression using a lower concentration of the inducer failed to noticeably improve the activity and solubility of the enzyme (data no shown), while removal of the 6 × His-tag (plasmid pVP1) improved the solubility of P450 BM3-LG23, resulting in the two-fold (to 32.75 mol%) increase in the efficiency of substrate bioconversion (Fig. 5).

However, even in the case of improved protein solubility, the conversion of AD to 7β-OH-AD was incomplete. To further increase the efficiency of substrate bioconversion, we investigated whether the activity of CYP102A1-LG23 could be elevated by providing a necessary level of reduced cofactors via coexpression of *gdh* (pVP2) or *zwf2* (pVP3).

In all the cases, 7β-OH-AD was accumulated as a major metabolite, while the content of 7α-OH-AD did not exceed 1.5 mol%. 7β-Hydroxylation of AD by the strains carrying the bicistronic constructs pVP2 and pVP3 was 9-15% more efficient (37.68 and 35.63 mol%, respectively) than by the strain with the monocistronic pVP1 construct (Fig. 5). The highest yield of 7β-OH-AD was achieved within 48 h, after which the concentration of the target product decreased together with a decrease in the AD content (Fig. 5).

We also observed introduction of the OH group at the C1β position (in addition to C7β hydroxylation) in the recombinant strains (Fig. 6, Table 2). Production of 1β-OH- and 1β,7β-diOH-steroids by P450-BM3 mutant variants has not been described before.

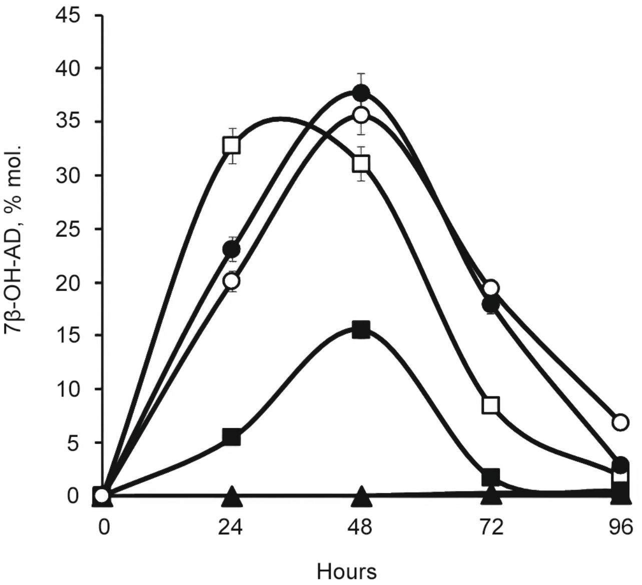


Fig. 5. Accumulation of 7β-OH-AD in the cultures of recombinant *M. smegmatis* BD strains carrying (–▲–) pMyNT, (–■–) pNS38, (–□–) pVP1, (–●–) pVP2, and (–○–) pVP3 plasmids.

