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Metabolic Engineering of *Escherichia coli* for 1,3-Butanediol Biosynthesis through the Inverted Fatty Acid β-Oxidation Cycle

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Abstract—The feasibility of 1,3-butanediol biosynthesis through the inverted cycle of fatty acid β-oxidation in *Escherichia coli* cells was investigated by the rational metabolic engineering approach. CoA-dependent aldehyde dehydrogenase MhpF and alcohol dehydrogenases FucO and YqhD were used as terminal enzymes catalyzing conversion of 3-hydroxybutyryl-CoA to 1,3-butanediol. Constitutive expression of the corresponding genes in *E. coli* strains, which are deficient in mixed acid fermentation pathways and expressing *fàd* regulon genes under control of $P_{trc\text{-ideal-4}}$ promoter, did not lead to the synthesis of 1,3-butanediol during anaerobic glucose utilization. Additional inactivation of *fadE* and *ydiO* genes, encoding acyl-CoA dehydrogenases, also did not cause synthesis of the target product. Constitutive expression of *aceEF-lpdA* operon genes encoding enzymes of pyruvate dehydrogenase complex led to an increase in anaerobic synthesis of ethanol. Synthesis of 1,3-butanediol was observed with the overexpression of acetyl-CoA C-acetyltransferase AtoB. Constitutive expression of *atoB* gene in a strain with a basal expression of alcohol/aldehyde dehydrogenase leads to synthesis of 0.3 mM of 1,3-butanediol.

Keywords: 1,3-butanediol, fatty acid β-oxidation, *Escherichia coli*, metabolic engineering

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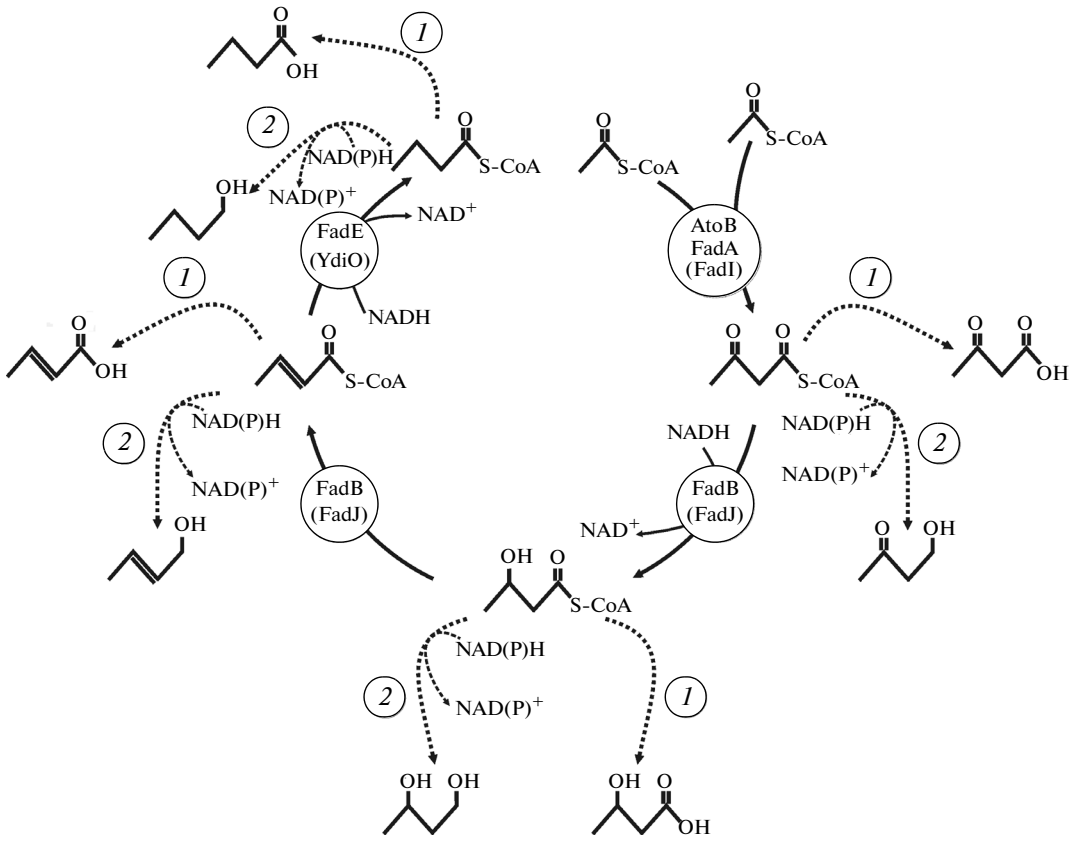
INTRODUCTION

