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Functional reconstruction of bovine P450scc steroidogenic system in *Escherichia coli*

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ABSTRACT

Mammalian cytochrome P450scc enzyme system catalyzes the initial step in steroid hormone biosynthesis—cholesterol hydroxylation followed by cleavage of the side-chain to yield pregnenolone. This system consists of three components—the cytochrome P450scc (CYP11A1), a flavoprotein (NADPH-adrenodoxin reductase, AdR) and an iron-sulfur protein (adrenodoxin, Adx). In this work, the three-component electron transport chain (AdR/Adx/CYP11A1) from bovine adrenal cortex has been implemented in *Escherichia coli* by co-expression of the corresponding coding sequences from a tricistronic plasmid. The cDNAs of AdR, Adx and CYP11A1 are situated in a single transcription unit and separated by ribosome binding sequences. The recombinant strain created was capable of synthesizing functional proteins identical to the bovine CYP11A1, AdR and Adx on molecular weights and immuno-specificity. The experiments *in vivo* showed pregnenolone production from cholesterol by the transformed bacteria. Maximal productivity of 0.42 ± 0.015 mg/l pregnenolone for 24 h has been reached for the induced cells in the presence of cholesterol solubilizing agent—methyl-*β*-cyclodextrin. Thus, a stable transgenic *E. coli* strain with the functional reconstructed bovine cholesterol side-chain cleavage system has been firstly generated in this work. The findings are of importance for studies of mammalian steroidogenic system features, and may open some perspectives for further generation of novel microbial biocatalysts.

Keywords: Cytochrome P450; CYP11A1; Adrenodoxin; Adrenodoxin Reductase; Steroid Hormone Biosynthesis; Heterologous Expression

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1. INTRODUCTION

Cytochromes P450 are ubiquitously distributed hemoproteins with broad field of catalytic activity towards various substances of exogenous and endogenous origin. As external monooxygenases, most of P450s functions as substrate binding terminal oxidases utilize external reductant, with electron transfer for oxygen activation and substrate conversion [1].

Cytochrome P450scc (CYP11A1, EC 1.14.15.6) catalyzes the side-chain cleavage of cholesterol in bovine adrenal cortex mitochondria. The mechanism involves three sequential monooxygenation reactions—production of 22R-hydroxycholesterol (22HC), 20*α*, and 22R-dihydroxycholesterol followed by the cleavage of the C20-C22 bond [2]. Natural partners of P450scc are adrenodoxin (Adx) and adrenodoxin reductase (AdR). The former is a [2Fe-2S] ferredoxin, and the latter is NADPH-dependent flavine reductase (EC 1.18.1.2). These three proteins (CYP11A1, Adx, AdR) are from cholesterol hydroxylase/20,22-lyase system (CH/L) which catalyzes the initial step of steroidogenesis in mammals: cholesterol conversion to pregnenolone—the key precursor of all steroid hormones (**Figure 1**).

Further steps include pregnenolone modification with at least five P450s, 3*β*-hydroxysteroid dehydrogenase/ $\Delta^{5,4}$-isomerase and 17*β*-hydroxysteroid dehydrogenase in the endoplasmic reticulum (ER) (CYP17, CYP21, CYP19) and mitochondria (CYP11B1, CYP11B2) of steroidogenic mammalian cells thus resulting in the formation of different steroid hormones [3].

The objective challenges with the studies of steroidogenic P450-systems in mammalian organs, such as the presence in the cells of few P450 isoforms on different topogenesis stages, stimulate creating of modeling systems which are based on heterologous protein expression in microorganisms for *in vitro* and *in vivo* investigations. Cloning and characterization of the individual steroido-

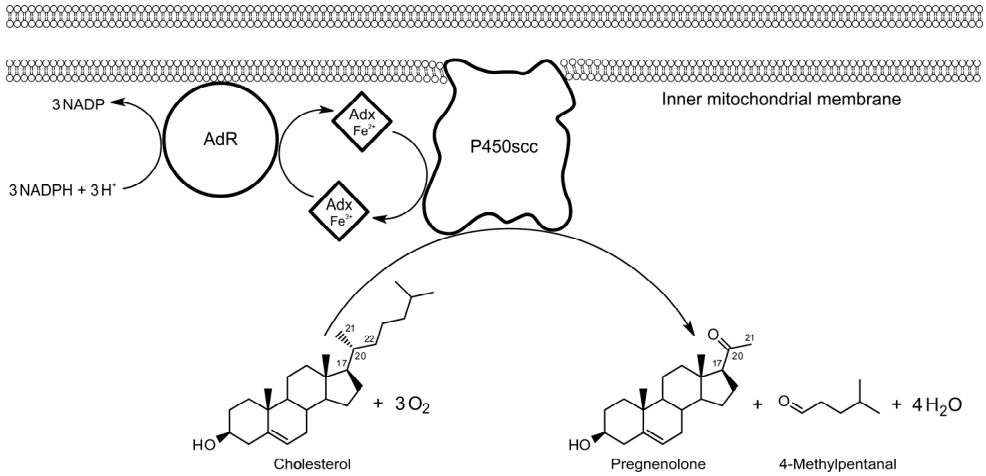

Figure 1. The general organization and function of mammalian cholesterol hydroxylase/lyase system. The original system is located in adrenocortical mitochondria and includes cytochrome P450scc (CYP11A1) and its redox partners: Adx (adrenodoxin, a member of [2Fe-2S] ferredoxins family) and AdR (adrenodoxin reductase, a NADPH-dependent flavin reductase). Membrane-bound cytochrome P450scc catalyzes cholesterol conversion to pregnenolone in three sequential steps including hydroxylation in positions C-22 and C-20 and C-C cleavage of the formed diol.

genic proteins in yeasts and bacteria were described earlier. Later on, the works were published on the improvement or modification of P450s features (e.g. membrane-binding, or substrate specificity change), as well as on the design of transgenic microorganisms with expression of multicomponent enzyme systems capable of performing few, or even cascade of mammalian steroidogenic reactions in one microorganism [4].