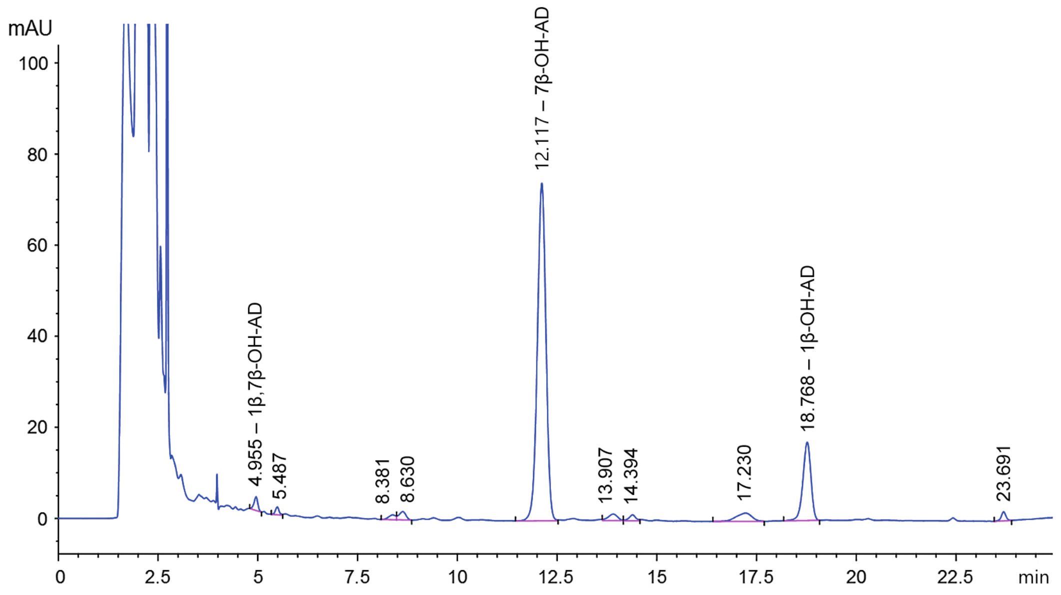
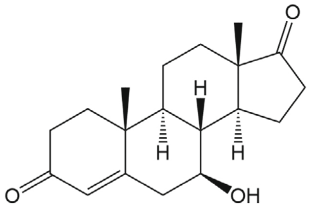
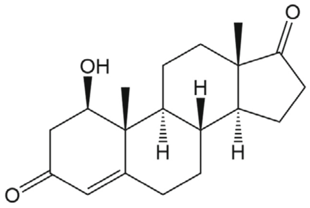
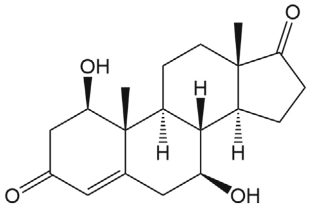


Fig. 6. HPLC profile of AD hydroxylation products from recombinant *M. smegmatis* BD strains.

Table 2. Products of AD bioconversion by recombinant *M. smegmatis* BD strains

Product	Chemical formula	Retention time, min (HPLC)	^{1}H-NMR (CDCl$_3$, δ, ppm)
7β-Hydroxy-androst-4-ene-3,17-dione (7β-OH-AD)		12.19 ± 0.13	5.78 (s, 1H, H-4) 3.60 (dt, J = 10.3, 5.2 Hz, 1H, H-7α) 1.24 (s, 3H, H-19) 0.95 (s, 3H, H-18)
1β-Hydroxy-androst-4-ene-3,17-dione (1β-OH-AD)		18.15 ± 0.88	5.82 (d, J = 1.1 Hz, 1H, H-4) 4.06 (dd, J = 8.2, 7.6 Hz, 1H, H-1α) 1.27 (s, 3H, H-19) 0.93 (s, 3H, H-18)
1β,7β-Dihydroxy-androst-4-ene-3,17-dione (1β,7β-diOH-AD)		4.97 ± 0.02	5.83 (br. s, 1H, H-4) 4.03 (dd, J = 9.2, 6.2 Hz, 1H, H-1α) 3.63–3.55 (m, 1H, H-7α) 1.30 (s, 3H, H-19) 0.95 (s, 3H, H-18)

DISCUSSION

Introduction of functional oxygen-containing groups into the cycloalkane core is one of the most important and difficult tasks in the synthesis of therapeutic steroids. New prospects in this area are associated with the creation and use of recombinant bacterial P450 cytochromes that have a higher solubility and activity than most eukaryotic cytochromes [5]. The most promising direction is the use of bacillary P450 BM3 cytochrome that possesses unique biocatalytic features [9, 11]. One of the outstanding achievements was the creation of its mutant variant CYP102A1-LG23 capable of hydroxylating steroids at C7β [13]. In this work, we optimized the nucleotide sequence of this mutant enzyme for expression in *Mycolicibacterium* cells. The optimized gene sequence was synthesized and used for the construction of plasmids for heterologous expression of *cyp102A1-LG23* as a part of either monocistronic operon or bicistronic operons with the *gdh* or *zwf2* genes coding for GDH and G6PD, respectively, in *M. smegmatis* BD cells.

