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Effect of Anaplerotic Pathways Activation on CO_2-dependent Anaerobic Glucose Utilization by *Escherichia coli* Strains Deficient in the Main Pathways of Mixed Acid Fermentation

A. Yu. Skorokhodova*, A. A. Stasenko, A. Yu. Gulevich, and V. G. Debabov

State Research Institute of Genetics and Selection of Industrial Microorganisms, National Research Center "Kurchatov Institute", Moscow, 117545 Russia

**e-mail: skorokhodova@genetika.ru*

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Abstract—The effect of anaplerotic pathways activation on CO_2-dependent anaerobic glucose utilization by *Escherichia coli* strains deficient in the main fermentation pathways and possessing a modified system of glucose transport and phosphorylation was studied. Intracellular CO_2 generation in the strains was ensured resulting from oxidative decarboxylation of pyruvic acid by pyruvate dehydrogenase. Sodium bicarbonate dissolved in the medium was used as an external source of CO_2. The genes of heterologous pyruvate carboxylase and native NADH-dependent malic enzyme were overexpressed in the strains to allow anaplerotic carboxylation of pyruvic acid to oxaloacetic or malic acid. The ability of the strains to reoxidize NADH utilizing carboxylation products was additionally increased due to enhanced expression of malate dehydrogenase gene. In the case of endogenous CO_2 formation, the activation of anaplerotic pathways did not cause a notable increase in the anaerobic glucose consumption by the constructed strains. At the same time, the expression of pyruvate carboxylase led to a pronounced decrease in the secretion of pyruvic acid with the concomitant increase in the yield of four-carbon metabolites. Further enhancement of NADH-dependent malic enzyme expression provoked activation of a pyruvate – oxaloacetate – malate – pyruvate futile cycle in the strains. The availability in the medium of the external CO_2 source sharply increased the anaerobic utilization of glucose by strains expressing pyruvate carboxylase. The activity of the futile cycle has raised with the increased malic enzyme expression and dropped upon enhancement of malate dehydrogenase expression. As a result, the efficiency of CO_2-dependent anaerobic glucose utilization coupled to the formation of four-carbon carboxylation products increased in the studied strains resulting from the primary anaplerotic conversion of pyruvic acid into oxaloacetic acid followed by the involvement of the precursor formed in NADH-consuming biosynthetic reactions dominating over the reactions of the revealed futile cycle.

Keywords: anaplerotic pathways, carbon dioxide, fermentation, glucose, *Escherichia coli*

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INTRODUCTION

The growing concerns about environmental pollution have stimulated the development of the microbial technologies for the synthesis of industrially valuable chemicals that can provide an ecologically reasonable alternative to the traditional resource- and energy-consuming petrochemical routes. The biosynthetic processes coupled to CO_2 fixation rather than its dissimilation attract special attention due to increasing industrial greenhouse gas emissions.

Many industrially valuable chemicals are more reduced than sugars, the traditional substrates for microbial biotechnology, suggesting the necessity of anaerobic conditions for the efficient biosynthesis of the respective compounds. Technological advantages in such processes can be achieved through the use of facultative anaerobic microorganisms as biocatalysts, which ensure rapid accumulation of biomass under aeration and efficient conversion of the substrate to the target product during anaerobiosis. *Escherichia coli*, a facultative anaerobic bacterium, is widely used as a chassis for development of producers of useful metabolites due to its well-studied metabolism, easy operation, and low nutrient requirements [1, 2].