The strains of *E. coli* are often used as a host microorganism for expression of recombinant P450s since these bacteria do not contain their own P450s [5]. Application of *E. coli* expression system often provides a high expression level of heterologous P450s, and in particular, that of steroidogenic P450s in their active forms. Besides, mature forms of mitochondrial and microsomal P450s lacking the N-terminal targeting sequence can be expressed [6]. Such systems are especially suitable for investigation of P450s' structure/function by site-directed mutagenesis and protein engineering.

Applications of *E. coli* expression systems and purified recombinant steroidogenic proteins are known for analyses of their topogenesis [7], interactions with redox partners [8] and substrates [9], and study of structural characteristics [10,11], etc. Moreover, such systems can also be used for medical purposes (for example, [12,13]). Recently, the effect of different therapeutic agents on CYP11A1 activity has been evaluated using purified recombinant P40scc (CYP11A1) in the *in vitro* reconstituted system [14].

The expression of mature form of mitochondrial bovine P450scc (mP450scc) as a spectrophotometrically and catalytically active protein in *E. coli* was firstly reported in 1991 [6]. The enzymatic activity of P450scc was estimated toward 25-hydroxycholesterol using solubilized membranes in the presence of purified bovine Adx and AdR. Similar results were later published for the mature form of P450scc from human placenta [15]. As shown, recombinant cytochromes P40scc inserted into the cytoplasmic membrane were basically similar to the native proteins. [6,15,16]. Besides, functional AdR [17,18] and Adx [15,19,20] of different origin were expressed in *E. coli*.

In our previous works, we have attempted to generate recombinant bacterial cells bearing heterologous CH/L system using two distinct approaches for P450scc co-expressing with Adx and AdR redox partners. One approach was based on the expression in *E. coli* of the fused side-chain cleavage system with catalytic domains being connected by short (2-5 amino acid residues) linkers [21]. However, the *in vitro* activity of this system was lower as compared with the system built of the separate purified bovine constituents, probably due to the steric hindrance for the interaction of active centers of the particular domains. The second approach involved expression in *E. coli* of the mammalian CH/L system constituents based on the polycistron (tandem) plasmid [22]. Cell-free homogenate was shown to transform cholesterol to pregnenolone, but the activity was again very low. It was possible that the low level of *in vitro* activity was related to the different origin of the co-expressed proteins—bovine P450scc and human Adx and AdR. The expression of bovine cholesterol hydroxylase system with the use of co-transformation by two plasmids was later described in [23], but no data on the activity, neither *in vivo* nor *in vitro*, were reported.

In this work, the vector including cDNA for all three

proteins of bovine CH/L system (P450scc, Adx and AdR) was constructed and the *in vivo* activity of the recombinant *E. coli* strain generated was examined.

2. MATERIALS AND METHODS

2.1. Materials

The following reagents were used in the work: δ-aminolevulonic acid (δ-ALA), isopropyl-β-D-thiogalactopyranoside (IPTG), diaminobenzidine tetrachloride hydrate, cholesterol oxidase and horse-radish peroxidase-conjugated anti-rabbit antibodies were purchased from Sigma (USA), Tween 80 was from Serva (Germany). Statistically methylated β-cyclodextrin (MCD) was obtained from Wacker Chemie (Germany); electrophoresis reagents—from Bio-Rad (USA).

Nutrient media (LB and TB [24]) for bacterial growth have been prepared using materials supplied by Difco (USA). Nitrocellulose filters Hybond-C extra were obtained from Amersham (USA).

All DNA modifying enzymes and DNA Extraction Kit have been purchased from MBI Fermentas (Lithuania). The reaction mixtures preparation, sample incubation and enzyme inactivation were carried out according to the manufacturer's instructions (Fermentas Catalogue & Product Application Guide).

Primary antibodies (IgG fraction) against bovine P450scc, AdR, and Adx were kindly provided by Prof. V. M. Shkumatov (Institute of Physico-Chemical Problems, Minsk State University, Belarus).

Steroids: cholesterol (98% purity) was purchased from Serva (Germany); pregnenolone (pregn-5-ene-3-ol-20-one) and progesterone (pregn-4-ene-3,20-dione) were from Sigma (USA), and Steraloids (USA), respectively. Gradient-grade HPLC solvents have been purchased from Panreac (Spain). Other reagents were of analytical grade and have been purchased from domestic companies (Russia).

2.2. Bacterial Strains and Plasmids

Plasmid pTrc99A/P450scc [6] containing cDNA for mature bovine cytochrome P450scc and plasmid pBar_Twin [25] containing cDNAs for mature forms of bovine AdR and Adx as a single expression cassette were used in this work. Plasmid pTrc99A/P450scc was kindly provided by Prof. M. R. Waterman (University of Texas, Southwestern Medical Center, Dallas, TX, USA). The pBar_Twin plasmid was kindly provided by Prof. R. Bernhardt (University of Saarlandes, Saabrucken, Germany).

Escherichia coli strain DH5αc (Gibco-BRL) and *E. coli* recombinant strains JM109/F2, JM109/D36, and JM109/E32 (collection names) were used in this work. *E. coli* strains JM109/F2, JM109/D36, and JM109/E32 have been generated earlier in our laboratory on the base of *E. coli* strain JM-109 (Promega, USA) as a result of transformation by pTrc99A/F2 (with artificial cDNA encoding for fusion CH/L system), and pTrc99A/P450scc/AdR.Adx, or pTrc99A/P450scc/AdR/Adx (with cDNAs encoding for all separate proteins of CH/L), respectively [7,21,22].

The strains and plasmids used in this study are summarized in **Table 1**.