The inverted fatty acid β-oxidation cycle can serve as biosynthetic platform for microbiological synthesis of a wide range of industrially important high value-added chemicals from sugars of plant biomass. Depending on terminal enzymes, which catalyze the final conversion of the corresponding cycle CoA-intermediates, the final products of biosynthesis may be carboxylic acids and their hydroxy- and keto-derivatives, as well as mono- and dihydric alcohols. A one-turn reversal of fatty acid β-oxidation cycle results in the following four-carbon compounds: 3-ketobutyric acid and 3-oxobutanol (acetoacetyl-CoA derivatives), 3-hydroxybutyric acid and 1,3-butanediol (derivatives of 3-hydroxybutyryl-CoA), crotonic acid and crotyl alcohol (derivatives of crotonyl-CoA), and butyric acid and 1-butanol (butyryl-CoA derivatives) (figure). A series of studies recently demonstrated successful anaerobic biosynthesis of 3-ketobutyric [1], 3-hydroxybutyric [1–3], crotonic [1], butyric [1–4] acids and 1-butanol [1, 5, 6] from glucose or glycerol resulting from the one-turn reversal of fatty acid β-oxidation in directly engineered *Escherichia coli* strains. In most cases the synthesis products were acids. For the synthesis of alcohols, more reduced compounds compared with the respective acids, the maintenance of the proper intracellular redox balance is required. However, biological synthesis of four-carbon β-functionalized nonnatural alcohols is of particular interest. For example, 1,3-butanediol, which is currently produced at industrial scale from petroleum, has significant market potential. Due to the high cost of 1,3-butanediol chemical synthesis [7], this product is now used mainly as a precursor for the synthesis of flavorings, insecticides, macrolides, and carbapenem beta-lactam antibiotics [8]. However, biological synthesis from renewable raw materials can reduce the price of 1,3-butanediol, allowing its use as an inexpensive and highly efficient substrate for the production of 1,3-butadiene, with an annual worldwide demand of about 10 million tons [9].

The aim of this work is to evaluate the feasibility of anaerobic biosynthesis of 1,3-butanediol from glucose by *E. coli* cells via an inverted fatty acid β-oxidation pathway.

EXPERIMENTAL CONDITIONS

Bacterial strains, plasmids, and media. The *E. coli* strain K-12 MG1655 (VKPM B-6195) and the previously constructed *E. coli* BOX-3 Δ3 and BOX-3 Δ4 strains [6] with an altered regulation of expression of genes encoding the aerobic fatty acid β-oxidation enzymes and with inactivated mixed acid fermentation pathways were used as the parent strains for the construction of all strains described in the work. The bacterial strains and plasmids used in this study are



Biosynthetic potential of the inverted fatty acid β-oxidation cycle in *E. coli*. Intermediates of the one-turn reversal of fatty acid β-oxidation cycle are as follows: in the clockwise direction—acetoacetyl-CoA, 3-hydroxybutyryl-CoA, crotonyl-CoA, and butyryl-CoA. Enzymes catalyzing reversible reactions of fatty acid β-oxidation are marked with the names of corresponding proteins: AtoB—acetyl-CoA C-acetyltransferase (EC 2.3.1.9); FadA, FadI—acetyl-CoA C-acyltransferase (EC 2.3.1.16); FadB, FadJ—3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) / enoyl-CoA hydratase (EC 4.2.1.17); FadE, YdiO—acyl-CoA dehydrogenase (EC 1.3.99.3). Enzymes of anaerobic fatty acid β-oxidation pathway are given in brackets. Enzymatic activities potentially catalyzing the conversion of fatty acid β-oxidation CoA-intermediates into corresponding acids and alcohols are numbered: *1*—thioesterases; *2*—alcohol/aldehyde dehydrogenases.

listed in Table 1. The bacteria were cultivated in rich LB, SOB, SOC, and minimal M9 media [10] with the addition of ampicillin (100 μg/mL) or chloramphenicol (30 μg/mL) as needed.

Reagents. Restrictases and T4 DNA ligase (Fermentas, Lithuania), Taq (Fermentas, Lithuania) and Phusion (Finnzymes, Finland) DNA polymerases were used. The oligonucleotides (Table 2) were provided by Syntol (Russia). The PCR products were purified by electrophoresis in agarose gel and isolated using a QIAquick Gel Extraction Kit from Qiagen (USA). Components of the culture media, salts, and other reagents were produced by Panreac (Spain) and Sigma (USA).