E. coli strains directly engineered as efficient anaerobic whole-cell biocatalysts can form required precursor metabolites, participating in both the maintenance of the intracellular redox balance and the biosynthesis of target products, during CO_2 fixation in a number of key cellular reactions of reversible de-/carboxylation. These reactions are refered to the anaplerotic pathways of the metabolic node phosphoenolpyruvate—pyruvic acid—oxaloacetic acid—malic acid [3], and generally are not subjected to inactivation during the

Table 1. Oligonucleotide primers used in the work

No.	Sequence
P1	5'-tgcgac-agatct-ctcacctaccaaacaatgccc-3'
P2	5'-atgtatatctccttc-acggccaatgcttcgtttc-3'
P3	5'-aataccgccagcagcgccgaggactgcgactttcat-atgtatatctccttc-acggccaatg-3'
P4	5'-tggttccatgtcactcactctttttgaatatccat-atgtatatctccttc-acggccaatg-3'
P5	5'- ctagta-agatct-tgaagcctgctttttatactaagttgg-3'
P6	5'-catatcttagtttatcaatataataaggagtttagg-cgctcaagttagtataaaaaagctgaac-3'
P7	5'-tggcggtaaagcaaagacgataaaagccccccaggg-cgctcaagttagtataaaaaagctgaac-3'
P8	5'-cttgcgtgactacacattcttg-3'
P9	5'-cggagtcgcatcttcaccag-3'
P10	5'-cgctgaaaagtaattcataaccatc-3'
P11	5'-gttacggcgttcttccatgc-3'

construction of anaerobic producers of reduced chemicals [4, 5].

An obligatory requirement for creation of *E. coli* strains suitable for the efficient anaerobic production of useful substances is the inactivation of mixed acid fermentation pathways, which compete with target biosynthetic pathways for precursor metabolites and reducing equivalents. However, *E. coli* strains deficient in mixed acid fermentation, which leads to the formation of ethanol, and acetic and lactic acids, dramatically decrease the anaerobic utilization of glucose in the absence of available CO_2 as a result of inability to efficiently reoxidize glycolytic NADH in residual fermentation pathways [6, 7].

We previously investigated the effect of CO_2 on the anaerobic utilization of glucose by recombinant *E. coli* strains deficient in the main fermentation pathways and possessing a modified system of glucose transport and phosphorylation [8], which increases the intracellular availability of phosphoenolpyruvate (PEP) for carboxylation reactions [9, 10]. The positive effect of extracellular CO_2 availability or its intracellular generation in the corresponding strains decreased upon impaired coordination between the formation of the intermediates of the metabolic node phosphoenolpyruvate—pyruvic acid—oxaloacetic acid—acetyl-CoA and their subsequent involvement in the residual reactions of the cellular redox balance maintenance. The spectrum of secreted metabolites indicated insufficient functional activity of the anaplerotic pathways in the constructed strains.

The goal of the work was to study the effect of the activation of anaplerotic pathways on CO_2-dependent anaerobic utilization of glucose by *Escherichia coli* strains deficient in the main pathways of mixed acid fermentation.

MATERIALS AND METHODS

Reagents. Restrictases, T4 DNA ligase, Taq DNA polymerase (Thermo Scientific, Lithuania) and Phusion DNA polymerase (Thermo Scientific, Finland) were used. PCR products were electrophoretically purified in an agarose gel and isolated with a QIAquick Gel Extraction Kit (Qiagen, United States). The oligonucleotides (Sintol, Russia) are presented in Table 1. The components of the nutrient media, salts and other reagents were produced by Panreac (Spain) and Sigma (United States).

Bacterial strains, plasmids and media. The *E. coli* strain K-12 MG1655 (VKPM B-6195) and the previously constructed *E. coli* strain MSG1.0 Δ*frdAB* Δ*pflB* [8] designated as FP, which possesses a modified system of glucose transport and phosphorylation, deleted pyruvate formate lyase (EC 2.3.1.54), and inactivated pathways of mixed acid fermentation, were used as the parent strains for the construction of all strains obtained in the work. The bacterial strains and plasmids used in this work are presented in Table 2. Bacteria were cultured using reach media LB, SOB, and SOC and minimal medium M9 [11] with addition of ampicillin (100 μg/mL) or chloramphenicol (30 μg/mL) was added to the medium if required.

