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A tricistronic human adrenodoxin reductase-adrenodoxin–cytochrome P450 27A1 vector system for substrate hydroxylation in *Escherichia coli*

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Abstract

Cytochrome P450 (P450) 27A1 catalyzes 27-hydroxylation of cholesterol and 25-hydroxylation of vitamin D₃, serving as an important component for the maintenance of lipid homeostasis. In eukaryotic cells P450 27A1 is a membrane-bound protein located on the inner mitochondrial membrane and requires two auxiliary reduction partners, adrenodoxin (Adx) and NADPH-adrenodoxin reductase (Adr), for catalysis in the bile acid biosynthesis pathway. A strategy was developed for the functional coexpression of P450 27A1 with Adr and Adx in a tricistronic fashion (single RNA, three proteins) in *Escherichia coli*, mimicking the mitochondrial P450 system. Intact bacterial cells coexpressing the P450 vector (pTC27A1) efficiently hydroxylated cholesterol at the 27 position as well as vitamin D₃ at the 25 position when supplemented with glycerol as a carbon source. Thus, *E. coli* containing pTC27A1 is able to hydroxylate cholesterol in a self-sufficient fashion and is suitable for further applications of protein interaction, drug discovery, and inhibitor evaluation and for the study of other mitochondrial P450s and oxysterol production in microorganisms without a need for membrane reconstitution, membrane simulation by detergents, or purification of the components.

Keywords

Cytochrome P450; Vitamin D; Cholesterol; Tricistronic vector; Adrenodoxin

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Supplementary data

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Introduction

P450 enzymes are present in both the endoplasmic reticulum (microsomes) and mitochondria in animal cells [3]. All of the P450s are membrane-bound or need a membrane environment to function, and all need at least one redox partner [4]. The components of microsomal P450 systems are all membrane-bound, whereas in the mitochondria this system consists of membrane-bound P450 and the soluble redox partners Adr and Adx. In bacteria both P450 and its accessory enzymes are often soluble.

Sterols are essential in the membranes of all eukaryotic organisms, although in prokaryotes their biosynthesis is completely absent with only a few exceptions [5–7]. In eukaryotes, sterol functions include control of membrane fluidity, permeability, cell proliferation, signal transduction, and modulation of membrane-bound enzymes [8, 9]. Cholesterol is a major sterol of mammalian cells and is required for viability and cell proliferation. More than 90% of cellular cholesterol is located in the plasma membrane [10], and its conversion into bile acids is of major importance in maintenance and homeostasis. In the adult human liver, ~500 mg of cholesterol is converted into bile acids each day [11]. Oxysterols are derivatives of enzymatic or nonenzymatic cholesterol oxidation, and their cellular concentrations are dependent on intracellular cholesterol biosynthesis and biosynthesis of bile acids and steroid hormones as well as on uptake from exogenous sources such as oxidized lipoproteins [12]. Even though oxysterols are heterogeneous in their biological activities, they have the common property of transfer between membranes at rates orders of magnitude faster than cholesterol due to their higher polarity and poorer membrane packing. Depending on the nature and location of the oxygen substitution, oxysterols have distinct effects on the biophysical properties of membranes [13].

Several P450 enzymes are involved in the metabolism of cholesterol, both by the classical (hepatic) and alternative (extrahepatic) pathways [14]. Mitochondrial sterol 27-hydroxylase (P450 27A1) is the key enzyme in the alternative pathway [15] and produces 27(OH)C, a biologically active metabolite whose levels are elevated in hypercholesterolemia and found in atherosclerotic lesions [1, 16]. Like other mitochondrial P450s, P450 27A1 is an integral membrane protein and receives electrons for the catalysis of monooxygenation reactions from NADPH via Adr, a 51 kD FAD-containing enzyme, the first component in the mitochondrial P450 electron transfer systems. Adr receives electrons from NADPH and transfers them via the [2Fe-2S]-ferredoxin-type carrier Adx, a 14 kDa soluble protein (128 amino acids). Adx carries electrons from AdR, via its Fe₂S₂ cluster, to several P450 enzymes including P450 27A1 [17, 18]. This P450 represents a bridge between hormone and oxysterol pathways, catalyzing the 25-hydroxylation of vitamin D₃ [19, 20] and bile acid precursors in mammals. At physiological concentrations 25(OH)D₃ does not seem to have a function of its own but rather is the precursor of the active hormone 1,25(OH)₂D₃ [21].