M. smegmatis BD was used as a host due to the presence of an efficient system for steroid transport [15] and possibility of one-step production of 7β-hydroxylated androstenes from phytosterols [23]. Previously, this strain has been successfully tested for the expression of genes of bacillary CYP106A1 and CYP106A2 cytochromes. The recombinant strain expressing the *cyp106A2* gene selectively hydroxylated AD at C15β [15].

Overexpression of CYP102A1-LG23 led to the generation of insoluble inclusion bodies and, consequently, low efficiency of C7β-hydroxylation *in vivo*. Presumably, protein aggregation was caused by the presence of the N-terminal polyhistidine tag that had a negative effect on the solubility, structure, and/or folding of P450 BM-LG23. It is known that addition of the His-tag can affect protein function and stability. Recently, there has been an increasing number of publications reporting a negative effect of His-tags on the structure, oligomeric state, kinetics, and catalytic activity of proteins [24]. Deletion of the hexahistidine tag from the N-terminus allowed up to increase the content of the soluble functionally active protein up to 50% without affecting its total yield, which increased the content of produced 7β-OH-AD two-fold.

Cofactor engineering is an efficient approach to improving the performance in production of valuable metabolites. For example, overexpression of NADPH-regenerating G6PD noticeably increased production of ε-caprolactone in *E. coli* [25]. The balance between $NADP^+$ and NADPH has been found to play a key role in the regulation of phytosterol conversion to 7β-OH-AD in *Mycolicibacterium neoaurum* [23]. To maintain the pool of reduced NADPH, GDH, or G6PD were coexpressed with CYP102A1-LG23 in *M. smegmatis* BD. Glucose was added to the fermentation medium as a cosubstrate to achieve more efficient NADPH regeneration [23, 26]. Introduction of enzymes for additional cofactor regeneration allowed to increase the activity of the recombinant system by 15%. In all the cases, 7β-OH-AD was accumulated as a major metabolite and 7α-OH-AD was detected in trace amounts, which was in agreement with the published data [13, 23]. Two new compounds (1β-OH-AD and 1β,7β-diOH-AD) were obtained for the first time as the products of hydroxylation reaction catalyzed by the mutant variants of P450 BM3 cytochrome. Microbiological C1-hydroxylation of steroids (in particular, at the C1β position) is a relatively rare phenomenon [27] that should be investigated further.

CONCLUSIONS

In this study, we achieved heterologous expression of the unique mutant cytochrome CYP102A1-LG23 in *M. smegmatis* BD cells for the purpose of 7β-OH-AD synthesis from AD. The enzymatic activity of the recombinant *Mycolicibacterium* bacteria was increased by increasing the content of protein active form and using a cofactor regeneration system, as both factors limited AD conversion to 7β-OH-AD in *M. smegmatis*. The maximum product yield (37.68 mol%) was achieved with the *M. smegmatis* BD (pVP2) strain expressing *cyp102A1-LG23* and *gdh*. These results contribute to the understanding of the catalytic features of recombinant P450 enzymes and can be used as a starting point to develop a system for producing valuable C7β-hydroxylated steroids from inexpensive precursors (phytosterols and cholesterol) via microbial transformation by genetically modified *Mycolicibacterium* bacteria.

Contributions. V.Yu.P. planned and performed experiments, discussed the results, and prepared the manuscript; N.I.S. developed the study concepted and designed genetic constructs; M.V.K. planned the experiments, discussed the results, and prepared the manuscript; V.M.N. purified steroids; A.V.K. performed NMR analysis; O.I.S. performed the experiments; A.A.Sh. conducted HPLC analysis; M.V.D. supervised the study and edited the manuscript.