Strain construction. All chromosomal modifications were performed by the method described earlier [12]. The construction of DNA fragments for the replacement of native regulatory regions of the *mdh* and *sfcA* genes by artificial genetic element P_L-SDφ10, which contains strong constitutive P_L promoter of the lambda phage and efficient ribosome-binding site of the φ10 gene from the T7 phage, was performed in several stages. At the first stage, DNA fragments containing the *Bgl*II recognition site, the P_L promoter, the SD sequence of the φ10 gene from the T7 phage and 36 nucleotides complementary to the 5'-ends of the coding regions of the *mdh* and *sfcA* genes were obtained by PCR. The fragments were obtained in two steps. Initially, a DNA fragment containing the *Bgl*II recognition site, the P_L promoter, and a part of the SD sequence of the φ10 gene from the T7 phage was obtained by PCR using genomic DNA of the lambda phage as a template and primers P1 and P2. The

Table 2. Strains and plasmids

Object	Genotype	Reference
Strain		
MG1655	Wild-type *E. coli* strain (VKPM B-6195)	VKPM
FP	*E. coli* MSG1.0 (MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L*glk*, P_{tac}*galP*) Δ*frdAB*, Δ*pflB*	[8]
FP P_L-*mdh*	*E. coli* MSG1.0 Δ*frdAB*, Δ*pflB*, P_L-$SD_{\varphi10}$-*mdh*	Present work
FP P_L-*sfcA*	*E. coli* MSG1.0 Δ*frdAB*, Δ*pflB*, P_L-$SD_{\varphi10}$-*sfcA*	"
FP P_L-*mdh* P_L-*sfcA*	*E. coli* MSG1.0 Δ*frdAB*, Δ*pflB*, P_L-$SD_{\varphi10}$-*mdh*, P_L-$SD_{\varphi10}$-*sfcA*	"
Plasmid		
pMW118-(λ*attL*-Cm-λ*attR*)	pSC101, *bla*, *cat,* λ*attL*-*cat*-λ*attR*	[13]
pKD46	pINT-ts, *bla*, P_{araB}-λ*gam-bet-exo*	[12]
pMWts-Int/Xis	pSC101-ts, *bla*, P_R-λ*xis-int*, c*I*ts857	[14]
pMW119	pSC101, *bla*, *cat*	GenBank AB005476.2
pPYC	pMW119 with cloned pyruvate carboxylase gene (*pycA*) from *B. subtilis*	[15]

resulting PCR product served as a template in the next rounds of PCR with primer pairs P1 and P3, P1 and P4. Primers P3 and P4 contained a region complementary to the 3'-end of the P_L promoter, the SD sequence of the φ10 gene from the T7 phage and the first 36 nucleotides from reading frames of the *mdh* and *sfcA* genes, respectively. In parallel, the second step of the construction of DNA fragments was carried out. DNA fragments containing the *Bgl*II recognition site, chloramphenicol resistance marker (the *cat* gene), and 36 nucleotides homologous to DNA regions upstream of the coding regions of the *mdh* and *sfcA* genes were obtained by PCR using primer pairs P5 and P6, P5 and P7, and plasmid pMW118-(λ*attL*-Cm-λ*attR*) [13] as a template. The resulting DNA fragments were treated with *Bgl*II restriction endonuclease and ligated with T4 DNA ligase. The ligation products were amplified using primer pairs P3 and P6, P4 and P7. The obtained PCR products were integrated into the chromosome of *E. coli* strain MG1655, which carries the pKD46 helper plasmid. The correspondence between the desired and experimentally obtained nucleotide sequences of the new regulatory element introduced upstream of the coding regions of the *mdh* and *sfcA* genes was confirmed by sequencing with primer pairs P8 and P9, P10 and P11.

FP P_L-*mdh*, FP P_L-*sfcA* and FP P_L-*mdh* P_L-*sfcA* strains were obtained by the introduction of individual modifications into the chromosome of the FP strain by P1-dependent transductions [11]. The removal of the marker, which is flanked by *att* sites of the lambda phage, from the chromosomes of the target strains was performed using the pMWts-Int/Xis plasmid as described previously [14]. Transformation of the strains with pMW119 and pPYC plasmids [15] was carried out according to a standard procedure.