Similarities between mitochondria and bacteria are known [22]. The use of bacteria as carriers of several mitochondrial genes is exemplified by the fact that *Escherichia coli* is able to stably maintain the entire mitochondrial genome isolated from mouse liver [23], demonstrating the potential of the concept of use of bacteria for mitochondrial gene expression and interactions for engineering and biochemical studies. Furthermore, the use of a physiological environment in a whole cell system has been shown to be advantageous as a biocatalyst in redox reactions when compared to applications with isolated enzymes [24].

¹Abbreviations used: 17-EE, 17 α -ethynylestradiol; 25(OH)D₃, 25-hydroxyvitamin D₃; 27(OH)C, 27-hydroxycholesterol; Adr, NADPH-adrenodoxin reductase; Adx, adrenodoxin; dansyl chloride, 5-dimethylaminonaphthalene-1-sulfonyl chloride; DMAP, 4,4-(dimethylamino)pyridine; HP β CD, 2-(hydroxypropyl)- β -cyclodextrin; IPTG, isopropyl β -D-1-thiogalactopyranoside; *m/z*, mass-to-charge ratio; P450, cytochrome P450; RBS, ribosome binding site; SRM, selected reaction monitoring; UPLC, ultra performance/pressure liquid chromatography

Published methods for evaluation of P450 27A1 function rely on the addition of Adr and Adx, typically purified from bovine adrenal glands or expressed heterologously in bacteria [25, 26]. Studies of this P450, together with Adx and Adr—required for the catalysis, have not been reported before using a whole cell environment resembling the mitochondrial context.

There are few examples in the literature of the use of tricistronic vectors for the expression of other P450s together with the redox partners, including studies of the metabolism of camphor by bacterial P450 101A1 [27] and camphor and limonene [28], the co-expression of Adx and Adr together with mitochondrial P450 11B1 and 11B2 in a V79-4 cell line used successfully for inhibitor screening [29], and the coexpression of human P450 2E1 in combination with cytochrome *b*₅, rat NADPH-P450 reductase and cytochrome *b*₅ reductase in *Salmonella typhimurium* for the evaluation of nitrosamine activation [30, 31]. From a synthetic biology point of view the RBS, promoter regions, and some other specific and regulatory sequences can be considered as parts to be combined into assemblies with varying functions [32, 33]. Here we describe the integration of three parts to produce a tricistronic vector that, when incorporated into *E. coli*, allows the bacteria to metabolize either cholesterol or vitamin D₃ through a P450 hydroxylation reaction.

Materials and methods

Chemical and reagents

27(OH)C (cholest-5-ene-3 β ,27-diol, 25*R*) (Avanti Polar Lipids, Alabaster, AL) was used as a standard for LC/MS analysis and 17-EE (Sigma-Aldrich, St. Louis, MO) was used as an internal standard. Cholesterol (solubilized in HP β CD, Sigma-Aldrich) and vitamin D₃ (Alfa Aesar, Ward Hill, MA) were used as substrates, incorporated in the culture medium. DMAP (Sigma-Aldrich) and dansyl chloride (Invitrogen, San Diego, CA) were used in chemical derivatization for LC/MS analysis. HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA)

Construction of plasmids

A scheme is presented of the series of plasmids constructed to obtain the final vector for tricistronic expression (Figure 1). Human P450 27A1 cDNA was obtained from construct FL27A1 (monocistronic vector), bovine Adx cDNA was obtained from construct pBA1159, and bovine Adr cDNA was obtained from construct pCW-Bar (all gifts from I. Pikuleva, Case Western Reserve University, Cleveland, OH) [26]. All cDNAs were modified by PCR and subcloned to include the restriction sites, sequence spaces, and RBS required to link them in the final functional tricistronic vector. Monocistronic expression plasmids for all cDNAs were first cloned into a pET22 vector (Novagen, San Diego, CA) and the *Adx* and *Adr* sequences were modified by the addition of (His)₆ tag sequences (C-terminal) using this plasmid. First, PCR of *Adx* was carried out using 20F (5'-CAGAATTCATGAGCTCAGAAGATA-3') and 14R (5'-CCAAGCTTGCATACGGTCGACTTTC-3') primers. The PCR product was cloned into pKK223 (commercially not available). Using this construction, a second PCR was carried out using 15F (5'-CATGCCATGGACTAGTCGTATAATGTGTGG-3') and 14R primers to amplify a sequence containing the RBS from pKK223, which was then subcloned into pET22 (p2KX vector). For *Adr*, the first PCR was carried out using 35F (5'-CAGAATTCATGAGCACTCAAGAAC-3') and 32R (5'-CGGTCGACAAGCTTACTAGTCAGTGGTGG-3') primers. The PCR product was cloned into p2KX replacing *Adx* using EcoRI and SalI sites (p2K32A). Using this construct a second PCR was carried out using 16F (5'-CCATATGTCGACTGAGCGGATAAC-3') and 32R primers and the PCR product was cloned downstream of *Adx* in p2KX using SalI and