Construction of strains. All chromosomal modifications were performed according to the previously described method [11]. The construction of DNA fragments for the replacement of the native regulatory regions of *mhpF*, *fucO*, *yqhD*, and *atoB* genes by the artificial genetic element P_L-SDφ10, which contains a P_L promoter of lambda phage and an effective ribosome binding site of φ10 gene from T7 phage, was performed in several stages. At the first stage, DNA fragments containing the *Bgl*II recognition site, P_L promoter, the SD sequence of φ10 gene from T7 phage, and 36 nucleotides (complementary to the 5'-end of coding regions of *mhpF*, *fucO*, *yqhD*, and *atoB* genes) were obtained by PCR. These fragments were prepared in two steps. Initially, a DNA fragment containing the *Bgl*II recognition site, P_L promoter, and a part of the SD sequence of φ10 gene from T7 phage was obtained by PCR using primers P1 and P2 and the genomic DNA of lambda phage as a template. Then, the resulting PCR product served as a template in the next PCR rounds with primer pairs P1, P3; P1, P4; P1, P5; and P1, P6. Primers P3, P4, P5, and P6 contained the region complementary to the 3'-end of P_L promoter, SD sequence of φ10 gene from T7 phage, and the first 36 nucleotides from reading frames of *mhpF*, *fucO*, *yqhD*, and *atoB* genes, respectively. At the same time, the second step of DNA fragment construction was performed. DNA fragments containing the *Bgl*II recognition site, marker of chloramphenicol resis-

Table 1. Strains and plasmids

Object	Genotype	Reference
Strain		
MG1655	Wild type *E. coli* strain (VKPM B-6195)	VKPM
BOX-3	MG1655 *lacI*^{Q} $P_{trc\text{-ideal-4}}$-$SD_{\varphi10}$-*adhE*(Glu568Lys), $P_{trc\text{-ideal-4}}$-$SD_{\varphi10}$-*atoB*, $P_{trc\text{-ideal-4}}$-$SD_{\varphi10}$-*fadB*, $P_{trc\text{-ideal-4}}$-$SD_{\varphi10}$-*fadE*	[5]
BOX-3 Δ3	BOX-3 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*	[6]
BOX-3 Δ4	BOX-3 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*fucO*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*fucO*	This study
BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*yqhD*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* Δ*fadE*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*fucO*, Δ*fadE*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* Δ*fadE*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*yqhD*, Δ*fadE*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* Δ*ydiO*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*fucO*, Δ*ydiO*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* Δ*ydiO*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*yqhD*, Δ*ydiO*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* P_L-*aceEF*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*fucO*, P_L-SD_{aceF}-*aceEF-lpdA*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* P_L- *aceEF*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*yqhD*, P_L-SD_{aceF}-*aceEF-lpdA*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* P_L-*atoB*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*fucO*, P_L-$SD_{\varphi10}$-*atoB*	"
BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* P_L- *atoB*	BOX-3 Δ4, P_L-$SD_{\varphi10}$-*mhpF*, P_L-$SD_{\varphi10}$-*yqhD*, P_L-$SD_{\varphi10}$-*atoB*	"
BOX-3 Δ3 P_L-*atoB*	BOX-3 Δ3, P_L-$SD_{\varphi10}$-*atoB*	"
Plasmid		
pMW118-(l*attL*-Cm-l*attR*)	pSC101, *bla*, *cat,* λ*attL-cat-*λ*attR* cassete	[12]
pKD46	pINT-ts, *bla*, P_{araB}-λ*gam-bet-exo*	[11]
pMWts-Int/Xis	pSC101-ts, *bla*, P_R-λ*xis-int*, c*I*ts857	[13]

tance (*cat* gene), and 36 nucleotides homologous to DNA regions upstream of the coding regions of *mhpF*, *fucO*, *yqhD*, and *atoB* genes were obtained by PCR using primer pairs P7, P8; P7, P9; P7, P10; P7, P11; and plasmid pMW118-(λ*attL*-Cm-λ*attR*) [12] as a template. The resulting DNA fragments were treated with restriction endonuclease *Bgl*II and ligated with T4 DNA ligase. The ligation products were amplified using primer pairs P3, P8; P9, P4; P5, P10; and P6, P11. The obtained PCR products were individually integrated into the chromosome of the *E. coli* MG1655 strain harboring the helper plasmid pKD46. The correspondence between the desired and experimentally obtained nucleotide sequences of a new regulatory element, which were introduced upstream of the coding regions of *mhpF*, *fucO*, *yqhD,* and *atoB* genes, was confirmed by sequencing with the primer pairs P12, P13; P14, P15; P16, P17; and P18, P19. Similarly, the native promoter of *aceEF-lpdA* operon was replaced by the P_L-SD*aceF* regulatory element, which contained P_L promoter of lambda phage and a ribosome binding site of *aceF* gene with a nucleotide sequence close to the *E. coli* canonical sequence. For this purpose, we used primer pairs P1, P20; P1, P21; P7, P22; and P21, P22; as well as genomic DNA from lambda phage and plasmid pMW118-(λ*attL*-Cm-λ*attR*) as templates. The correspondence between the desired and experimentally obtained nucleotide sequence of a new regulatory element introduced upstream the coding region of *aceE* gene was confirmed by sequencing of obtained clones using primers P23 and P24.