Construction of pBar_Triple Plasmid

To derive a suitable expression vector containing cDNA encoding for three cholesterol hydroxylase proteins, two initial plasmids—pTrc99A/P450scc and pBar_Twin, were used. The plasmid pBar_Twin was sequentially digested with restriction endonuclease *Eco*RI (the site located after the termination codon cDNA encoding for Adx), filled in with Klenow fragment, and treated with thermosensitive alkaline phosphatase. The DNA insert (1583 bp) encoding for mature bovine P450scc with ribosome binding site (RBS) in front of it was excised by *Mbi*I and *Sal*I from pTrc99A/P450scc plasmid, filled in using Klenow polymerase and ligated with linearized and blunt ended pBar_Twin, so that ribosome-binding site (RBS) and cytochrome P450scc cDNA were inserted into the expression cassette beyond cDNA for Adx.

The DNA fragments obtained by restriction were separated using electrophoresis in 1% agarose gel. Extraction of DNA was carried out using DNA Extraction Kit. Plasmid DNA for cloning procedures was isolated from bacteria by the alkaline lysis method [24]. Transformation of *E. coli* cells was performed in accordance with known protocol [24] thus resulting in a recombinant *E. coli* DH5αc/Triple (**Table 1**).

2.3. Expression of Recombinant Proteins in *E. coli* Cells

In order to express the recombinant proteins, the cells of individual colonies were grown overnight in 5 ml of liquid LB (Luria-Bertani broth) medium containing ampicillin (100 μg/ml) aerobically on a rotary shaker GH-4103 Bottmingen HT (Germany) (140 rpm) at 37˚C, diluted 1:200 with TB (Terrific Broth) medium, and again cultivated at 37˚C for 3 - 4 h. Synthesis of recombinant proteins was then induced by an addition of IPTG to 0.5 mM, and the cell growth was continued in the presence of ampicillin (100 μg/ml) and δ-ALA (0.5 mM) for 48 h at 24 - 28˚C with constant shaking (140 rpm).

2.4. Ds-Na-PAAG Electrophoresis and Western Immunoblotting

The cells of *E. coli* DH5αc/Triple25 obtained as described above (as per 2.3.) were harvested by centrifugation, re-suspended in sample buffer [26] and disrupted by heating for 2 minutes at 100˚C. Cell homogenates were

Table 1. *Escherichia coli* strains and plasmids used in this study.

Recipient strain used for construction pBar_Triple	Genotype		Source
DH5αc	supE44 Δlac U169 (ϕ 80 lacZ ΔM15) hsdR17 recA1end A1 gyrA96 thi-1 relA1		Gibco-BRL
Initial vectors used for construction pBar_Triple[a]	Characteristics		References
pTrc99A/mP450scc	*bla* (Amp^R), *trp/lac/trc* promoter, cDNA for P450scc(b)		[6]
pBar_Twin	*bla* (Amp^R), *tac1/tac2/lacUV5* promoter, cDNAs for AdR(b) and Adx(b) inserted in one expression cassette		[25]
Recombinant strains used for cholesterol conversion	Expression vector	Insert[a]	References
JM-109/F2	pTrc99A/F2	cDNA for P450scc(b)-AdR(h)-Adx(h) fusion	[21]
JM-109/D36	pTrc99A/P450/AdR/Adx	cDNAs for P450scc(b) and AdR(h) inserted in the first expression cassette and cDNA for Adx(h) inserted in the second expression cassette	[7]
JM-109/E32	pTrc99A/P450/AdR.Adx	cDNAs for P450scc(b), AdR(h), Adx(h) inserted in one expression cassette	[22]
DH5αc/Triple25	pBar_Triple	cDNAs for P450scc(b), AdR(b), Adx(b) inserted in one expression cassette	This study

[a]The plasmids indicated in the table contain cDNAs encoding the human (h) or bovine (b) mature forms of proteins.

subjected to SDS-PAGE in 10, or 15% gel [26] and Western blotting [27]. Upon SDS-PAGE and protein transfer from gel onto nitrocellulose membrane, the latter was consecutively treated by a primary antibody (IgG fraction) to P450scc, AdR, or Adx and a secondary antibody conjugated with horseradish peroxidase. As it was shown earlier [16,22], the primary antibodies against bovine P450scc, AdR, and Adx used in this work bind effectively with respective either native or recombinant proteins of P450scc system expressed in *E. coli*. Protein bands were visualized using diaminobenzidine tetrachloride hydrate.

Protein in homogenates was measured by the Lowry method [28].

2.5. *In Vivo* Activity of Cholesterol Hydroxylase/Lyase System

In order to determine the activity of bovine P450scc/Adx/AdR system in recombinant E. coli cells *in vivo*, the bacteria were grown and expression was induced as described above (in 2.2) with some modifications. The overnight culture (1%, v/v) was inoculated in 50 ml TB-medium supplemented with 100 μg/ml ampicillin, and cultivated at 37˚C aerobically (160 rpm) for 4 h. Then, IPTG (1 mM), δ-ALA (0.5 mM) and microelement solutions (each of 50 μl) were added. The microelement solutions were composed according to [25]. The microelement solution 1 contained (g/l): $FeCl_2 \cdot 6H_2O$—4.07; $CaCl_2 \cdot 2H_2O$—0.28; $CoCl_2 \cdot 6H_2O$—0.28; $ZnCl_2 \cdot 4H_2O$—0.19; $CuSO_4 \cdot 5H_2O$—0.26; H_3BO_4—0.07. Solution 2 contained 0.28 g/l $Na_2MoO_4 \cdot 2H_2O$. After the additions, A_{600} was ~1.5. The cells were further incubated for 2 h at 29˚C. The culture obtained was used for cholesterol bioconversion.