HindIII flanking sites to obtain the p2KXAH vector (this strategy was followed because the restriction sites in the original sequences that match with frequently restriction sites for cloning, e.g. BamHI (*Adr*), XbaI, NdeI (*Adx*), and HindII (*CYP27A1*). In order to complete the final vector, PCR of *27A1* was carried out using 33F (5'-CCATATGGCTCTTCCATCTGATAAAGC-3') and 34R (5'-CGGACTAGTGATCCCCTCAGTGG-3'). The PCR product was cloned into a modified version of pCW containing NdeI and SpeI sites together with the fragment of *Adx* and *Adr* (RBS-*Adx*-RBS-*Adr*) obtained from the p2KXH vector after SpeI restriction. The final construct, pTC27A1 (Fig. 1) contains the functional order: *lacUV*/two copies of *tac* promoter/cia linker/translation initiation region derived from the phage T4 lysozyme gene including RBS/*CYP27A1* coding region/translational stop/RBS/*Adx* coding region/translational stop/RBS/*Adr* coding region/translational stop/trpA terminator (*27A1:Adx:Adr*). In parallel, a control vector containing P450 and *Adx* was designed by excision of *Adr* segment (pCWx). C-Terminal (His)₆ tags were added to all three proteins for immunoquantitation (using a single anti-(His)₆ antibody) and to facilitate any purification in the future. All constructions were analyzed by their restriction patterns using Fermentas enzymes (Life Sciences, Glen Burnie, MD) and verified by nucleotide sequence analysis in the Vanderbilt facility.

Protein expression and immunodetection

E. coli DH5 α cells containing the chaperone system pGro12 ES/EL were used for the expression trials of the pTC27A (*27A1:Adx:Adr*) construct. Both the plasmids pGro12 ES/EL and pTC27A1 were transformed into *E. coli* DH5 α competent cells and selected on Luria-Bertani medium plates containing 100 μ g ampicillin/mL and 50 μ g kanamycin/mL. Single colonies were grown overnight in Luria-Bertani liquid medium (200 μ g ampicillin/mL and 50 μ g kanamycin/mL) at 37 °C (225 rpm). Each overnight culture was then inoculated into a 250-mL flask containing 100 mL of Terrific Broth supplemented with 0.4% glycerol (v/v), 2 mg/mL bactopectone, 1 mM NaCl, 1 mM thiamine, a mixture of trace elements (50 μ M FeCl₃, 1 mM MgCl₂, 2.5 mM (NH₄)₂SO₄), 200 μ g of ampicillin/mL, and 50 μ g of kanamycin/mL. The cultures were incubated at 37 °C and 250 rpm for 6 h. Expression of *27A1-Adx-Adr* (under control of the *lac* promoter) was induced by the addition of 1.0 mM IPTG and 1.0 mM 5-aminolevulinic acid (w/v), the latter used to support heme synthesis. For the expression of the chaperone system by pGro12 ES/EL, 0.1% arabinose (w/v) was added to the cultures. The incubations continued for 48 h at 29 °C and 150 rpm. Expression levels were monitored at 24 and 48 h after induction by immunoblotting. Briefly, cellular lysates were collected from the bacterial cultures and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (pre-cast gels, 10% w/v, Bio-Rad Laboratories, Hercules, CA) and then transferred to a polyvinylidene fluoride membrane. The membrane was then incubated with mouse monoclonal anti-(His)₆-peroxidase (50 U/mL, Roche Diagnostics, Indianapolis, IN) at a 1:1,500 dilution (v/v). After the membrane was washed, it was incubated for 1 h at room temperature with IR-Dye800 goat anti-mouse IgG (1 mg/mL) at a dilution of 1:5,000 (1 mg/mL, LI-COR Biosciences, Lincoln, NE). The membrane was visualized in an Odyssey Imager (LI-COR Biosciences).