To inactivate *fadE* and *ydiO* genes, linear DNA fragments containing the chloramphenicol resistance marker (*cat* gene) were obtained by PCR with primer pairs P25 and P26, P27 and P28, and plasmid pMW118-(λ*attL*-Cm-λ*attR*) as a template. The resulting DNA fragments were individually integrated into the chromosome of *E. coli* MG1655 strain, carrying the helper plasmid pKD46. The correspondence between the expected and experimentally obtained chromosome structures of selected strains with individually inactivated *fadE* and *ydiO* genes was confirmed by PCR analysis with pairs of locus-specific primers P29, P30 and P31, P32.

Table 2. The oligonucleotide primers used in the study

Designation	Sequence
P1	5'-tgcgac-agatct-ctcacctaccaaacaatgccc-3'
P2	5'-atgtatatctccttc-acggccaatgcttcgtttc-3'
P3	5'-gccagaaccgataatggcgactttacgcttactcat-atgtatatctccttc-acggccaatg-3'
P4	5'-tgccgtttcgttcagaatcattctgttagccatcat-atgtatatctccttc-acggccaatg-3'
P5	5'-aatgcgggttggggtgtgcagattaaagttgttcat-atgtatatctccttc-acggccaatg-3'
P6	5'-agtacgtaccgcactgacgatgacacaatttttcat-atgtatatctccttc-acggccaatg-3'
P7	5'-ctagta-agatct-tgaagcctgctttttatactaagttgg-3'
P8	5'-cacccttcctatactgagcgcacaataaaaaatcat-cgctcaagttagtataaaaaagctgaac-3'
P9	5'-attgaagagtaatttcgtaaagcaacaaggagaagg-cgctcaagttagtataaaaaagctgaac-3'
P10	5'-ttaattcccctgcatcgcccgcattcttgccgcatc-cgctcaagttagtataaaaaagctgaac-3'
P11	5'-ttctgacggcacccctacaaacagaaggaatataaa-cgctcaagttagtataaaaaagctgaac-3'
P12	5'-ccgtctgctcattgttctgc-3'
P13	5'-gtaccaatgttgccagaaccg-3'
P14	5'-gacgaccctggcgattacg-3'
P15	5'-gcattgcaccagcgttttatc-3'
P16	5'-ggtaatgacgttcctgatgatc-3'
P17	5'-caataccgccaaattccag-3'
P18	5'-catgggctactgcatcactg-3'
P19	5'-caggtcgatggcgctggt-3'
P20	5'-tattcttttacctctta-acggccaatgcttcgtttc-3'
P21	5'-gatcggatccacgtcatttgggaaacgttctgacat-tattcttttacctctta-acggccaatg-3'
P22	5'-aaaactcaacgttattagatagataaggaataaccc-cgctcaagttagtataaaaaagctgaac-3'
P23	5'-gcaactaaacgtagaacctgtc-3'
P24	5'-tgagcacgctcaacaccttc-3'
P25	5'-agtggtcagacctcctacaagtaaggggcttttcgt-cgctcaagttagtataaaaaagctgaac-3'
P26	5'-ttacgcggcttcaactttccgcactttctccggcaa-tgaagcctgctttttatactaagttgg-3'
P27	5'-tttaactgaagaacaagaactgctgctggccagtat-cgctcaagttagtataaaaaagctgaac-3'
P28	5'-ttatttgttctgatagtctttcaggatctgccgacc-tgaagcctgctttttatactaagttgg-3'
P29	5'-gaagtacgggcaggtgctatg-3'
P30	5'-ggtaaaacggtgttgttcgcg-3'
P31	5'-gaagcggctcattaacaggag-3'
P32	5'-tcaacggcgtatgacggaag-3'

Target recombinant strains were obtained by introducing individual modifications into chromosomes of BOX-3 Δ3 and BOX-3 Δ4 strains by sequential P1-dependent transductions [10]. Removal of the marker flanked by *att* sites of lambda phage from chromosomes of strains was performed using plasmid pMWts-Int/Xis, as described previously [13].