Strain cultivation. Recombinant strains were grown overnight in M9 medium containing 2 g/L glucose at 37°C. Five milliliters of the obtained night cultures was tenfold diluted by the addition of 45 mL of M9 medium containing 10 g/L of glucose and 10 g/L of yeast extract. The resulting cultures were grown in 750-mL flasks on a rotary shaker at 250 rpm for 8 h at 37°C and then 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 or 10 g/L of glucose and 10 g/L of $NaHCO_3$. The cultures were incubated in 15-mL tubes closed with screw caps on a rotary shaker at 250 rpm for 24 h at 37°C. All media additionally contained 100 μg/mL of ampicillin (Sintez, Russia). The cell suspensions were centrifuged at 10000 g for 10 min. The concentrations of secreted metabolites and residual glucose were determined in the obtained supernatants. All experiments were repeated at least three times; the results of repeated experiments varied within a range not exceeding 10%.

Analytical methods. The concentrations of organic acids in culture liquids freed from biomass by centrifugation were determined by HPLC on a Rezex ROA-Organic Acid H+ (8%) ion-exclusion column (300 × 7.8 mm, 8 μm, Phenomenex, United States) with a Waters HPLC system (Waters, United States). An aqueous solution of sulfuric acid (2.5 mM) was used as the mobile phase with a flow rate of 0.5 mL/min. Detection was performed at 210 nm. A Spherisorb-NH2 column (4.6 × 250 mm, 5 μm, Waters, United States) and a Waters 2414 refractive detector were used to determine the glucose concentration. An acetonitrile-water mixture (75 : 25 volume ratio) served as the mobile phase at a flow rate of 1 mL/min. The ethanol concentrations in culture liquids were determined by gas chromatography using an OmegaWax column

(30 m, 0.25 mm i.d., 0.25 μm film thickness, Supelco, United States). A GC-17A chromatograph (Shimadzu, Japan) equipped with flame ionization detector and AOC-20i autosampler was used.

RESULTS AND DISCUSSION

The previously constructed FP strain (Table 2) was used as the chassis to study the effect of anaplerotic pathways activation on CO_2-dependent anaerobic utilization of glucose by *Escherichia coli* strains deficient in the main pathways of mixed acid fermentation. In this strain, mixed acid fermentation pathways responsible for the formation of acetic and lactic acids, as well as ethanol, were inactivated by the deletion of the *ackA*, *pta*, *poxB*, *ldhA* and *adhE* genes, which encode key enzymes catalyzing corresponding reactions. The functionality of the reductive branch of the tricarboxylic acid (TCA) cycle, providing the only way to reoxidize NADH anaerobically during the sequential conversion of oxaloacetic acid (OAA) to malic and succinic acids, was impaired in the strain by the inactivation of fumarate reductase (EC 1.3.5.4) due to deletion of the *frdAB* genes. At the same time, the intracellular availability of PEP, the substrate for anaplerotic OAA formation by phosphoenolpyruvate carboxylase Ppc (EC 4.1.1.31) and phosphoenolpyruvate carboxykinase PckA (EC 4.1.1.49), was increased due to PEP-independent glucose transport and phosphorylation. Intracellular generation of CO_2, which is necessary for PEP carboxylation, was resulted from the residual activity of pyruvate dehydrogenase [8, 16], which oxidatively decarboxylates pyruvic acid with the formation of acetyl-CoA, CO_2 and NADH. Excessive acetyl-CoA formation in the strain was prevented by inactivation of the *pflB* gene, encoding pyruvate formate lyase (EC 2.3.1.54).

Both intracellular CO_2 generation and its extracellular availability caused only a slight positive effect on the anaerobic utilization of glucose by the FP strain resulting from the impaired coordination between the formation of intermediates of the metabolic node phosphoenolpyruvate—pyruvic acid—oxaloacetic acid—acetyl-CoA and their subsequent involvement in residual fermentation reactions. Pyruvic acid was the main metabolite secreted by the strain, and its portion among the fermentation products exceeded the total portion of four-carbon metabolites, malic and succinic acids, synthesized in the strain with the participation of carboxylation reactions. These observations could indicate insufficient activity of anaplerotic pathways in the constructed strain.

