Evaluation of the Efficiency of Functional Reversal of Fatty Acid B-Oxidation in *Escherichia coli* upon the Action of Various Native Acyl-CoA Dehydrogenases

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Abstract—Using *Escherichia coli* strain MG1655 lacI, ΔackA-pta, ΔpoxB, ΔldhA, ΔadhE, ΔfadE, P<sub>1</sub>-SD<sub>10</sub>-atoB, P<sub>trs-ideal-4</sub>-SD<sub>10</sub>-fadB, P<sub>L</sub>-SD<sub>10</sub>-tesB, ΔyciA as a core strain, the efficiency of the reversal of fatty acid β-oxidation upon the action of native cellular enzymes capable of serving as acyl-CoA dehydrogenases was examined. Increased expression of *fadE*, *fabI*, and *ydiO/ydiQRST* genes encoding the corresponding enzymes was ensured in derivatives of the core strain by substituting their native regulatory regions with artificial regulatory element P<sub>trs-ideal-4</sub>-SD<sub>10</sub>-A three-turn reversal of the cycle in the engineered recombinants was demonstrated that was accompanied by considerable secretion of butyric, caproic, and caprylic acids. The highest level of six- and eight-carbon carboxylates production was achieved upon the overexpression of the *fabI* gene, while the lowest levels of secretion of the corresponding compounds were demonstrated by the strain with the enhanced expression of the *ydiO* and *ydiQRST* genes. The recombinant with the individually enhanced expression of *ydiO* did not produce detectable amounts of the derivatives of the complete and successful β-oxidation reversal.

Keywords: acyl-CoA dehydrogenase, fatty acid β-oxidation, metabolic engineering, *Escherichia coli*

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INTRODUCTION

The current trend in modern biotechnology is the development of microbial biosynthetic platforms to produce industrially valuable chemicals. Indeed, many of these substances can be obtained as a result of a series of relatively simple transformations of a limited set of intermediates of the cellular central metabolism, such as pyruvic acid, oxaloacetic acid, and acetyl-CoA. Thus, the biosynthesis of various structurally related compounds can be ensured by involving one or another key intermediate of central metabolism in the target platform biochemical pathway using a single core recombinant strain, instead of creating a series of specialized producers.

To date, the prospects of this approach has been demonstrated using *Escherichia coli* cells engineered for the synthesis of methyl ketones [1], hydroxy acids [2], aliphatic alcohols [3], and aliphatic functionalized carboxylic acids [4, 5]. In general, the platform biosynthetic pathway is a restricted and defined sequence of reactions one of the intermediates of which can serve as a key precursor in the biosynthesis of target products [6]. However, potentially, iterative biochemical pathways can be used as such pathways, providing an elongation of the carbon chain of the formed molecules at each new functional round. This property of iterative biochemical pathways represents their additional advantage, which greatly expands the range of possible end products of biosynthesis. One of the few convenient and efficient iterative biosynthetic platforms whose potential for the microbiological synthesis of target groups of substances of various classes has been experimentally confirmed is reversed fatty acid β-oxidation (FAO) [7].