Strain cultivation for 1,3-butanediol biosynthesis. Cells of recombinant strains were grown overnight at 37°C in M9 medium containing 2 g/L of glucose. 5 mL of the overnight cultures was diluted 10 times with 45 mL of M9 medium containing 10 g/L of glucose, 10 mM of $ZnSO_4$, and 10 mM of $FeSO_4$. The cultures were grown in 750 mL flasks on a rotary shaker (250 rpm) for 6 h at 37°C. To induce the expression of genes encoding enzymes of aerobic fatty acid β-oxidation pathway, isopropyl-β-D-thiogalactoside (IPTG) was added into culture medium of BOX-3 strain and its derivatives after 3 h of incubation to a final concentration of 1 mM. The obtained cell suspensions were centrifuged for 15 min at 2000 g and 4°C. The pellets were resuspended in 15 mL of M9 medium containing 10 g/L of glucose, 10 mM of $ZnSO_4$ and 10 mM $FeSO_4$. Expression of genes under the control of $P_{trc\text{-ideal-4}}$ promoter was induced by addition of IPTG (1 mM) to the culture medium. The obtained cell cultures were incubated for 24 hours in 15 mL screw cap tubes at 37°C on a rotary shaker (150 rpm). All experiments were performed in triplicate. The variations among the experimental results were less than 10%.

Analytical methods. The organic acid concentrations were determined by HPLC on an ion-exclusion column (7.8 × 300 mm, 8 μm) Rezex ROA-Organic Acid H+ (8%) (Phenomenex, USA) with detection at 210 nm using Waters HPLC system (Waters, USA). Sulfuric acid (2.5 mM) was used as a mobile phase at a flow rate of 0.5 mL/min. Identification and quantitative analysis of alcohols in culture media were per-

formed by gas chromatography with FID and MSD detection, as described previously [5, 6].

RESULTS AND DISCUSSION

It was previously shown that directly engineered *E. coli* BOX-3 strain and its derivatives are able to synthesize 1-butanol as the end product of glucose fermentation resulting from the one-turn reversal of fatty acid β-oxidation pathway. It was found that the efficiency of the target alcohol formation from butyryl-CoA, an intermediate of fatty acid β-oxidation, depends on the nature of alcohol/aldehyde dehydrogenases, which catalyze the terminal steps of corresponding CoA derivative reduction [6]. In particular, the *E. coli* BOX-3 Δ4 strain, deficient in the mixed acid fermentation pathways and lacking the activity of the main alcohol/aldehyde dehydrogenase AdhE (EC 1.1.1.1/1.2.1.3), was not able to synthesize 1-butanol and accumulated only little ethanol upon dramatically decreased anaerobic glucose utilization. This strain lost the ability to ferment glucose due to insufficiency of the basal activity of alternative alcohol/aldehyde dehydrogenases for efficient alcohol biosynthesis and glycolytic NADH reoxidation. Therefore, the BOX-3 Δ4 strain was chosen as a chasis for studying the feasibility of anaerobic 1,3-butanediol biosynthesis from glucose through the inverted fatty acid β-oxidation pathway. It was supposed that the strain would restore the ability to reoxidate glycolytically formed reducing equivalents and gain the capacity to produce 1,3-butanediol upon constitutive expression of alternative alcohol/aldehyde dehydrogenase genes.

CoA-dependent aldehyde dehydrogenase MhpF (EC 1.2.1.10) was selected as an enzyme catalyzing the reduction of 3-hydroxybutyryl-CoA into 3-hydroxy butyraldehyde. This choice was caused by the fact that the enzyme is able to use four-carbon compounds, such as butyryl-CoA as substrates [1]. To catalyze the terminal stage of the 3-hydroxybutyraldehyde reduction to 1,3-butanediol, L-1,2-propanediol oxidoreductase FucO (EC 1.1.1.77) and aldehyde reductase YqhD (EC 1.1.1.2), which forms 1,3-propanediol [14], were selected due to their ability to form diols as reaction products.

The constitutive expression of gene pairs *mhpF*, *fucO*, or *mhpF*, *yqhD* in the parent BOX-3 Δ4 strain was ensured resulting from replacement of the native regulatory regions of the corresponding genes by the artificial genetic element P_L-SDφ10 containing P_L promoter of lambda phage and the efficient ribosome binding site of φ10 gene from T7 phage.

The resulting BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* strains restored anaerobic glucose consumption, synthesizing significant quantities of ethanol as the end product of fermentation (Table 3). However, upon IPTG induction of expression of genes encoding enzymes of fatty acid β-oxidation cycle, which are under the control of LacI-dependent promoter, these strains did not synthesize 1,3-butanediol, while the level of ethanol synthesis remained unchanged. The absence of 1,3-butanediol among fermentation products formed by BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* strains in the presence of inducer (IPTG) could be explained by the activity of the intact acyl-CoA dehydrogenases (EC 1.3.99.3) FadE and YdiO, which are able to channel the key precursor metabolite (3-hydroxybutyryl-CoA) towards further reactions of inverted fatty acid β-oxidation. Indeed, the multifunctional protein FadB, which is responsible for the formation of 3-hydroxybutyryl-CoA from acetoacetyl-CoA, possess not only 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) activity, but also enoyl-CoA hydratase (EC 4.2.1.17) activity, which is responsible for the interconversion of 3-hydroxybutyryl-CoA and crotonyl-CoA. The presence of acyl-CoA dehydrogenase activity in the cells can shift the equilibrium of the latter reaction toward the formation of crotonyl-CoA through the involvement of the corresponding product in subsequent butyryl-CoA formation. While the native regulatory region of *ydiO* in the parent BOX-3 Δ4 strain has not been modified, expression of the *fadE* gene in the strain is controlled by artificial genetic element $P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$ and induced by IPTG. Although the contribution of the respective enzymes in functional reversal of the fatty acid β-oxidation cycle in *E. coli* has previously been shown to be insignificant [2, 3], *ydiO* and *fadE* were inactivated in BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* strains. However, these modifications did not affect the distribution of the fermentation end products synthesized by strains (Table 3).