Measurement of metabolism of cholesterol and vitamin D₃ with whole cell systems

Single colonies of *E. coli* DH5 α containing pTC27A1 (P450 *27A1:Adx:Adr* plus pGroEl/Es plasmid) and *E. coli* DH5 α containing FL*27A1* (monocistronic vector, plus pGroEl/Es plasmid) were incubated overnight in Luria-Bertani liquid media (200 μ g ampicillin/mL and 50 μ g kanamycin/mL) at 37 °C, 250 rpm. Each overnight culture was then inoculated into a 50-mL flask containing 15 mL of Terrific Broth medium supplemented with 0.4% glycerol (v/v), bactopectone (2 mg/mL), NaCl (1 mM), thiamine (1 mM), a mixture of trace elements

(50 μM FeCl_3 , 1 mM MgCl_2 , 2.5 mM $(\text{NH}_4)_2\text{SO}_4$), 200 μg ampicillin/mL, and 50 μg kanamycin/mL. The cultures were incubated at 37 °C and 250 rpm for 6 h. Expression was induced by the addition of 1.0 mM IPTG, 1.0 mM 5-aminolevulinic acid, and 0.1% arabinose (w/v). Cholesterol (0.3 mM, solubilized in HP β CD, 0.45%, w/v) or vitamin D₃ (solubilized in 95% ethanol, added to a final concentration of $\leq 1\%$, w/v) was also added to the medium at this time. P450 expression was assessed by the appearance of CO-reduced difference spectra using an extinction coefficient of 91 $\text{mM}^{-1} \text{cm}^{-1}$ for the absorbance difference between 450 and 490 nm. Bacterial cultures (1 mL) were extracted with 3.0 mL of CH_2Cl_2 including 1.0 μmol 17-EE (as an internal standard), mixed with a vortex device for 2 min, and then centrifuged ($3,200 \times g$, 5 min). The organic layer (1 mL) was taken to dryness under an N_2 stream. A modified dansylation method for improving the sensitivity of MS detection of unactivated alcohols [34] was used for quantification of 27(OH)C using 17-EE as an internal standard for LC-MS analysis. Briefly, the samples were dissolved in 200 μL of CH_2Cl_2 containing 2 mg of dansyl chloride, 0.5 mg of DMAP, and 2 μL of triethylamine and then incubated at 65 °C for 1 h. For analysis, the samples were dried under an N_2 stream, dissolved in 250 μL CH_3CN , and placed in amber glass vials. 27(OH)C detection (LC-MS) was performed on a Waters Acquity UPLC system connected to an TSQ Vantage triple-stage quadrupole mass spectrometer (Thermo Scientific) using a Hypersil GOLD column (3 μm , 150 mm $\times$ 2.1 mm). For LC, the solvent system was composed of solvent A (5% CH_3CN and 0.1% HCO_2H , v/v) and solvent B (85% CH_3CN , 10% $(\text{CH}_3)_3\text{COCH}_3$, and 0.1% HCO_2H , v/v) using a gradient: 50% A (v/v) from 0–0.5 min, linear gradient to 0% A from 0.5–2.5 min, hold at 0% A from 2.5–10 min, linear gradient to 50% A (v/v) from 10–10.5 min, hold at 80% A (v/v) from 10.5–13.5 min. The temperature of the column was maintained at 40 °C. Samples (15 μL) were stored at 4 °C and infused with an autosampler. MS analyses were performed in the positive ion mode. Electrospray ionization (ESI) conditions for dansylated 27(OH)C were as follows: spray voltage, 4.6 kV; auxiliary gas flow rate, 25 arbitrary units; sheath gas, 43 arbitrary units; capillary temperature, 270 °C; and tube lens voltage, 161 V. ESI conditions for dansylated 25(OH)D₃ were as follows: spray voltage, 5.0 kV; auxiliary gas flow rate, 25 arbitrary units; sheath gas, 35 arbitrary units; capillary temperature, 270 °C; and tube lens voltage, 185 V. SRM conditions are shown in Table 1. On the basis of the MS fragmentation analysis, the characteristic ion fragments for dansylated 27(OH)C, 25(OH)D₃ and the internal standard were m/z 252, 236, and 170, respectively, which were selected as the product ions for further LC-MS/MS analysis of the dansylated derivatives.