Upon enforced inversion of FAO towards biosynthesis, the sequence of the corresponding reactions is initiated by the condensation of two molecules of acetyl-CoA, a direct derivative of pyruvic acid, and proceeds through the sequential formation of 3-keto-, 3-hydroxyacyl-, 2-enoyl-, and acyl-derivatives of CoA, which implies the feasibility of conversion of the corresponding cycle intermediates into linear aliphatic 3-keto-, 3-hydroxy-functionalized, saturated and unsaturated carboxylic acids, as well as normal alcohols and 1,3-diols resulting from hydrolysis of the thioether bond and/or reduction of the carboxylic group by the suitable terminating thioesters or aldehyde/alcohol dehydrogenases. The primary and consequent stages of condensation of acetyl-CoA and acyl-CoA in *E. coli* can be catalyzed by acetyl-CoA-C-acetyltransferase AtoB (EC 2.3.1.9), followed by further reduction of 3-ketoacyl-CoA to 3-hydroxyacyl-...
CoA and the formation 2-enoyl-CoA by bifunctional (S)-3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA reductase FadB (EC 1.1.1.35/EC 4.2.1.17). The reduction of 2-enoyl-CoA to the corresponding acyl-CoA derivative can be catalyzed by both heterologous trans-enoyl-CoA reductases Ter (EC 1.3.1.38) from Treponema denticola and Euglena gracilis, and native E. coli acyl-CoA dehydrogenase FadE (EC 1.3.99.3) and enoyl-ACP reductase/acyl-CoA dehydrogenase FabI (EC 1.3.1.9) [8–10]. Acyl-CoA dehydrogenase YdiO can also potentially participate in the catalysis of the corresponding reaction [11].

Previously published studies repeatedly confirmed the efficiency of using E. coli AtoB and FadB proteins as enzymes catalyzing the initial stages of reversed FAO. At the same time, the feasibility and efficiency of repeated cycle reversal directly depend on the activity of the enzymes that catalyze its final stage. Meanwhile, the data on the efficiency of the participation of Ter, FadE, FabI, and YdiO in the reversion of FAO differ, precluding an unambiguous choice in favor of one of these enzymes. This is due to the fact that the effect of increased expression of genes, encoding the corresponding proteins on the production of marker compounds by recombinant producers, was evaluated using different carbon sources in non-isogenic strains [8–10].

**Objective**—To evaluate the efficiency of functional FAO reversal in Escherichia coli upon the action of various native acyl-CoA dehydrogenases in a single previously constructed core strain capable of efficient production of 3-hydroxybutyric acid from glucose as a result of a partial one-turn cycle reversal.

**MATERIALS AND METHODS**

**Reagents.** Restriction enzyme BglII, Taq DNA polymerase, T4 DNA ligase (Thermo Scientific, Lithuania), and high-fidelity Kapa HiFi DNA polymerase (Roche, Switzerland) were used in the study. PCR products were purified using agarose gel electrophoresis and isolated using a QIAquick Gel Extraction Kit (Qiagen, United States). Oligonucleotides (Evrogen, Russia) are presented in Table 1. Nutrient media components, salts, and other reagents were produced by Panreac (Spain) and Sigma (United States).

**Bacterial strains, plasmids and media.** E. coli strain K-12 MG1655 (VKPM B-6195) and previously constructed E. coli strain BOX3.1 ∆Δ P<sub>L</sub>-atoB P<sub>L</sub>-tesB AycIΔ [12], designated as BOX3.3 ∆Δ, with altered regulation of expression of genes encoding the key enzymes of aerobic β-oxidation of fatty acids and thioesterase II, lacking mixed-acid fermentation pathways and the activity of nonspecific YciA thioesterase, were used as the core strains for the construction of all strains obtained in the study. The bacterial strains and plasmids used here are presented in Table 2. Rich LB, SOB, SOC, and minimal M9 media [13] were used for cultivation of bacteria; if necessary, ampicillin (100 µg/mL) or chloramphenicol (30 µg/mL) were added to them.

**Table 1. Oligonucleotide primers used in the work**

| No. | Sequence |
|-----|----------|
| P1  | 5'-tgccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P2  | 5'-ctcgccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P3  | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P4  | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P5  | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P6  | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P7  | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P8  | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P9  | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P10 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P11 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P12 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P13 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P14 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P15 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P16 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P17 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P18 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
| P19 | 5'-tggccgacagatctgaatgttagcgcctcaacaatggatc-3' |
were introduced into the native regulatory regions of the technique described earlier [14].