Another factor limiting the production of 1,3-butanediol by constructed strains could be deficiency of the reducing equivalents required for the synthesis of the target substance. Indeed, two NADH molecules are required to form one ethanol molecule from acetyl-CoA, while two molecules of acetyl-CoA and three reducing equivalents are needed to form one 1,3-butanediol molecule. Glycolysis generates two moles of NADH per mole of consumed substrate, and under anaerobic conditions it is the only source of reducing equivalents in *E. coli* cells. Depending on the aeration conditions, oxidation of glycolytically formed pyruvic acid to acetyl-CoA in *E. coli* cells is catalyzed by either pyruvate dehydrogenase multyenzyme complex (EC 1.2.4.1./2.3.1.12/1.8.1.4) or pyruvate formate lyase (EC 2.3.1.54). Pyruvate formate lyase converts pyruvate to acetyl-CoA without NADH formation, while pyruvate dehydrogenase is known to generate additional NADH per synthesized acetyl-CoA molecule. Under anaerobic conditions, the conversion of pyruvate to acetyl-CoA in *E. coli* is catalyzed by pyruvate formate lyase, while the expression of pyruvate dehydrogenase complex (*aceEF-lpdA*)

Table 3. Representative concentrations of metabolites produced by constructed strains during anaerobic glucose utilization

Basic strain	Additional modifications	Acid, mM				Alcohol, mM			
		pyruvic		succinic		ethanol		1,3-butanediol	
		– IPTG	+ IPTG	– IPTG	+ IPTG	– IPTG	+ IPTG	– IPTG	+ IPTG
BOX-3 Δ4		3.6	3.5	0.7	0.7	0.7	0.6	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*fucO*	7.3	7.0	3.7	3.5	13.7	13.4	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*yqhD*	7.4	7.2	3.8	3.6	13.5	13.6	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*fucO* Δ*fadE*	7.2	7.1	3.6	3.4	13.4	13.1	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*yqhD* Δ*fadE*	7.3	6.9	3.3	3.3	13.7	13.5	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*fucO* Δ*ydiO*	7.3	7.2	3.5	3.2	13.9	13.6	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*yqhD* Δ*ydiO*	7.2	7.0	3.5	3.4	13.5	13.2	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*fucO* P_L-*aceEF*	7.3	7.5	7.0	6.9	17.6	18.1	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*yqhD* P_L-*aceEF*	7.6	7.5	7.1	6.8	17.3	17.7	–	–
BOX-3 Δ4	P_L-*mhpF* P_L-*fucO* P_L-*atoB*	9.6	9.5	4.1	3.9	14.5	14.4	–	0.1
BOX-3 Δ4	P_L-*mhpF* P_L-*yqhD* P_L-*atoB*	9.3	9.4	3.8	3.8	13.9	14.1	–	0.1
BOX-3 Δ3	P_L-*atoB*	3.0	10.5	2.2	3.9	3.7	14.6	0.3	–

* Data are the mean value of three independent experiments. The variations among the experimental results were less than 10%.

genes is repressed during anaerobiosis [15]. Thus, anaerobic utilization of glucose molecule in BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* strains led to the formation of two acetyl-CoA molecules with concomitant generation of two NADH molecules and did not meet the requirements for redox balanced 1,3-butanediol biosynthesis.