Funding. This work was supported by the Russian Science Foundation (project no. 21-64-00024).

Ethics declarations. The authors declare no conflict of interest. This article does not contain experimental studies involving animals or human subjects performed by any of the authors.

REFERENCES

1. Donova, M. V. (2017) Steroid bioconversions, *Methods Mol. Biol.*, **1645**, 1-13, doi: 10.1007/978-1-4939-7183-1_1.
2. Wojtal, K., Trojnar, M. K., and Czuczwar, S. J. (2006) Endogenous neuroprotective factors: neurosteroids, *Pharmacol. Rep.*, **58**, 335-340.
3. Fegan, K. S., Rae, M. T., Critchley, H. O. D., and Hillier, S. G. (2008) Anti-inflammatory steroid signalling in the human peritoneum, *J. Endocrinol.*, **196**, 369-376, doi: 10.1677/joe-07-0419.
4. Ali Shah, S. A., Sultan, S., and Adnan, H. S. (2013) A whole-cell biocatalysis application of steroidal drugs, *Orient. J. Chem.*, **29**, 389-403, doi: 10.13005/ojc/290201.
5. Szaleniec, M., Wojtkiewicz, A. M., Borowski, T., Bernhardt, R., and Donova, M. (2018) Bacterial steroid hydroxylases: enzyme classes, their functions and comparison of their catalytic mechanisms, *Appl. Microbiol. Biotechnol.*, **102**, 8153-8171, doi: 10.1007/s00253-018-9239-3.