If a fragment containing the end, then the P<sub>trc</sub>-genes were obtained. Fragments were obtained in two stages. In the first stage, using PCR, DNA fragments containing the 36 nucleotides complementary to the 5'-ends of the ϕID promoter and an efficient ribosome-binding site of the genetic element containing a strong LacI-dependent gene SD sequence from T7 phage were obtained. PCR was performed using primers P1 and P2. The resulting gene sequence from the T7 phage, was conducted in several stages. In the first stage, using PCR, DNA fragments containing the BglII recognition site at the 5'-end, then the P<sub>trc</sub>-ideal-4 promoter, the sequence of the new regulatory element introduced was planned and experimentally obtained nucleotide sequences of the new regulatory element introduced before the coding regions of the fadE, fabI, ydiO, and ydiQRST genes were confirmed by sequencing using plasmid pKD46. The correspondence between the planned and experimentally obtained nucleotide sequences of the new regulatory element introduced before the coding regions of the fadE, fabI, ydiO, and ydiQRST genes was confirmed by sequencing using plasmid pKD46.

Respective individual genetic modifications were introduced into the chromosomes of target

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**Table 2. Strains and plasmids**

| Strain          | Genotype                           | Link   |
|-----------------|------------------------------------|--------|
| MG1655          | Wild type E. coli strain (VKPM B-6195) | VKPM   |
| BOX3.3 Δ4       | E. coli MG1655 lac<sup>P</sup>, ΔackA-p<sub>pta</sub>, ΔpoxB, ΔldhA, ΔadhE, ΔfadE, P<sub>L</sub>-SD<sub>q<sup>10</sup>-atoB</sub>, P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup>-fadB</sub>, P<sub>L</sub>-SD<sub>q<sup>10</sup>-tesB</sub>, ΔyciA | [12]   |
| BOX3.1 Δ4 P<sub>trc</sub>-id-4-fadE | E. coli MG1655 lac<sup>P</sup>, ΔackA-p<sub>pta</sub>, ΔpoxB, ΔldhA, ΔadhE, P<sub>L</sub>-SD<sub>q<sup>10</sup>-atoB</sub>, P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup>-fadB</sub>, P<sub>L</sub>-SD<sub>q<sup>10</sup>-tesB</sub>, ΔyciA, P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup>-fadE</sub> | Current work |
| BOX3.3 Δ4 P<sub>trc</sub>-id-4-fabI | E. coli MG1655 lac<sup>P</sup>, ΔackA-p<sub>pta</sub>, ΔpoxB, ΔldhA, ΔadhE, P<sub>L</sub>-SD<sub>q<sup>10</sup>-atoB</sub>, P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup>-fadB</sub>, ΔfadE, P<sub>L</sub>-SD<sub>q<sup>10</sup>-tesB</sub>, ΔyciA, P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup>-fabI</sub> | Current work |
| BOX3.3 Δ4 P<sub>trc</sub>-id-4- ydiO | E. coli MG1655 lac<sup>P</sup>, ΔackA-p<sub>pta</sub>, ΔpoxB, ΔldhA, ΔadhE, P<sub>L</sub>-SD<sub>q<sup>10</sup>-atoB</sub>, P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup>-fadB</sub>, ΔfadE, P<sub>L</sub>-SD<sub>q<sup>10</sup>-tesB</sub>, ΔyciA, P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup>-ydiO</sub> | Current work |

**Plasmid**

| pMW18-(λattL-Cm-λattR) | pSC101, bla, cat, λattL-cat-λattR | [16]   |
| pMW-O<sub>lac</sub>-ideal-P<sub>trc</sub>/O<sub>lac</sub>-ideal-lacZ | pSC101, bla, O<sub>lac</sub>-ideal-P<sub>trc</sub>/O<sub>lac</sub>-ideal-lacZ | [15]   |
| pKD46 | pINT-ts, bla, P<sub>aram</sub>-argam-ber-exo | [14]   |
| pMWts-Int/Xis | pSC101-ts, bla, P<sub>R</sub>-λxis-int, cfts857 | [17]   |

**Construction of the strains.** Target modifications were introduced into the E. coli chromosome using the technique described earlier [14].