The key bacterial anaplerotic enzymes responsible for the formation of OAA from glycolytic precursors include PEP-carboxylating phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase as well as pyruvate carboxylase (EC 6.4.1.1), which uses pyruvic acid as a substrate [3]. Phosphoenolpyruvate carboxylase and pyruvate carboxylase irreversibly carboxylate corresponding substrates to form OAA, while the reaction catalyzed by phosphoenolpyruvate carboxykinase is reversible. *E. coli* cells possess both PEP carboxylating enzymes, whereas pyruvate carboxylase activity is absent in this bacterium. Consequently, during utilization of glycolytic substrates phosphoenolpyruvate carboxylase serves in *E. coli* as the main anaplerotic enzyme [3]. Since phosphoenolpyruvate carboxylase competes for the common precursor metabolite, PEP, with pyruvic acid synthesizing glycolytic pyruvate kinases PykA and PykF (EC 2.7.1.40), the presence of heterologous pyruvate carboxylase activity could significantly increase the flexibility and efficiency of anaplerotic processes in the FP strain.

Indeed, during anaerobic glucose utilization the FP [pPYC] strain expressesing cloned into pMW119 plasmid *Bacillus subtilis pycA* gene, which encodes pyruvate carboxylase, secreted significantly less pyruvic acid than the control strain FP [pMW119] (Fig. 1a). Concomitantly, the yield of four-carbon fermentation products formed by the FP [pPYC] strain was 1.5 times higher than that of the FP [pMW119] strain (Table 3). Anaerobic biosynthesis of the corresponding metabolites in the FP strain and its derivatives suggested the involvement of OAA in a series of residual fermentation reactions, including, in particular, those catalyzed by malate dehydrogenase (EC 1.1.1.37), fumarases (EC 4.2.1.2) and aerobically synthesized succinate dehydrogenase (EC 1.3.5.1) [8]. Therefore, an elevated malic and succinic acids formation indicated an increased intensity of carboxylation in the FP [pPYC] strain. Nevertheless, upon the intracellular generation of CO_2 by pyruvate dehydrogenase the expression of heterologous pyruvate carboxylase did not lead to notable increase in anaerobic glucose utilization by the recombinant strain [8]. At the same time, the total portion of acetyl-CoA derivatives among the products formed by the FP [pPYC] strain during anaerobic glucose utilization was higher than that of four-carbon metabolites, whose biosynthesis via the reductive branch of the TCA cycle would require equimolar intracellular CO_2 generation. Thus, anaerobic glucose utilization by the strain could have been limited by factors other than the intracellular CO_2 availability.

The low anaerobic glucose consumption by the initial FP strain was presumably caused by the inability to efficiently reoxidize glycolytic NADH in residual fermentation reactions due to imbalance in the required precursor metabolites formation [8]. In the case of the FP [pPYC] strain, an increased yield of four-carbon metabolites indicated an enhanced generation of OAA, which is required for the operation of the subsequent reactions of the reductive branch of the TCA cycle. At the same time, the portion of four-carbon metabolites among the reduced fermentation products formed by the strain changed insignificantly (Table 3). The latter could indicate that the activity of malate