Cholesterol was added to the final concentration 0.5 mM (193 mg/l) in a form of 100-fold aqueous concentrates: 1) as a solution in MCD (250 mM), or 2) as fine suspension stabilized with Tween 80 (100 g/l) and homogenized on ultrasonic bath (Cole-Parmer, USA) at 42 kHz, 100 Wt, for 5 min. After cholesterol addition, the cells were incubated at 24˚C and 180 rpm for 74 h. The samples were taken every 24 h since cholesterol addition. In controls, the following variants were used: a) expression was not induced; b) no cholesterol was added; c) recipient *E. coli* strains were used.

2.6. Steroid Analyses

The samples of cultivation broth (10 ml) were twice extracted with ethyl acetate (firstly—with double, then with equal solvent volume), the organic phases were combined and vacuum-evaporated to dryness. The residue was re-dissolved in 1 ml of 50% aqueous acetonitrile and insolubles formed were separated by centrifugation at 5,000 × *g*, 15 min. Steroids were analyzed by high-pressure liquid chromatography (HPLC) on the HPLC system Series 1200 (Agilent, USA) at 50˚C and eluents flow rate of 1 ml/min. Components were separated on a Symmetry column (Waters, USA) 250 mm × 4.6 mm (with a guard column 20 mm × 3.9 mm) packed with reverse phase ODS (5 μm) by three different methods: 1) isocratic—in a system composed of 52% acetonitrile, 48% H_2O and 0.01% acetic acid, 2) in a system containing 64% acetonitrile, 36% H_2O and 0,01% acetic acid; 3) in a linear gradient of acetonitrile (50% from 0 to 10 min; 50% - 88% from 10 to 20 min; 88% from 20 to 25 min).

Peak detection was carried out by UV absorbance at 200 and 240 nm. Identification of the peaks and the quantification of pregnenolone and progesterone were carried out using external standard technique.

2.7. Enzymatic Treatment of Extracted Steroids

The evaporated organic extracts of cultivation broth sam-

ples obtained as described above (2.6) were suspended in 0.5 ml of 0.05 M sodium phosphate buffer (pH 7.5) supplemented with MCD (50 μM). In control, the same buffer containing 2 mg/l pregnenolone was used. The mixtures obtained were incubated with 8 U/ml of recombinant cholesterol oxidase (Sigma, USA) at 30˚C for 20 h. Then, the samples were diluted with equal volume of acetonitrile and centrifuged (5,000 × g, 15 min). The supernatants were applied for HPLC analyses as described above (2.6).

3. RESULTS AND DISCUSSION

3.1. Plasmid Construction for Co-Expresssion of Three Bovine Proteins

For expression of more than one protein in bacteria, a plasmid which contains cDNAs encoding for different proteins and RBSs before the each of heterologous cDNA in a single transcription unit can be constructed. Therefore, the heterologous genes reading should be controlled by one promoter and one terminator. One fusion (hybrid) mRNA should be formed, with an independent translation of the individual proteins. Several functional monooxygenase systems were published to be constructed using similar polycistrone plasmids for protein co-expression in bacterial cells [29-31].

In the present work, the vector was created for co-expression in *E. coli* cells of three bovine system proteins, -CYP11A1/Adx/AdR.

To express cytochrome P450scc cDNA alongside with the cDNAs for AdR and Adx as a single transcription unit, DNA fragment encoding the P450scc and RBS in front of it was cloned into the pBar_Twin [25]. The cells of *E. coli* were transformed with ligase mixture, and after restriction analysis of the recombinant plasmids the clones were isolated which did contain the vector where RBS and cDNA encoding for P450scc were situated downstream of Adx cDNA seguence. Selected plasmid contained the nucleotide sequences of the heterologous proteins and RBSs in a single expression cassette in the following order [RBS-AdR-RBS-Adx-RBS-P450scc].

The resulting tricistronic co-expressing vector was designated as pBar_Triple (**Figure 2**) and used for transformation of *E. coli* DH5αc cells and subsequent IPTG-controlled co-expression of the contained cDNAs for all constituents of the CH/L system.

3.2. Co-Expression of Bovine CH/L System Proteins in *E. coli* Cells

Co-expression of bovine P450scc, AdR and Adx in *E. coli* DH5αc transformed with pBar_Triple was carried out using medium supplemented with ampicillin upon induction of transcription of heterologous cDNAs from the recombinant plasmid (as per 2.3). Recombinant proteins were identified in the cell homogenates (as per 2.4) using electrophoretic analysis in polyacrylamide gel followed by Western-blotting with antibodies against P450scc, AdR or Adx. The immunodetection results are presented in **Figure 3**.

According to **Figures 3A-C**, recombinant protein molecular weights (mP450scc, 54 kD; mAdR, 53 kD; mAdx, 12 kD) and their immunospecificity corresponded to the mature forms of P450scc, AdR and Adx from bovine adrenal cortex.

3.3. Activity of Bovine CH/L System Reconstructed in Bacterial Cells

The recombinant strain *E. coli* DH5αc/Triple25 was tested for the activity towards cholesterol.

As shown in **Figure 4**, pregnenolone was formed both

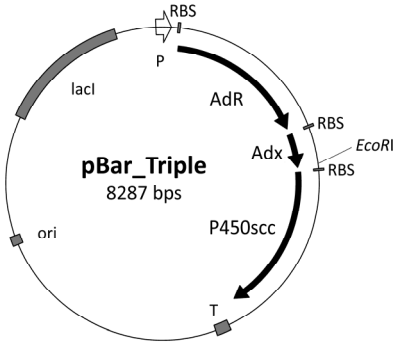

Figure 2. Structure of the tricistronic plasmid pBar_Triple for co-expression of the cholesterol hydroxylase/lyase system proteins. pBar_Triple (8.287 kb) contains cDNAs for bovine cytochrome P450scc, AdR and Adx in a single expression cassette. The unique EcoRI site was used during the construction. An ampicillin resistance gene allows selection for plasmid uptake. Expression of inserted cDNAs is driven by the tac1/tac2/lacUV5 promoter.