The construction of DNA fragments to replace the native regulatory regions of the fadE, fabI, ydiO, and ydiQRST genes with the P<sub>trc</sub>-ideal-4-SD<sub>q<sup>10</sup></sub> artificial genetic element containing a strong LacI-dependent promoter and an efficient ribosome-binding site of the ϕ10 gene from the T7 phage, was conducted in several stages. In the first stage, using PCR, DNA fragments containing the BglII recognition site at the 5'-end, then the P<sub>trc</sub>-ideal-4 promoter, the sequence of the SD sequence of gene q10 from the T7 phage, and, finally, 36 nucleotides complementary to the 5'-ends of the coding regions of the fadE, fabI, ydiO, and ydiQRST genes were obtained. Fragments were obtained in two stages. In the first stage, using the pMW-O<sub>lac</sub>-ideal-P<sub>trc</sub>/O<sub>lac</sub>-ideal-lacZ plasmid as a template [15], a DNA fragment containing the BglII recognition site at the 5'-end, then the P<sub>trc</sub>-ideal-4 promoter and part of the ϕ10 gene SD sequence from T7 phage were obtained. PCR was performed using primers P1 and P2. The resulting PCR product served as a template in the next rounds of PCR using primer pairs P1 and P3, P1 and P4, P1 and P5, and P1 and P6. Primers P3, P4, P5, and P6 contained the region complementary to the 3'-end of the P<sub>trc</sub>-ideal-4 promoter, the SD sequence of the ϕ10 gene from the T7 phage, and the first 36 nucleotides from the reading frames of the fadE, fabI, ydiO, and ydiQRST genes, respectively. In parallel, the second stage of constructing DNA fragments was carried out. DNA fragments containing the BglII recognition site, a chloramphenicol resistance marker (cat gene), and 36 nucleotides homologous to the DNA regions upstream the coding regions of the fadE, fabI, ydiO, and ydiQRST genes were obtained by PCR using primer pairs P7 and P8, P7 and P9, P7 and P10, P7 and P11, and plasmid pMW18-(attL-Cm-attR) [16] as a template. The resulting DNA fragments were treated with BglII restriction endonuclease and ligated with T4 DNA ligase. The ligation products were amplified using primer pairs P3 and P8, P4 and P9, P5 and P10, and P6 and P11. The constructed DNA fragments were individually integrated into the chromosome of E. coli strain MG1655 carrying helper plasmid pKD46. The correspondence between the planned and experimentally obtained nucleotide sequences of the new regulatory element introduced before the coding regions of the fadE, fabI, ydiO, and ydiQRST genes was confirmed by sequencing using primer pairs P12 and P13, P14 and P15, P16 and P17, and P18 and P19.
nant strains using P1-dependent transductions [13]. Removal of the marker flanked by lambda phage att-sites from chromosomes of the target strains was performed using the pMWts-Int/Xis plasmid, as described previously [17]. The transformation of strains with plasmids was carried out according to the standard method.

**Cultivation of strains.** Recombinant strains were grown overnight in M9 medium containing 2 g/L glucose at 37°C. Five mL of the obtained overnight cultures were diluted 10 times by adding 45 mL of M9 medium containing 10 g/L of glucose and 10 g/L of yeast extract. The obtained cultures were grown in 750 mL flasks on a rotary shaker at 250 rpm for 8 h at 37°C. To induce the expression of genes controlled by LacI-dependent P_{trc-ideal-3} and P_{trc-ideal-4} promoters, isopropyl-β-D-thiogalactoside (IPTG) was added to the culture media 3 h after the start of incubation to a final concentration of 1.0 mM. Cell suspensions were centrifuged for 15 min of 2000 g at 4°C. The pellets were resuspended in 15 mL of M9 medium containing 10 g/L glucose and 1.0 mM IPTG. Subsequently, the cultures were incubated anaerobically for 24 h in 15 mL tubes sealed with screw caps at 37°C on a rotary shaker at 250 rpm. Cell suspensions were centrifuged of 10000 g for 10 min, and the concentrations of secreted metabolites and residual glucose were determined in the obtained supernatants. All experiments were repeated at least three times.