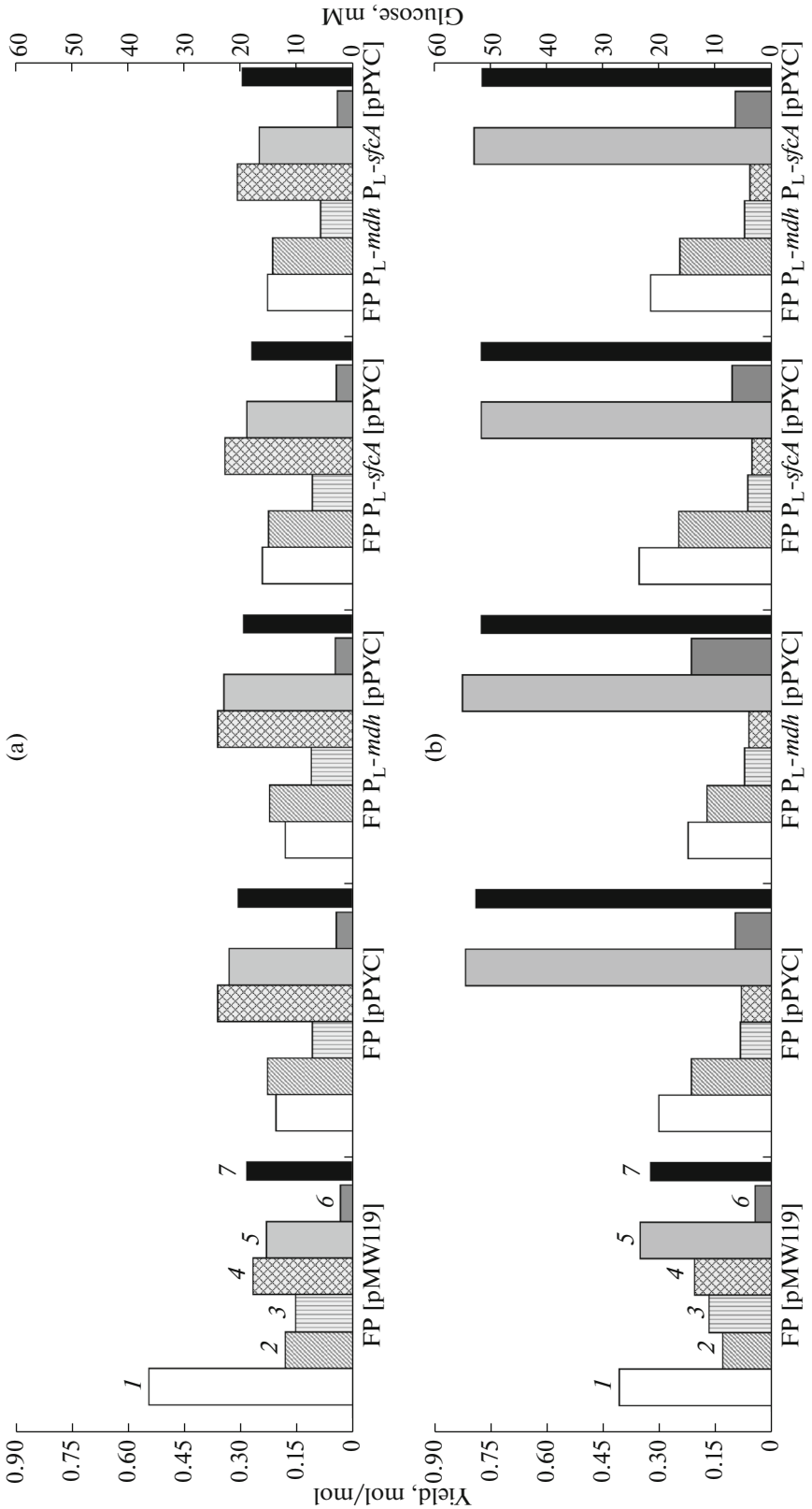

Fig. 1. Molar yields of metabolites: pyruvate (*1*), lactate (*2*), acetate (*3*), ethanol (*4*), succinate (*5*) and malate (*6*), secreted by the studied strains during anaerobic glucose utilization (*7*) in the absence (a) and presence (b) of external CO_2 source ($NaHCO_3$) in the medium.

Table 3. Characteristics of fermentative metabolism in constructed strains during anaerobic glucose utilization in the absence and presence of external CO_2 source in the medium

Strain	Yield of secreted four-carbon metabolites, mol/mol		Portion of four-carbon metabolites among detected reduced fermentation products, %	
	without $NaHCO_3$	with $NaHCO_3$	without $NaHCO_3$	with $NaHCO_3$
FP [pMW119]	0.26	0.40	36.6	54.1
FP [pPYC]	0.38	0.92	39.2	76.0
FP P_L-*mdh* [pPYC]	0.39	1.03	40.2	81.7
FP P_L-*sfcA* [pPYC]	0.32	0.89	35.9	74.8
FP P_L-*mdh* P_L-*sfcA* [pPYC]	0.29	0.89	35.4	74.2

dehydrogenase, catalyzing NADH-dependent conversion of OAA to malic acid, was insufficient in the strain.