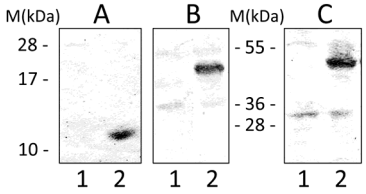

Figure 3. Co-expression of P450scc, AdR and Adx in *E. coli* cells. A cellular homogenates (60 μg of total protein each), from *E. coli* culture were subjected to SDS-PAGE (15% (A) or 10% (B and C) acrylamide) and Western-blotting. Recombinant proteins were detected with antisera to Adx (A), AdR (B) and P450scc (C). In each case, lane 1 corresponds to homogenate of non-transformed cells, and lane 2 corresponds to homogenate *E. coli* DH5αc/pBar_Triple.

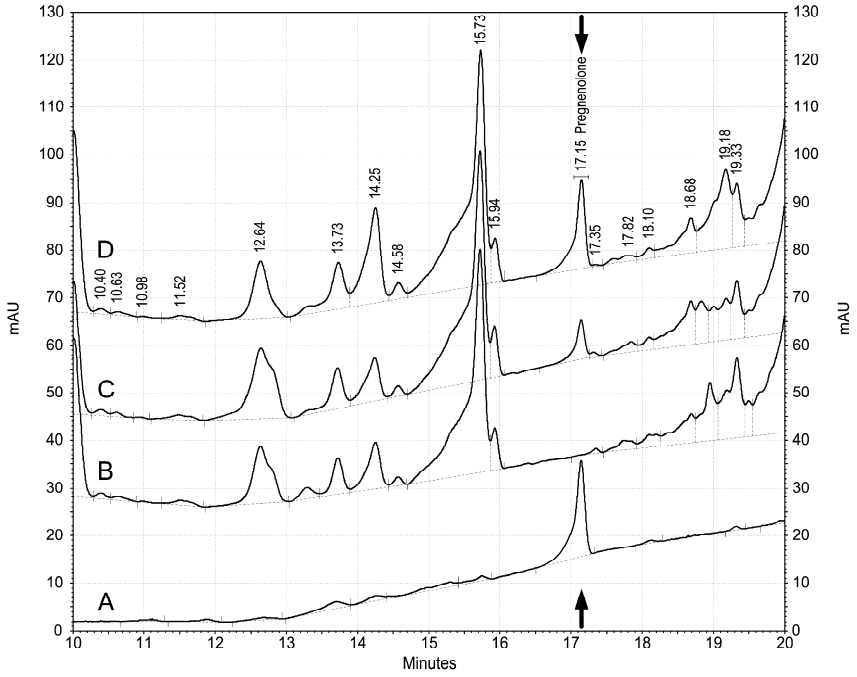

Figure 4. Pregnenolone formation from cholesterol by *E. coli* DH5*α*c/Triple. The cells were grown as described in 2.3 and incubated during 48 h with 0.5 mM cholesterol which was added as an aqueous MCD-solution. Reversed-phase HPLC profiles of cultivation broth extracts were obtained in a linear gradient of acetonitrile as described in 2.6, method 3 at 200 nm. Retention time of pregnenolone (17.15 min) is indicated by arrows. A—pregnenolone external standard injection (4.5 mg/l); B—control profile (at incubation of recipient *E. coli* DH5*α*c strain with cholesterol); C—*E. coli* DH5*α*c/Triple without induction; D—*E. coli* DH5*α*c/Triple cells induced with 0.5 mM IPTG.

by IPTG-induced (A) and non-induced cells (B) thus evidencing that promoter which controls transcription of heterologous cDNA is not a "strongly inducible" one.

It is well-known that cholesterol is a poorly soluble substrate with an aqueous solubility of 2 - 10 mg per liter [32]. This extremely poor solubility may be a reason of low substrate availability to microbial cell enzymes. Different approaches are used in order to provide cholesterol availability to microbial cells (for review, see [33]). We assumed that cholesterol micronization with detergents, or its solubilization using cyclodextrins (CDs) are the most suitable modes of substrate addition which can facilitate cholesterol conversion in our case.

The dependence of pregnenolone formation by recombinant *E. coli* DH5*α*c/Triple on the mode of cholesterol addition and IPTG-induction is illustrated by **Figure 5**. As shown, the amount and rate of pregnenolone formation by IPTG-induced cells was 2 - 3 times higher than in the case of non-induced cells. Pregnenolone concentration by IPTG-induced cells reached its maximal level for 24 h, while this period was no less than 48 h for non-induced cells (**Figure 5**).

As shown in **Figure 5**, addition of cholesterol as a MCD solution resulted in higher pregnenolone production by the non-induced cells, while very low activity

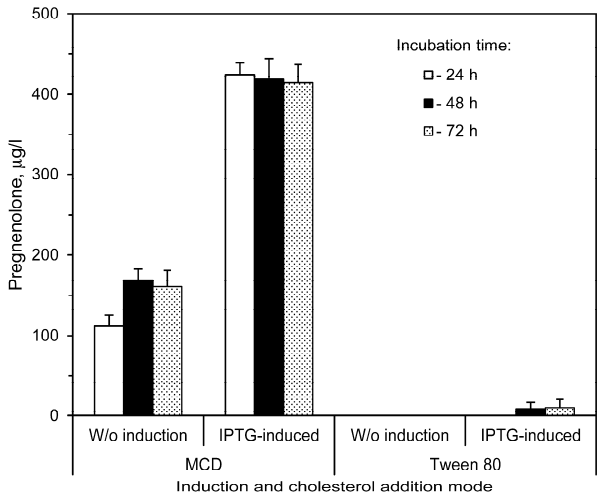

Figure 5. Influence of IPTG-induction and cholesterol addition mode (in aqueous MCD solution or as Tween 80—stabilized suspension) on pregnenolone formation by recombinant *E. coli* DH5*α*c/Triple at different incubation times. The average values of three measurements are presented.