**Analytical methods.** The concentrations of organic acids in culture liquids freed from biomass by centrifugation were determined by HPLC using the Waters HPLC system (United States). A Rezex ROA-Organic Acid H+ ion-exclusion column (8%) (Phenomenex, United States) with detection at a wavelength of 210 nm was used. The mobile phase was an aqueous solution of sulfuric acid (2.5 mM) with a flow rate of 0.5 mL/min. To measure the glucose concentration, the system was equipped with a Waters 2414 refractive index detector and a Stabilwax-DA capillary 30 m column (Shimadzu, Japan) equipped with a flame ionization detector and an AOC-20i autosampler was used.

A GC-2010 Plus gas chromatograph (Shimadzu, Japan) equipped with a Stabilwax-DA capillary 30 m column (Restek, United States), with an inner diameter of 0.25 mm and 0.25 μm film thickness was used to quantify the amounts of carboxylic acids—products of FOX reversal. Helium served as the carrier gas at a constant flow rate of 1.2 mL/min. Samples with a volume of 0.5 μL were introduced into the evaporator in the split-flow mode 1:20. The temperature of the evaporator and flame ionization detector were 150 and 250°C, respectively. The temperature program of the column thermostat: initial isotherm, 2 min at 90°C, followed by a linear gradient up to 200°C at a rate of 10°C/min, and a final isotherm, 2 min at 200°C.

**RESULTS AND DISCUSSION**

The previously constructed strain *E. coli* BOX3.3 ∆4 [12] was used as a core for evaluating the efficiency of the functional reversal of FAO upon the action of various native acyl-CoA dehydrogenases [12] (Table 2). In this strain, the ackA, pta, poxB, ldhA, and adhE genes encoding enzymes of the mixed-acid fermentation pathways involving pyruvic acid and its direct derivative, acetyl-CoA, a key metabolic precursor in the reactions of inverted FAO, were inactivated. In addition, the expression of the *atoB* and *fadB* genes encoding the FAO enzymes, which ensure the formation of 3-hydroxybutyryl-CoA from acetyl-CoA, was enhanced in the strain, as well as the expression of the thioesterase II gene, *tesB*, which can serve as a terminating enzyme that ensures the formation of carboxylic acids from acyl-CoA intermediates of FAO. In the strain, the nonspecific thioesterase YciA gene was inactivated in order to further reduce the competitive conversion of acetyl-CoA to acetic acid, and the main acyl-CoA dehydrogenase gene, *fadE*, was inactivated in order to prevent multiple reversal of FAO. As a result, during test-tube fermentation, the strain was capable of synthesizing 3-hydroxybutyric acid from glucose up to ~6 mM under microaerobic and 3-hydroxybutyric acid up to ~4 mM under anaerobic conditions resulting from partial one-turn inversion of FAO [12].

Reversed FAO reactions catalyzed by 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA dehydrogenase are NADH-consuming. Thus, anaerobic conditions that exclude intense oxidation of reduced equivalents in the respiratory electron transport chain with the participation of oxygen as a terminal electron acceptor are more preferable to support multiple cycle reversal. Therefore, the efficiency of FAO reversal in the studied strains was evaluated by the concentrations of marker compounds secreted by recombinants in the anaerobic stage of two-phase aerobic–anaerobic fermentation. The corresponding cultivation process, including an aerobic stage of biomass accumulation followed by an anaerobic productive stage, was chosen because *E. coli* strains deficient in mixed acid fermentation pathways are not capable of anaerobic growth [17], but retain metabolic activity in the absence of aeration.