Anaerobic expression of genes encoding the components of pyruvate dehydrogenase complex supplies the synthesis of acetyl-CoA derivatives with additional reducing power [16–18]. At the same time, anaerobic formation of acetyl-CoA exclusively by pyruvate dehydrogenase would lead to the formation of NADH in amounts excessive for the synthesis of 1,3-butanediol. Therefore, constitutive expression of *aceEF-lpdA* operon genes providing both aerobic and anaerobic activity of pyruvate dehydrogenase complex was ensured in BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* strains with intact pyruvate formate lyase. During anaerobic glucose utilization, irrespective of the presence of a specific inducer in the medium, the modified BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* P_L-*aceEF* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* P_L-*aceEF* strains formed similar profiles of the fermentation end products, which differed from those of the parental strains (Table 3). As shown in Table 3, constitutive expression of *aceEF-lpdA* operon resulted in a significant increase in amounts of ethanol and succinic acid synthesized by the strains. The increased production of succinic acid by the strains was probably caused by the activation of the glyoxylate shunt resulted from intensified acetyl-CoA synthesis [17, 19]. The high level of ethanol synthesis, which does not depend on the presence of IPTG in the medium, and the absence of 1,3-butanediol among the fermentation products, can be explained by the properties of enzymes that catalyze the conversion of acetyl-CoA to acetaldehyde and acetoacetyl-CoA. Indeed, the Km value of aldehyde dehydrogenase to acetyl-CoA is about 0.01 mM, and the specific activity of the enzyme is 14.1 μmol/min/mg, whereas the corresponding values for acetyl-CoA acetyltransferase AtoB (EC 2.3.1.9) are 0.47 mM and 1078 μmol/min/mg, respectively [20]. Since P_L promoter, which controls *mhpF* gene expression in the constructed strains, is much stronger than $P_{trc\text{-ideal-4}}$, which controls *atoB* gene expression, such catalytic properties of the respective enzymes could favor acetyl-CoA reduction to acetaldehyde rather than its involvement in acetoacetyl-CoA formation.

To compensate the imbalance in levels of *mhpF* and *atoB* gene expression in BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* strains, the artificial regulatory element $P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$ upstream *atoB* gene was replaced by a regulatory element P_L-SDφ10. 1,3-Butanediol was found in the culture medium of the modified BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* P_L-*atoB* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* P_L-*atoB* strains, while ethanol synthesis was slightly decreased (Table 3). At the same time, ethanol remained the main product formed by the strains during fermentation. Thus, it can be concluded that aldehyde dehydrogenase activity in the strains predominated over that of acetyl-CoA C-acetyltransferase despite the enhancement of *atoB* gene expression.

In the previously constructed BOX-3 Δ3 strain, the main CoA-dependent alcohol/aldehyde dehydrogenase of *E. coli* – AdhE was not inactivated. Meanwhile, the *adhE* gene in the strain is located under the control of the regulatory element $P_{trc\text{-ideal-4}}$-$SD_{\varphi 10}$, and the activity of the corresponding enzyme was reduced due to the directed introduction of a point mutation G → A into 1702 position of the gene coding region leading to Glu568Lys substitution in the protein product [6]. Thus, the ability of the BOX-3 Δ3 strain to synthesize 1,3-butanediol was studied upon enhancement of *atoB* gene expression. During anaerobic glucose utilization in the presence of the inducer, the BOX-3 Δ3 P_L-*atoB* strain formed a metabolite profile similar to those of strains BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* P_L-*atoB* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* P_L-*atoB*, except that 1,3-butanediol was not found among the products of fermentation (Table 3). In the absence of the inducer, the BOX-3 Δ3 P_L-*atoB* strain synthesized more 1,3-butanediol compared with BOX-3 Δ4 P_L-*mhpF* P_L-*fucO* P_L-*atoB* and BOX-3 Δ4 P_L-*mhpF* P_L-*yqhD* P_L-*atoB* strains; while ethanol formation by the strain was significantly decreased (Table 3). It can be assumed that the high substrate specificity of alcohol/aldehyde dehydrogenase AdhE prevented its effective use of 3-hydroxybutyryl-CoA as a substrate, thereby promoting ethanol synthesis upon induction of *adhE* gene expression and, accordingly, contributing to the decrease in availability of acetyl-CoA for acetoacetyl-CoA and 3-hydroxybutyryl-CoA formation. In the absence of induction, basal transcription from $P_{trc\text{-ideal-4}}$ promoter could provide cells with activity of intact 3-hydroxyacyl-CoA dehydrogenase FadB, but not mutant $AdhE^{E568K}$ activity. Therefore, the residual ethanol biosynthesis by the BOX-3 Δ3 P_L-*atoB* strain in the absence of inducer could have resulted from the basal activity of alternative alcohol/aldehyde dehydrogenases, not excluding MhpF, FucO, and YqhD. The significantly decreased activity of these alcohol/aldehyde dehydrogenases, compared with overexpressed acetyl-CoA C-acetyltransferase AtoB activity, allowed the strain to synthesize increased amounts of 1,3-butanediol.

The results of the current study indicated that efficient and balanced biosynthesis of 1,3-butanediol requires precise intracellular coordination of the activities of acetyl-CoA C-acetyltransferase, 3-hydroxyacyl-CoA dehydrogenase and alcohol/aldehyde dehydrogenases, the key enzymes responsible for the conversion of acetyl-CoA to the target product. As shown previously, such coordination can be attained as a result of fine tuning of the expression levels of the corresponding genes [12, 21]. The revealed ability of *E. coli* to synthesize

1,3-butanediol resulting from partial inversion of the native fatty acid β-oxidation cycle allows further targeted and rational engineering of the efficient microbial strains producing this nonnatural compound.