(almost on the lowest level of reliable detection range) was observed when cholesterol was added as a suspension with Tween 80. When using IPTG-induced culture and MCD-solubilized cholesterol, pregnenolone yield

reached 0.192 μmol/l, while more than 3-fold less pregnenolone concentration was detected when cholesterol micronization with Tween 80 was applied.

Thus, the results evidence that the mode of cholesterol addition is of importance for the activity of the recombinant cells. CD-mediated enhancement of microbial sterol side chain cleavage was reported earlier [34-36].

The enhancement effect can be mainly attributed to steroid solubilization by the formation of water-soluble inclusion complex of CDs with steroids. Besides, CDs may facilitate the transport of poorly soluble hydrophobic substances to and from microbial cells, thus functioning as effective substrate/product delivery systems. As reported, CDs themselves do not penetrate through bacterial cell membranes, but can disrupt the outermost cell wall layers of the gram-positive bacteria [36]. The detail study of MCD effect on the cells of *E. coli* is out of the purposes of the current work, and can be investigated especially.

3.4. Comparison of *E. coli* DH5αc/Triple with Analogous Strains

In our previous works, several recombinant strains have been created on the base of *E. coli* JM-109, bearing CH/L system proteins [7,21,22] (**Table 1**). Three genetic constructs were designed for co-expression of mature proteins (lacking of N-terminal addressing sequences) of the CH/L system and used at the construction of the strains. The first construct was pTrc99A/P450scc/AdR/Adx containing (similar to pBar_Triple) P450scc, AdR, and Adx cDNA within the same expression cassette. The other construct was pTrc/P450scc/AdR.Adx containing P450scc and AdR cDNA within the same expression cassette, and the gene of Adx was inserted into the same plasmid within a separate transcription unit (regulated by its own promoter and terminator). The third plasmid—pTrc99A/F2, contained hybrid cDNA encoding the fusion protein P450scc-AdR-Adx. For re-construction of mammalian CH/L in these cases, cDNAs of different origin were applied—bovine P450scc, and human AdR and Adx.

As evidenced by immune-enzyme analysis (ELISA), the cell-free homogenates of the recombinants containing these plasmids demonstrated *in vitro* hydroxylase/lyase activity towards 22(R)-hydroxy cholesterol [7,21,22]. However, no *in vivo* activity was detected. In the current study, we compared cholesterol conversion by these strains with newly constructed *E. coli* DH5αc/Triple25. The experiments were carried out at the conditions optimized for *E. coli* DH5αc/Triple25 as described above.

Pregnenolone was detected in very low amounts at cholesterol incubation with the D36 and E32 strains (**Table 1**) cultured under conditions of induced expression of heterologous cDNAs. In order to provide reliable quantitative detection, the extracts were 10 - 30-fold concentrated before analysis. The approximately two-fold higher level of pregnenolone production was observed in the case of D36 strain as compared to E32. It is well correlated with the expected higher level of Adx expression in D36 which evidently enhanced functioning of the whole system.

Besides, in order to confirm the identity of pregnenolone formed, it was converted to more reliably detected progesterone by commercial cholesterol oxidase (as per 2.6.2). The control experiment confirmed complete enzymatic conversion of pregnenolone to progesterone. Thus, both methods (as per 2.6 and 2.7) confirmed the formation of pregnenolone by the strains of D36 and E32.

The strain DH5αc/Triple25 produced up to 420 μg/ml pregnenolone, thus demonstrating 7 - 13-fold higher activity as compared with D36 and E32 strains, correspondingly (**Table 2**).

Low efficiency of cholesterol conversion by *E. coli*

Table 2. Conversion of cholesterol to pregnenolone by recombinant *E. coli* strains.

Recombinant *E. coli* strain[a]	IPTG induction	Mode of substrate addition	Pregnenolone		Time, h
			μg/l	μM	
DH5αc/Triple25	+	MCD	420 ± 15	1.33 ± 0.047	24
	-	MCD	168 ± 14	0.53 ± 0.044	48
	+	Tween 80	Traces[b]	Traces[b]	72
JM-109/D36	+	MCD	60.5 ± 3	0.192 ± 0.009	72
	+	Tween 80	18.35 ± 3.5	0.058 ± 0.011	72
JM-109/E32	+	MCD	30.8 ± 7	0.097	72
	+	Tween 80	0	0	72
JM-109/F2	+	MCD	Traces[b]	Traces[b]	72
	+	Tween 80	0	0	72

The average values of three measurements are presented. [a]The plasmids indicated in the table contain cDNAs encoding the human (h) or bovine (b) mature forms of proteins; [b]Pregnenolone amount was lower than detection reliability.

JM-109/F2 strain which expressed fusion CH/L system did not allow reliable detection of pregnenolone. This result indicated that *in vivo* activity of the fused recombinant CH/L is much lower than that of the D36 and E32 strains which were transformed with plasmids, allowing simultaneous expression of individual CH/L system proteins. This is consistent with the data reported on *in vitro* activity of the recombinant CH/L systems. Probably, the catalytic centers of the fused domains in this CH/L were either unable to interact, or misfolded thus leading to low cholesterol side-chain cleavage activity [21].

Much higher *in vivo* activity of *E. coli* DH5αc/Triple25 synthesizes the three component bovine CH/L system, as compared with JM109 strain synthesizing the proteins of different origin (bovine P450scc and human AdR and Adx) evidence the preference of homologous P450scc system over its heterologous analog. But the reason of this difference is not fully clear: either heterologous system with proteins of different origin is less active, or the more optimal stoichiometry of the expressed proteins is provided in *E. coli* DH5αc/Triple25. The latter can be particularly caused by different location of cDNA-sequences of the component proteins in the expression cassette of the recombinant plasmids used. For instance, the order shift in the location of cDNAs encodes for cytochrome P45027B1, Adx and NADPH; cytochrome P450-reductase in the expression cassette of the tandem plasmids resulted in 3-5-fold change of protein content [29]. Besides, pBar_Triple vector directs the synthesis of the truncated bovine Adx (4-108) [25]. Different aggregation of foreign protein molecules also can not be excluded—the formation of inactive forms of the recombinant P450scc, AdR and Adx, *i.e.*, the so called "inclusion bodies", in *E. coli* was reported earlier [22].