During anaerobic utilization of glucose, core strain BOX3.3 ∆4 synthesized acetic and lactic acids, as well as ethanol (Table 3) as the main products of the carbon substrate consumption, without secreting detectable amounts of products of complete and moreover, repeated reversal of FAO due to the deletion of the *fadE* gene (Table 4).
It should be noted that the strain did not secrete acyl-CoA derived carboxylic acids, despite the presence of intact fabI and ydiO genes in the chromosome. This indicated that the native levels of expression of the corresponding genes could not provide sufficient acyl-CoA dehydrogenase activity in the cell for successful functional reversal of FAO. Indeed, in the case of FabI, acyl-CoA dehydrogenase activity is secondary to the main enoyl-ACP reductase activity of this protein [18], and to ensure efficient reduction of crotornylo-CoA, which is required for FAO reversal in recombinant strains, it is necessary to increase the expression of the fabI gene [10]. On the other hand, the expression of the ydiO gene encoding the acyl-CoA dehydrogenase of anaerobic FAO [11] can be repressed in the presence of oxygen [19]. The aerobic conditions used for the accumulation of the biomass of the BOX3.3 Δ4 strain prevented the formation of such a level of the corresponding protein in the cells during the growth stage that would be sufficient for its effective action in the subsequent biosynthetic stage. In addition, in the absence of fatty acids in the medium, the expression offad regulon genes, including fadE, is repressed inE. coli by the transcriptional regulator FadR, and the activity of the corresponding enzymes in the cell is severely limited [20]. Taken together, this indicated that in order to ensure the activities of acyl-CoA dehydrogenases in the recombinants, which are necessary for the efficient reversal of FAO, an increase in the expression of the corresponding genes was an essential condition.

In the BOX3.3 Δ4 strain, the expression of the genes for acetyl-CoA-C-acetyltransferase, atoB, and thioesterase II, tesB, was controlled by the lambda phage P_L promoter, which is one of the “strongest” for E. coli, while the P_trc-ideal promoter, somewhat inferior to PL promoter, was located upstream from the 3-hydroxyacyl-CoA dehydrogenase gene, fadB [15]. The corresponding promoters in the artificial regulatory elements containing an effective ribosome-binding site ofφ10 gene from the T7 phage were located before the indicated genes to ensure, first of all, the possibility of efficient initiation of FAO reversal and the subsequent formation of detectable products from the CoA intermediates of the cycle, while the intensity of the intermediate reactions could be somewhat decreased. Thus, in order to prevent a potential imbalance between the initiation of FAO reversal, the formation of cycle intermediates, and the synthesis of end products, the native regulatory regions of the fadE, fabI, and ydiO genes in BOX3.3 Δ4 derived strains were also replaced by the artificial regulatory element P_trc-ideal-4-SDφ10.

All relevant recombinants formed profiles of the main secreted metabolites during anaerobic glucose utilization similar to those demonstrated by the parent strain BOX3.3 Δ4 (Table 3). The main glucose utiliza-
tion products formed by the strains were acetic acid and ethanol, the yields of which were ~0.5 mol/mol and ~0.3 mol/mol, as well as lactic acid, the yield of which reached ~0.35 mol/mol.

The secretion by strains of a significant portion of consumed glucose in the form of acetic acid and ethanol, which are direct derivatives of acetyl-CoA, a key precursor in the reactions of reversed FAO, as well as lactic acid, which, along with ethanol, is a product of NADH-consuming reactions, indicated a low intensity of functioning in recombinants of the target biosynthetic pathway. However, strains BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-fadE} and BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-fabI} secreted significant amounts of butyric, caproic, and caprylic acids (Table 4), which are four-, six-, and eight-carbon products of a full-fledged one-, two- and three-turns FAO reversal. At the same time, the amount of butyric acid synthesized by the strains was considerably greater than the amount of six- and eight-carbon carboxylates formed by them. Apparently, this was due, first of all, to the insufficient specificity of acetyl-CoA-C-acyltransferase AtoB to acyl-CoA substrates containing more than 4 carbon atoms in the hydrocarbon chain [21]. Indeed, this enzyme is preferentially involved in the catalysis of the terminal stages of lipid degradation, while 3-ketoacyl-CoA thiolase FadA is involved in the cleavage of higher molecular weight intermediates [21].