The enhanced expression of the *mdh* gene, encoding malate dehydrogenase, was ensured in the FP [pPYC] strain resulting from the replacement of the native regulatory region of the corresponding gene with an artificial genetic element P_L-SDφ10, which contains strong constitutive lambda phage P_L promoter and efficient ribosome binding site of the φ10 gene from the phage T7. However, anaerobic glucose utilization and metabolite production by the obtained strain FP P_L-*mdh* [pPYC] were comparable to those demonstrated by the parent strain (Fig. 1a, Table 3). The secretion of pyruvic acid by the FP P_L-*mdh* [pPYC] decreased insignificantly.

Reversible carboxylation of pyruvic acid into malic acid, which is catalyzed by malic enzymes, can also be regarded an anaplerotic reaction [3]. *E. coli* possesses two malic enzymes, NADH-dependent SfcA (EC 1.1.1.39) and NADPH-dependent MaeB (EC 1.1.1.40). Due to its catalytic characteristics, NADH-dependent malic enzyme SfcA is more capable of reductive carboxylation of pyruvic acid than NADPH-dependent MaeB [17]. In addition, overexpression of the *sfcA* gene was shown to increase the anaerobic production of succinic acid by engineered *E. coli* strains [18, 19]. Therefore, the native regulatory region of the *sfcA* gene in the FP [pPYC] strain was replaced by an artificial genetic element P_L-SDφ10 as well as in the case of *mdh* gene.

Despite the comparable anaerobic glucose consumption (Fig. 1a), the FP P_L-*sfcA* [pPYC] strain synthesized pyruvic acid with a slightly higher while four-carbon fermentation products with a notably lower yield than the strains FP P_L-*mdh* [pPYC] and FP [pPYC] (Table 3). Moreover, the portion of four-carbon metabolites among the reduced fermentation products formed by the strain was close to that of the FP [pMW119] strain. These data suggest that NADH-dependent malic enzyme in the the FP P_L-*sfcA* [pPYC] strain preferably catalyzed decarboxylation of malic acid, rather than its formation through the pyruvic acid carboxylation.

It is interesting to note that, besides the positive effect of the *sfcA* overexpression on anaerobic production of succinic acid by *E. coli* strains, both the absence of such effect due to respective modification [20] and elevated biosynthesis of the intermediates of the reductive branch of the TCA cycle caused by *sfcA* inactivation have also been reported [21, 22]. It could be supposed that, in the FP P_L-*sfcA* [pPYC] strain expressing pyruvate carboxylase, the decarboxylating activity of NADH-dependent malic enzyme provoked appearance of the pyruvic acid—OAA—malic acid—pyruvic acid futile cycle. Accordingly, increased expression of malate dehydrogenase would lead to the cycle intensification and concomitant decrease in anaerobic production of four-carbon metabolites by the respective strain. Indeed, the total yield of four-carbon metabolites and their portion among the reduced fermentation products formed by the strain FP P_L-*mdh* P_L-*sfcA* [pPYC] dropped (Fig. 1a, Table 3), confirming the emergence of the futile cycle in the constructed strains with enhanced expression of the NADH-dependent malic enzyme gene and possessing heterologous pyruvate carboxylase activity.

Since the introduction of modifications activating anaplerotic pathways did not increase anaerobic glucose consumption by the constructed strains upon intracellular CO_2 generation, their effect was further evaluated in the presence of sodium bicarbonate dissolved in the medium.