In conclusion, the bovine CH/L system was firstly reconstructed in *E. coli* using pBar_Triple vector. The recombinant strain created is capable to produce up to 420 μg/l of pregnenolone for 24 h, and the level of the productivity was higher than hitherto reported for the similar *E. coli* recombinants. The strain can be applied as a modeling system in the basic research of mammalian steroidogenic system features.

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REFERENCES

[1] Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsulus, I.C. and Nebert, D.W. (1996) P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, **6**, 1-42. http://dx.doi.org/10.1097/00008571-199602000-00002

[2] Orme-Johnson, N.R. (1990) Distinctive properties of adrenal cortex mitochondria. *Biochimica et Biophysica Acta*, **1020**, 213-231. http://dx.doi.org/10.1016/0005-2728(90)90151-S

[3] Miller, W.L. (2008) Steroidogenic enzymes. *Endocrine Development*, **13**, 1-18.

[4] Szczebara, F.M., Chandelier, C., Villeret, C., Masurel, A., Bourot, S., Duport, C., Blanchard, S., Groisillier, A., Testet, E., Costaglioli, P., Cauet, G., Degryse, E., Balbuena, D., Winter, J., Achstetter, T., Spagnoli, R., Pompon, D. and Dumas, B. (2003) Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nature Biotechnology*, **21**, 143-149. http://dx.doi.org/10.1038/nbt775

[5] Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsulus, I.C., Gotoh, O.,Okuda, K. and Nebert, D.W. (1993) The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA and Cell Biology*, **12**, 1-51. http://dx.doi.org/10.1089/dna.1993.12.1

[6] Wada, A., Mathew, P.A., Barnes, H.J., Sanders, D., Estabrook, R.W. and Waterman, M.R. (1991) Expression of functional bovine cholesterol side-chain cleavage cytochrome P450 (P450scc) in *E. coli*. *Archives of Biochemistry and Biophysics*, **290**, 376-380. http://dx.doi.org/10.1016/0003-9861(91)90554-V

[7] Novikova, L.A., Faletrov, Y.V., Kovaleva, I.E., Mauersberger, S., Luzikov, V.N. and Shkumatov, V.M. (2009) From structure and functions of steroidogenic enzymes to new technologies of gene engineering. *Biochemistry* (Moscow), **74**, 1482-1504. http://dx.doi.org/10.1134/S0006297909130057

[8] Heinz, A., Hannemann, F., Müller, J.J., Heinemann, U. and Bernhardt, R. (2005) The interaction domain of the redox protein adrenodoxin is mandatory for binding of the electron acceptor CYP11A1, but is not required for binding of the electron donor adrenodoxin reductase. *Biochemical and Biophysical Research Communications*, **338**, 491-498. http://dx.doi.org/10.1016/j.bbrc.2005.08.077

[9] Pikuleva, I.A., Mackman, R.L., Kagawa, N., Waterman, M.R. and Ortiz de Montellano, P.R. (1995) Active-site topology of bovine cholesterol side-chain cleavage cytochrome P450 (P450scc) and evidence for interaction of tyrosine 94 with the side chain of cholesterol. *Archives of Biochemistry and Biophysics*, **322**, 189-197. http://dx.doi.org/10.1006/abbi.1995.1451

[10] Headlam, M. J., Wilce, M. C. and Tuckey, R. C. (2003) The F-G loop region of cytochrome P450scc (CYP11A1) interacts with the phospholipid membrane. *Biochimica et Biophysica Acta*, **1617**, 96-108. http://dx.doi.org/10.1016/j.bbamem.2003.09.007

[11] Strushkevich, N., MacKenzie, F., Cherkesova, T., Grabovec, I., Usanov, S. and Park, H.W. (2011) Structural basis for pregnenolone biosynthesis by the mitochondrial monooxygenase system. *Proceedings of the National Academy of Sciences of USA*, **108**, 10139-10143. http://dx.doi.org/10.1073/pnas.1019441108

[12] Shet, M.S., Fisher, C.W., Tremblay, Y., Belanger, A., Conley, A.J., Mason, J.I. and Estabrook, R.W. (2007) Comparison of the 17 alpha-hydroxylase/C17,20-lyase activities of porcine, guinea pig and bovine P450c17 using purified recombinant fusion proteins containing P450c17 linked to NADPH-P450 reductase. *Drug Metabolism Reviews*, **39**, 289-307. http://dx.doi.org/10.1080/03602530701468391

[13] Oyama, T., Kagawa, N., Sugio, K., Uramoto, H., Hatano, O., Harada, N., Kaneko, K., Kawamoto, T. and Yasumoto, K. (2009) Expression of aromatase CYP19 and its relationship with parameters in NSCLC. *Frontiers in Bioscience*, **14**, 2285-2292. http://dx.doi.org/10.2741/3379

[14] Mast, N., Linger, M. and Pikuleva, I.A. (2012) Inhibition and stimulation of activity of purified recombinant CYP11A1 by therapeutic agents. *Molecular and Cellular Endocrinology*, **371**, 100-106. http://dx.doi.org/10.1016/j.mce.2012.10.013

[15] Woods, S.T., Sadleir, J., Downs, T., Triantopoulos, T., Headlam, M.J. and Tuckey, R.C. (1998) Expression of catalytically active human cytochrome P450scc in *Escherichia coli* and mutagenesis of isoleucine-462. *Archives of Biochemistry and Biophysics*, **353**, 109-115. http://dx.doi.org/10.1006/abbi.1998.0621