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REFERENCES

1. Dellomonaco, C., Clomburg, J.M., Miller, E.N., and Gonzalez, R., *Nature*, 2011, vol. 476, no. 7360, pp. 355–359.
2. Clomburg, J.M., Vick, J.E., Blankschien, M.D., Rodriguez-Moya, M., and Gonzalez, R., *ACS Synth. Biol.*, 2012, vol. 1, no. 11, pp. 541–554.
3. Vick, J.E., Clomburg, J.M., Blankschien, M.D., Chou, A., Kim, S., and Gonzalez, R., *Appl. Environ. Microbiol.*, 2015, vol. 81, no. 4, pp. 1406–1146.
4. Seregina, T.A., Shakulov, R.S., Debabov, V.G., and Mironov, A.S., *Biotekhnologiya*, 2009, no. 6, pp. 24–35.
5. Gulevich, A.Y., Skorokhodova, A.Y., Sukhozhenko, A.V., Shakulov, R.S., and Debabov, V.G., *Biotechnol. Lett.*, 2012, vol. 34, no. 3, pp. 463–469.
6. Gulevich, A.Yu., Skorokhodova, A.Yu., Morzhakova, A.A., Antonova, S.V., Sukhozhenko, A.V., Shakulov, R.S., and Debabov, V.G., *Appl. Biochem. Microbiol.*, 2012, vol. 48, no. 4, pp. 344–349.
7. Jiang, Y., Liu, W., Zou, H., Cheng, T., Tian, N., and Xian, M., *Microb. Cell Fact.*, 2014, vol. 13, no. 165.
8. Rosen, T.C., Daussmann, T., and Stohrer, J., *Speciality Chemicals Magazine*, 2004, vol. 24, no. 4, pp. 39–40.
9. Makshina, E.V., Dusselier, M., Janssens, W., Degreve, J., Jacobs, P.A., and Sels, B.F., *Chem. Soc. Rev.*, 2014, vol. 43, no. 22, pp. 7917–7953.
10. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Lab. Press, 1989.
11. Datsenko, K.A. and Wanner, B.L., *Proc. Natl. Acad. Sci. USA*, 2000, vol. 97, no. 12, pp. 6640–6645.
12. Katashkina, Zh.I., Skorokhodova, A.Yu., Zimenkov, D.V., Gulevich, A.Yu., Minaeva, N.I., Doroshenko, V.G., Biryukova, I.V., and Mashko, S.V., *Mol. Biol.*, 2005, vol. 39, no. 5, pp. 823–831.
13. Gulevich, A.Yu., Skorokhodova, A.Yu., Ermishev, V.Yu., Krylov, A.A., Minaeva, N.I., Polonskaya, Z.M., Zimenkov, D.V., Biryukova, I.V., and Mashko, S.V., *Mol. Biol.*, 2009, vol. 43, no. 3, pp. 547–557.
14. Nakamura, C.E. and Whited, G.M., *Curr. Opin. Biotechnol.*, 2003, vol. 14, no. 5, pp. 454–459.
15. Soini, J., Falschlehner, C., Liedert, C., Bernhardt, J., Vuoristo, J., and Neubauer, P., *Microb. Cell. Fact.*, 2008, vol. 7, no. 30. doi:10.1186/1475-2859-7-30
16. Zhou, S., Iverson, A.G., and Grayburn, W.S., *Biotechnol. Prog.*, 2010, vol. 26, no. 1, pp. 45–51.
17. Skorokhodova, A.Yu., Gulevich, A.Yu., Morzhakova, A.A., Shakulov, R.S., and Debabov, V.G., *Appl. Biochem. Microbiol.*, 2011, vol. 47, no. 4, pp. 373–380.
18. Bond-Watts, B.B., Bellerose, R.J., and Chang, M.C., *Nat. Chem. Biol.*, 2011, vol. 7, no. 4, pp. 222–227.
19. Skorokhodova, A.Y., Gulevich, A.Y., Morzhakova, A.A., Shakulov, R.S., and Debabov, V.G., *Biotechnol. Lett.*, 2013, vol. 35, no. 4, pp. 577–583.
20. Duncombe, G.R. and Frerman, F.E., *Arch. Biochem. Biophys.*, 1976, vol. 176, no. 1, pp. 159–170.
21. Mutalik, V.K., Guimaraes, J.C., Cambray, G., Lam, C., Christoffersen, M.J., Mai, Q.A., Tran, A.B., Paull, M., Keasling, J.D., Arkin, A.P., and Endy, D., *Nat. Methods*, 2013, vol. 10, no. 4, pp. 354–360.

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