6. Julsing, M. K., Cornelissen, S., Bühler, B., and Schmid, A. (2008) Heme-iron oxygenases: powerful industrial biocatalysts? *Curr. Opin. Chem. Biol.*, **12**, 177-186, doi: 10.1016/j.cbpa.2008.01.029.
7. Bureik, M., and Bernhardt, R. (2007) in *Modern Biooxidation. Enzymes, Reactions and Applications* (Schmid, R. D., and Urlacher, V. B., eds) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp. 155-176.
8. Fernández-Cabezón, L., Galán, B., and García, J. L. (2018) New insights on steroid biotechnology, *Front. Microbiol.*, **9**, 958, doi: 10.3389/fmicb.2018.00958.
9. Munro, A. W., Leys, D. G., McLean, K. J., Marshall, K. R., Ost, T. W., et al. (2002) P450 BM3: the very model of a modern flavocytochrome, *Trends Biochem. Sci.*, **27**, 250-257, doi: 10.1016/s0968-0004(02)02086-8.
10. Guengerich, F. P. (1991) Reactions and significance of cytochrome P-450 enzymes, *J. Biol. Chem.*, **266**, 10019-10022, doi: 10.1016/S0021-9258(18)99177-5.
11. Munro, A. W., Daff, S., Coggins, J. R., Lindsay, J. G., and Chapman, S. K. (1996) Probing electron transfer in flavocytochrome P-450 BM3 and its component domains, *Eur. J. Biochem.*, **239**, 403-409, doi: 10.1111/j.1432-1033.1996.0403u.x.
12. Finnigan, J. D., Young, C., Cook, D. J., Charnock, S. J., and Black, G. W. (2020) Cytochromes P450 (P450s): A review of the class system with a focus on prokaryotic P450s, *Adv. Protein Chem. Struct. Biol.*, **122**, 289-320, doi: 10.1016/bs.apcsb.2020.06.005.
13. Li, A., Acevedo-Rocha, C. G., D'Amore, L., Chen, J., Peng, Y., et al. (2020) Regio- and stereoselective steroid hydroxylation at C7 by cytochrome P450 monooxygenase mutants, *Angew. Chem. Int. Ed.*, **59**, 12499-12505, doi: 10.1002/ange.202003139.
14. Tang, R., Ren, X., Xia, M., Shen, Y., Tu, L., et al. (2021) Efficient one-step biocatalytic multienzyme cascade strategy for direct conversion of phytosterol to C-17-hydroxylated steroids, *Appl. Environ. Microbiol.*, **87**, e00321-00321, doi: 10.1128/AEM.00321-21.
15. Karpov, M. V., Nikolaeva, V. M., Fokina, V. V., Shutov, A. A., Kazantsev, A. V., et al. (2022) Creation and functional analysis of *Mycolicibacterium smegmatis* recombinant strains carrying the bacillary cytochromes CYP106A1 and CYP106A2 genes, *Appl. Biochem. Microbiol.*, **58**, 947-957, doi: 10.1134/S0003683822090058.
16. Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., and Jacobs, W. R. Jr. (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*, *Mol. Microbiol.*, **4**, 1911-1919, doi: 10.1111/j.1365-2958.1990.tb02040.x.
17. Poulsen, C., Holton, S., Geerlof, A., Wilmanns, M., and Song, Y.-H. (2010) Stoichiometric protein complex formation and over expression using the prokaryotic native operon structure, *FEBS Lett.*, **584**, 669-674, doi: 10.1016/j.febslet.2009.12.057.
18. Strizhov, N., Karpov, M., Sukhodolskaya, G., Nikolayeva, V., Fokina, V., et al. (2016) Development of mycobacterial strains producing testosterone, *Proc. Nat. Acad. Sci. Belarus*, *Chem. Series*, **3**, 57-58.
19. Bertani, G. (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*, *J. Bacteriol.*, **62**, 293-300, doi: 10.1128/jb.62.3.293-300.1951.
20. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, N.Y.
21. Daugelat, S., Kowall, J., Mattow, J., Bumann, D., Winter, R., et al. (2003) The RD1 proteins of *Mycobacterium tuberculosis*: expression in *Mycobacterium smegmatis* and biochemical characterization, *Microbes Infect.*, **5**, 1082-1095, doi: 10.1016/s1286-4579(03)00205-3.
22. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**, 680-685, doi: 10.1038/227680a0.
23. Zhao, Y.-Q., Liu, Y.-J., Ji, W.-T., Liu, K., Gao, B., et al. (2022) One-pot biosynthesis of 7β-hydroxyandrost-4-ene-3,17-dione from phytosterols by cofactor regeneration system in engineered *Mycolicibacterium neoaurum*, *Microb. Cell Fact.*, **21**, 59, doi: 10.1186/s12934-022-01786-5.
24. Booth, W. T., Schlachter, C. R., Pote, S., Ussin, N., Mank, N. J., et al. (2018) Impact of an N-terminal polyhistidine tag on protein thermal stability, *ACS Omega*, **3**, 760-768, doi: 10.1021/acsomega.7b01598.
25. Lee, W.-H., Park, J.-B., Park, K., Kim, M.-D., and Seo, J.-H. (2007) Enhanced production of ε-caprolactone by overexpression of NADPH-regenerating glucose 6-phosphate dehydrogenase in recombinant *Escherichia coli* harboring cyclohexanone onooxygenase gene, *Appl. Microbiol. Biotechnol.*, **76**, 329-338, doi: 10.1007/s00253-007-1016-7.
26. Wu, Y., Li, H., Zhang, X. M., Gong, J. S., Li, H., et al. (2015) Improvement of NADPH-dependent P450-mediated biotransformation of 7α, 15α-diOH-DHEA from DHEA by a dual cosubstrate-coupled system, *Steroids*, **101**, 15-20, doi: 10.1016/j.steroids.2015.05.005.
27. Dodson, R. M., Kraychy, S., Nicholson, R. T., and Mizuba, S. (1962) Microbiological transformations. IX. The 1β-hydroxylation of androstenedione, *J. Org. Chem.*, **27**, 3159-3164, doi: 10.1021/jo01056a043.