Results and discussion

Construction of plasmids and proteins expression

Because of the relevance of cholesterol, bile acid precursors, and vitamin D₃ and the importance of mitochondrial P450 27A1 in the maintenance of lipid homeostasis, we designed and constructed a whole-cell bioconversion system that stably expresses recombinant human P450 27A1 in *E. coli*, as well the mitochondrial electron transfer system Adr and Adx, using a tricistronic vector that produces the three components from a single mRNA, in addition to the development of strategies that will allow characterization of other mitochondrial P450 enzymes. The coding region was connected to the *cla* linker sequence in pCW including the Shine-Delgarno RBS (Fig. 1). Spaces and RBS were included between the three cDNAs as well the *trpA* terminator, providing a highly reproducible system with the advantage of protein expression in a bacterial environment resembling the mitochondrial context of the proteins and able to hydroxylate both cholesterol and vitamin D₃.

The approach with *E. coli* host cells as a stable system (rather than traditional phospholipid-based reconstitution) was done by transforming *E. coli* DH5 α with the plasmid pTC27A1 [P450 27A1:Adx;Adr], followed by selection on plates containing 100 μg ampicillin and 50

µg kanamycin. Expression of P450:Adx:Adr was induced with a *lac* promoter using IPTG after 6 h. Further incubation for 24 and 48 h produced time dependent expression, with a maximum P450 level after 48 h (Fig. 2). This result suggests that the tricistronic expression vector in *E. coli* was effective for the simultaneous expression of the three components between 24 and 48 h following incubation.

It has been reported elsewhere that P450 expression can be modulated by co-expression together with other proteins, and its content levels are higher when the P450 is expressed monocistronically [35]. Using the multiple expression method with the tricistronic vector, it was possible to express P450 27A1 to a level of 330 nmol/L of culture medium when coexpressed with Adx and Adr in the tricistronic system (as judged by spectral analysis after 48 h, Supplementary Data Fig. S-1), which we considered adequate for further studies (the level was 300 nmol/L in a separate experiment). The levels of expression of Adx and Adr were 920 and 500 nmol/L culture medium in same culture, as estimated by immunoquantitation with an anti-(His)₆ antibody method and based on the response of recombinant P450 2B6 containing a C-terminal (His)₆ tag (see Materials and methods and Supplementary Data Fig. S-2)).

Oxidation of substrates

After the expression of the components of the system was established, the ability of the bacteria to metabolize cholesterol (27 position) and vitamin D₃ (25 position) was evaluated. After 48 h, *E. coli* containing P45027A1:Adx:Adr was able to hydroxylate either cholesterol and vitamin D₃ when added to the culture medium at the same time as the inducer IPTG. After this time, the bacteria generated 30 µM 27(OH)C (10% of total cholesterol added to the culture medium, 300 µM) or 13 µM 25(OH)D₃ (4% of total vitamin D₃ added to the culture medium, 300 µM). For detection of products, a dansyl chloride protocol was used as described in Materials and methods. All dansylated derivatives of the selected compounds were detected using different SRM transitions in the ESI positive mode (Table 1). The total ion chromatograms for 27(OH)C (Fig. 3) and 25(OH)D₃ (Fig. 4) and values of 27(OH)C and 25(OH)D₃ (normalized using 17-EE as internal standard) are shown (Fig. 5). A negative control using the monocistronic vector showed that other reductases in *E. coli* apparently cannot support P450 27A1 oxidation.

Other issues

Two concerns regarding biotransformation in living cells are i) transport of the substrate into the cell and the product out of the cell and ii) the availability of an energy source [28]. Glycerol was included in the culture medium as an energy source for *E. coli*. Other concerns were the effective transit of cholesterol to the interior of the bacterium, the requirement of the β-cyclodextrin HPβCD to solubilize cholesterol, and the location of the hydroxylated product. One of the structural characteristics of cholesterol is its weak hydrophilicity and amphipathicity [10], as opposed to the oxysterols in which oxygen substitution makes them markedly more hydrophilic [13]. After bacterial growth in culture medium containing cholesterol, 95% of the 27(OH)C was found outside of the bacterial cells (results not shown), suggesting that the cholesterol is effectively solubilized by HPβCD and mobilized across the *E. coli* polysaccharide cell wall, reaching the cytosol where the three recombinant proteins are located. We also incubated the bacteria with cholesterol in a M9 minimal salts media [36] after 48 h of induction with IPTG, and the results were very similar to those obtained when cholesterol was added directly to the Terrific Broth culture medium at the time of IPTG inducer (results not shown).