The presence of an external source of CO_2 in the medium sharply increased the anaerobic glucose consumption by strains expressing pyruvate carboxylase (Fig. 1b). In this case, the yield of four-carbon compounds formed by the corresponding strains increased by more than two-times compared with the strain FP [pMW119] and approached the theoretical maximum for biosynthesis of the respective metabolites in the reductive branch of the TCA cycle (Table 3). The portion of acetyl-CoA derivatives among the products secreted by the strains during anaerobic glucose utilization was significantly decreased, while the portion

of four-carbon metabolites among the reduced fermentation products increased (Table 3). Altogether, these data indicated that, in the presence of exogenous CO_2, pyruvate carboxylase in the constructed strains efficiently competed with pyruvate dehydrogenase for the common substrate, pyruvic acid, redirecting the carbon flux in the metabolic node pyruvic acid—oxaloacetic acid—acetyl-CoA, through OAA formation, towards residual NADH-dependent reactions of the reductive branch of the TCA cycle, while the contribution of acetyl-CoA-dependent reactions to the formation of malic and succinic acids was insignificant. The continued secretion of pyruvic acid by the strains was apparently resulted from the action of the revealed pyruvic acid—OAA—malic acid—pyruvic acid futile cycle. It should be noted that the basal activity of NADH-dependent malic enzyme in *E. coli* cells is relatively high [23]. Therefore, with the increased CO_2 availability, the functionality of the corresponding cycle in the strains expressing pyruvate carboxylase could manifest even upon intact expression of *sfcA* gene.

Under these conditions, increased expression of the malate dehydrogenase gene diminished the activity of the futile cycle. The FP P_L-*mdh* [pPYC] strain synthesized notably greater amount of malic acid than the parent strain FP [pPYC], mainly at the expense of decreased pyruvic acid secretion (Fig. 1b). As a result, the portion of four-carbon metabolites among the reduced fermentation products formed by the strain FP P_L-*mdh* [pPYC] in the presence of an external CO_2 source reached about 82% (Table 3) and was the maximum for all the studied strains.

In strains with enhanced expression of the NADH-dependent malic enzyme gene, the activity of the pyruvic acid—OAA—malic acid—pyruvic acid futile cycle was expectedly increased. The pyruvic acid secretion by the corresponding strains FP P_L-*sfcA* [pPYC] and FP P_L-*mdh* P_L-*sfcA* [pPYC] notably increased (Fig. 1b), while the production of four-carbon metabolites and their portion among the reduced fermentation products dropped below the levels demonstrated by the parent strain FP [pPYC] (Table 3). Meanwhile, in the presence of dissolved bicarbonate in the medium, the portion of four-carbon metabolites among the reduced fermentation products formed by the FP P_L-*mdh* P_L-*sfcA* [pPYC] strain did not fall to the level of the initial strain FP [pMW119]. This indicated that upon increased CO_2 availability the intensity of anaplerotic carboxylation of pyruvic acid into OAA followed by its reduction exceeded the intensity of oppositely directed reactions of the activated futile cycle in strains possessing pyruvate carboxylase activity.

Nevertheless, due to the residual activity of the revealed futile cycle CO_2-dependent anaerobic glucose utilization by the constructed strains did not reach optimum that would potentially be characterized by the formation of only reduced four-carbon fermentation products. Thus, it can be assumed that inactivation of genes encoding malic enzymes would favor more efficient anaerobic glucose utilization by strains deficient in the main fermentation pathways and possessing pyruvate carboxylase activity due to the prevention of the wasteful expenditure of pyruvic acid. The effect of such modifications on the characteristics of the respective recombinants requires further evaluation.

CONCLUSIONS

The results of this study indicate that CO_2-dependent anaerobic glucose utilization in *E. coli* strains deficient in the main fermentation pathways can be enhanced resulting from the primary anaplerotic carboxylation of pyruvic acid into OAA, in the presence of exogenous CO_2, followed by the efficient channeling of the precursor metabolite formed towards the subsequent NADH-consuming biosynthetic reactions.

Nevertheless, optimization of the processes of anaerobic glucose utilization and subsequent synthesis of target substances by the engineered platform strains will undoubtedly require the identification and inactivation of potential futile cycles consuming key intermediates of the central metabolism.

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