[16] Shkumatov, V.M., Radyuk, V.G., Faletrov, Y.V., Vinogradova, A.A., Luzikov, V.N. and Novikova, L.A. (2006) Expression of cytochrome P450scc in *Escherichia coli* cells: Intracellular location and interaction with bacterial redox proteins. *Biochemistry* (Moscow), **71**, 884-892. http://dx.doi.org/10.1134/S0006297906080104

[17] Brandt, M.E. and Vickery, L.E. (1992) Expression and characterization of human mitochondrial ferredoxin reductase in *Escherichia coli*. *Archives of Biochemistry and Biophysics*, **294**, 735-740. http://dx.doi.org/10.1016/0003-9861(92)90749-M

[18] Sagara, Y., Wada, A., Takata, Y., Waterman, M.R., Sekimizu, K. and Horiuchi, T. (1993) Direct expression of adrenodoxin reductase in *Escherichia coli* and the functional characterization. *Biological & Pharmaceutical Bulletin*, **7**, 627-630. http://dx.doi.org/10.1248/bpb.16.627

[19] Uhlmann, H., Beckert, V., Schwarz, D. and Bernhardt, R. (1992) Expression of bovine adrenodoxin and site-directed mutagenesis of [2Fe-2S] cluster ligands. *Biochemical and Biophysical Research Communications*, **188**, 1131-1138. http://dx.doi.org/10.1016/0006-291X(92)91349-U

[20] Sagara, Y. and Aramaki, H. (1998) Overproduction in *Escherichia coli* and characterization of the precise mature form of rat adrenodoxin. *Biological & Pharmaceutical Bulletin*, **21**, 1106-1109. http://dx.doi.org/10.1248/bpb.21.1106

[21] Nazarov, P.A., Drutsa, V.L., Miller, W.L., Shkumatov, V.M., Luzikov, V.N. and Novikova, L.A. (2003) Some features of formation and functioning of fused cholesterol hydroxylase/lyase. *DNA and Cell Biology*, **22**, 243-252. http://dx.doi.org/10.1089/104454903321908638

[22] Shashkova, T.V., Luzikov, V.N. and Novikova, L.A. (2006) Coexpression of all constituents of the cholesterol hydroxylase/lyase system in *Escherichia coli* cells. *Biochemistry* (Moscow), **71**, 810-814. http://dx.doi.org/10.1134/S0006297906070145

[23] Kembaren, R.F. and Janssen, D.B. (2008) Coexpression of three-component cytochrome P450scc in *E. coli*. In: 9th International symposium on Cytochrome P450 Biodiversity and Biotechnology, Abstracts, Nice, June 8-12, 2008, p. 70.

[24] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

[25] Hannemann, F., Virus, C. and Bernhardt, R. (2006) Design of an *Escherichia coli* system for whole cell mediated steroid synthesis and molecular evolution of steroid hydroxylases. *Journal of Biotechnology*, **124**, 172-181. http://dx.doi.org/10.1016/j.jbiotec.2006.01.009

[26] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685. http://dx.doi.org/10.1038/227680a0

[27] Sidhu, R.S. and Bollon, A.P. (1987) Analysis of alpha-factor secretion signals by fusing with acid phosphatase of yeast. *Gene*, **54**, 175-184. http://dx.doi.org/10.1016/0378-1119(87)90485-9

[28] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randal, R.J. (1951) Protein measurement with folin-phenol reagent. *The Journal of Biological Chemistry*, **193**, 265-275.

[29] Sawada, N., Sakaki, T., Kitanaka, S., Takeyama, K., Kato, S. and Inouye, K. (1999) Enzymatic properties of human 25-hydroxyvitamin D_3 1α-hydroxylase. *European Journal of Biochemistry*, **265**, 950-956. http://dx.doi.org/10.1046/j.1432-1327.1999.00794.x

[30] Salamanca-Pinzón, S.G. and Guengerich, F.P. (2011) A tricistronic human adrenodoxin reductase-adrenodoxin-cytochrome P450 27A1 vector system for substrate hydroxylation in *Escherichia coli*. *Protein Expression and Purification*, **79**, 231-236. http://dx.doi.org/10.1016/j.pep.2011.05.008

[31] Ringle, M., Khatri, Y., Zapp, J., Hannemann, F. and Bernhardt, R. (2012) Application of a new versatile electron transfer system for cytochrome P450-based *Escherichia coli* whole-cell bioconversions. *Applied Microbiology and Biotechnology*, **97**, 7741-7754. http://dx.doi.org/10.1007/s00253-012-4612-0

[32] Haberland, M.E. and Reynolds, J.A. (1973) Self-association of cholesterol in aqueous solution. *Proceedings of the National Academy of Sciences of USA*, **70**, 2313-2316. http://dx.doi.org/10.1073/pnas.70.8.2313

[33] Donova, M.V. and Egorova, O.V. (2012) Microbial steroid transformations: Current state and prospects. *Applied Microbiology and Biotechnology*, **94**, 1423-1447.

http://dx.doi.org/10.1007/s00253-012-4078-0

[34] Hesselink, P.G.M., van Vliet, S., de Vries, H. and Witholt, B. (1989) Optimization of steroid side-chain cleavage by *Mycobacterium* sp. in the presence of cyclodextrins. *Enzyme and Microbial Technology*, **11**, 398-404. http://dx.doi.org/10.1016/0141-0229(89)90133-6

[35] Jadoun, J. and Bar, R. (1993) Microbial transformations in a cyclodextrin medium. Part 4. Enzyme vs microbial oxidation of cholesterol. *Applied Microbiology and Biotechnology*, **40**, 477-482. http://dx.doi.org/10.1007/BF00175734

[36] Donova, M.V., Nikolayeva, V.M., Dovbnya, D.V., Gulevskaya, S.A. and Suzina, N.E. (2007) Methyl-β-cyclodextrin alters growth, activity and cell envelope features of sterol transforming mycobacteria. *Microbiology*, **153**, 1981-1992. http://dx.doi.org/10.1099/mic.0.2006/001636-0