P450, Adx, and Adr were able to interact catalytically and allow *E. coli* to oxidize both cholesterol and vitamin D₃, results not observed in *E. coli* with or without the monocistronic

vector. To our knowledge, the expression and purification of Adr and Adx in bacteria and their use in reconstituted systems has been done using only His tag-free proteins [25], and the results shown here suggest that apparently the inclusion of His tags at the C-terminal in the three components of the system is not an issue for the protein interaction required for electron transfer during catalysis.

Conclusions

In general, whole cell systems are more popular systems for biotransformations than isolated enzymes [37], and the design of bacterial cells that are specialized for drug discovery and production of metabolites are promising tools from the synthetic biology point of view, especially as a strategy that enables the functional characterization of different genes [38]. Our results reported here demonstrate that *E. coli* containing pTC27A1 is able to hydroxylate cholesterol and vitamin D₃ in a self-sufficient fashion, suggesting that this model mimics the mitochondrial P450 context in *E. coli* and may be suitable for further applications in protein characterization (e.g. other P450s) for protein interaction studies, and substrate/inhibitor evaluation in microorganisms, as an alternative and efficient way without membrane reconstitution, membrane simulation by detergents, or further purification of P450 and accessory enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 2.

Immunoblot of PVDF membrane after anti-(His)₆/goat anti-mouse IRD800CW Li-COR incubation of *E. coli* lysate for the detection of P450 27A1, Adr, and Adx expression after 24 and 48 h of IPTG (1.0 mM) induction. Lane MW, protein ladder marker (Kaleidoscope, BioRad); lanes 1 and 3, lysates of bacteria containing pCWx (P450:Adx); lanes 2 and 4, lysates of bacteria containing pTC27A1 (P450:Adx:Adr). Lanes 1 and 2, lysates of bacteria after 24 h of induction; lanes 3 and 4, lysates of bacteria after 48 h of induction. Because the M_r values of P450 27A1 (57.6 kDa) and Adr (52 kDa) are similar, an alternative vector (pCWx) expressing only P450 and Adx (15.2 kDa) was used to correct for the Adr contribution, as seen in the 52–58 kDa band range (lanes 2 and 4). (See also Supplemental Data Fig. S-2.)

Fig. 3. Separation of 27(OH)C standard and products of *E. coli* metabolism after 5, 24, and 48 h of incubation. Products were derivatized using the dansyl chloride method and analyzed by UPLC-MS-SRM (ESI, positive mode) with a 13.5 min gradient. 27(OH)C formed from cholesterol by *E. coli* containing FL27A1 (—) and pTC27A1 (27A1:Adx:Adr) (---). The insert shows the characteristic ion fragment expected after the SRM (m/z 252 for 27(OH)C product)

Fig 4. Separation of 25(OH)D₃ standard and products of vitamin D₃ from *E. coli* containing pTC27A1 (P450 27A1:Adx:Adr) after 5, 24, and 48 h of incubation and derivatized using the dansyl chloride method on a UPLC-MS-SRM (ESI, positive mode) with a 13.5 min gradient. The insert shows the characteristic ion fragment expected after the SRM (m/z 252 for 25(OH)D₃ product)

Fig. 5. Metabolism of cholesterol and vitamin D₃ by *E. coli* expressing pTC27A1 (P450 27A1, Adx, Adr) and FL27A1 (P450 27A1 alone). The values for hydroxylated products of cholesterol and vitamin D₃ were calculated using 17-EE as an internal standard.

TABLE 1

SRM and optimum parameters for dansylated hydroxylated products of the oxidation of cholesterol and vitamin D₃ by *E. coli* expressing P450 27A1:Adr:Adx

SRM transitions		Collision energy (v)	Scan time (s)	Compound detected
Parent mass	Product mass			
530 →	170.9	40	0.05	Dansylated 17-EE
636 →	252	40	0.05	Dansylated 27(OH)C
636 →	357	20	0.05	Dansylated 27(OH)C
634 →	252	28	0.05	Dansylated 25(OH)D ₃
634 →	236	60	0.05	Dansylated 25(OH)D ₃