The recombinant producers used in this study were model ones, while when constructing industrial strains, this problem can be solved by jointly enhancing the expression of the \textit{atoB} and \textit{fadA} genes in cells under the control of promoters with different strengths and regulation. Nevertheless, the obtained data allowed us to conclude that the use of FabI as acyl-CoA dehydrogenase promotes the multiple reversal of FAO to a greater extent than the use of FadE for this purpose. Indeed, the amount of caproic and caprylic acids synthesized by the BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-fabI} strain was 3.8 and 2.6 times higher than that of the BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-fadE} strain. However, nowadays, the enzymatic properties of FadE and FabI, as acyl-CoA dehydrogenases/enoyl-CoA reductases, have been poorly studied. Significantly different rates of specific acyl-CoA dehydrogenase activity for these proteins were reported, amounting to 0.019 μmol/mg/min and 0.001 μmol/mg/min for FadE and FabI against butyryl-CoA for strains expressing the corresponding genes in identical plasmids [10]. At the same time, data on the Micheales constant in relation to crotonyl-CoA obtained for the purified variant of recombinant FabI indicate its extremely low affinity for the corresponding substrate.

Thus, from the point of view of applied biotechnology, the achievement of FAO reversal should be based, in our opinion, on targeted manipulation of the expression levels of key genes with an analysis of the efficiency of substrate conversion into target products for the subsequent choice of a rational design strategy for industrial producers, and not on data on their enzymatic activity. At the same time, the specific activity of YdiO against butyryl-CoA was reported at 0.003 μmol/mg/min, which is comparable with the FabI properties [10]. However, strain BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-ydiO} during anaerobic utilization of glucose did not synthesize noticeable amounts of marker compounds, which could indicate complete functional reversal of FAO (Table 4). Acyl-CoA dehydrogenase, which is involved in \textit{E. coli} anaerobic FAO, is, by analogy with \textit{Clostridium}, a complex enzymatic complex that involves flavoproteins in its functioning providing electron transport to the terminal acceptor. The corresponding proteins in \textit{E. coli} encode the genes of the \textit{ydiQRST} operon [11], therefore, to ensure the activity of the anaerobic acyl-CoA dehydrogenase YdiO, the expression of the genes of this operon was additionally increased in the BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-ydiO} strain. The final recombinant BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-ydiO} P\textsubscript{trc-id-4}\textsubscript{-ydiQRST} synthesized the full range of products of three-turn reversal of FAO (Table 4) with efficiency, however, decreased compared to strains BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-fadE} and BOX3.3 Δ4P\textsubscript{trc-id-4}\textsubscript{-fabI}. In this regard, the need for the participation of other collateral enzymes in ensuring the maximum functional activity of the anaerobic acyl-CoA dehydrogenase for the reversal of FAO in recombinant cells, the data obtained indicated the preferential use of aerobic enzymes for the enforced inversion of this pathway to the biosynthetic direction.

As a result of the conducted studies, the ability of native acyl-CoA dehydrogenases to ensure multiple reversal of FAO in recombinant \textit{E. coli} strains for potential production of industrially valuable compounds was characterized. It has been shown that the maximum efficiency of cycle turnover is achieved by increasing the level of expression of the gene encoding enoyl-ACP reductase/acyl-CoA dehydrogenase FabI in recombinant strains. An alternative could be an increase in the expression of the acyl-CoA dehydrogenase FadE gene, while maintaining the optimal balance between the activities of the enzymes involved in the target biosynthetic pathway.

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EVALUATION OF THE EFFICIENCY OF FUNCTIONAL REVERSAL

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