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PRODUCING ORGANISMS, BIOLOGY, SELECTION, AND GENETIC ENGINEERING

Inactivation of Malic Enzymes Improves the Anaerobic Production of Four-Carbon Dicarboxylic Acids by Recombinant *Escherichia coli* Strains Expressing Pyruvate Carboxylase

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Abstract—The genes *maeA* and *maeB*, encoding NADH- and NADPH-dependent malic enzymes, have been deleted in a recombinant *Escherichia coli* strain with inactivated mixed-acid fermentation pathways and a modified system of glucose transport and phosphorylation upon the heterological expression of the pyruvate carboxylase gene. During anaerobic glucose utilization, the parental strain synthesized malic, fumaric, and succinic acids as the main fermentation end products, while pyruvic acid was accumulated as the main by-product resulting from the functioning of the pyruvate–oxaloacetate–malate–pyruvate futile cycle. Upon individual deletions of the *maeA* and *maeB* genes, the mutant strains converted glucose into four-carbon dicarboxylic acids with increased efficiency still secreting notable amounts of pyruvic acid. The combined inactivation of both malic enzymes in the constructed strain significantly elevated the portion of malic, fumaric, and succinic acids among the fermentation end products with a concomitant decrease in the secretion of pyruvic acid and other by-products due to the abolishment of the action of the futile cycle competing with the target biosynthetic processes.

Keywords: glucose, *Escherichia coli*, fermentation, malic enzyme, metabolic engineering, pyruvate carboxylase, fumaric acid, malic acid, succinic acid

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INTRODUCTION

Four-carbon dicarboxylic acids, in particular, malic, fumaric, and succinic, are industrially important chemicals that can serve as universal building blocks in the organic synthesis of a wide range of high value-added products [1]. At present, these acids are synthesized petrochemically. However, being conservative intermediates of the metabolism of huge variety of organisms, the corresponding compounds can be produced by microbial synthesis from renewable plant raw materials that can represent an ecologically reasonable alternative to traditional resource- and energy-consuming processes.

Aspergillus flavus, *Aspergillus niger* [2], *Rhizopus oryzae* [3], *Actinobacillus succinogenes* [4], *Anaerobiospirillum succiniciproducens* [5], and *Mannheimia succiniciproducens* are natural producers of malic, fumaric, and succinic acids [6]. However, nowadays the characteristics of known natural producers [7] cannot ensure economic feasibility for bio-based production of the corresponding dicarboxylates at industrial scale. At the same time, due to the convenience and availability of precise genetic editing tools, significant progress has been achieved in the development of recombinant producers of the respective compounds using *Escherichia coli* as a biosynthetic chassis [8].

The facultative anaerobic bacterium *E. coli* can form malic, fumaric, and succinic acids during carbon substrate utilization under both anaerobic and aerobic conditions. In the absence of oxygen, the reactions of the reductive branch of the tricarboxylic acid (TCA) cycle serve in *E. coli* as the main pathway of the biosynthesis of these acids whereas upon aeration the respective compounds are synthesized through the entire oxidative TCA cycle. The anaerobic synthesis of four-carbon dicarboxylates is more advantageous, since it can ensure a high yield of the target products due to CO_2 fixation of at the stage of oxaloacetate (OAA) formation, the key precursor metabolite of the reductive branch of the TCA cycle.

Nevertheless, up to date the anaerobic conversion of glucose, the traditional substrate of microbial biotechnology, to the target dicarboxylic acid with yield values close to the theoretical maximum has been achieved using engineered *E. coli* strains only in the case of succinic acid, whose efficient biosynthesis implies participation of not only the reductive branch of the TCA cycle, but also the reactions of the glyoxyl-

Table 1. Strains, plasmids, and oligonucleotide primers used in this work

Name	Genotype/sequence 5' → 3'	Source
	Strains	
MG1655	*E. coli* wild-type (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*	[17] Laboratory collection
FP Δ*maeA*	*E. coli* MSG1.0 Δ*frdAB*, Δ*pflB*, Δ*maeA*	"
FP Δ*maeB*	*E. coli* MSG1.0 Δ*frdAB*, Δ*pflB*, Δ*maeB*	"
FP Δ*maeA* Δ*maeB*	*E. coli* MSG1.0 Δ*frdAB*, Δ*pflB*, Δ*maeA*, Δ*maeB*	"
	Plasmids	
pMW118-(λ*attL*-Cm-λ*attR*)	pSC101, *bla*, *cat,* λ*attL-cat-*λ*attR*	[19]
pKD46	pINT-ts, *bla*, P_{araB}-λ*gam-bet-exo*	[20]
pMWts-Int/Xis	pSC101-ts, *bla*, P_R-λ*xis-int*, c*I*ts857	[21]
pPYC	pMW119 with cloned *B. subtilis* pyruvate carboxylase gene (*pycA*)	[22]
	Primers	
P1	gatggatattcaaaaaagagtgagtgacatggaacccgctcaagttagtataaaaaagctgaac	Current work
P2	ttagatggaggtacggcggtagtcgcggtattcggctgaagcctgctttttttatactaagttgg	"
P3	aatggatgaccagttaaaacaaagtgcacttgatttcgctcaagttagtataaaaaagctgaac	"
P4	ttacagcggttgggtttgcgcttctaccacggccagtgaagcctgctttttttatactaagttgg	"
P5	cgctgaaaagtaattcataaccatc	"
P6	gttctgcatagcaggtgaggc	"
P7	gacaggcatggtattgctgg	"
P8	gagagatattcgctgtggtgc	"

ate shunt (GS) [9–11]. Thus, the development of approaches for the construction of highly effecient microbial producers of not only succinic but also malic and fumaric acids remains an actual task.

Standard approaches aimed to ensure efficient anaerobic production of the four-carbon dicarboxylic acids in *E. coli* include increasing intracellular OAA availability for target biosynthetic reactions of the reductive branch of the TCA cycle. The corresponding effect is usually achieved resulting from the overexpression of genes encoding anaplerotic enzymes [12, 13]. The key bacterial anaplerotic enzymes responsible for the formation of the OAA from glycolytic precursors include phosphoenolpyruvate (PEP)-carboxylating PEP carboxylase (EC 4.1.1.31) and PEP carboxykinase (EC 4.1.1.49) as well as pyruvate carboxylase (EC 6.4.1.1), which uses pyruvic acid as a substrate [14].

E. coli cells possess both PEP carboxylating enzymes, whereas pyruvate carboxylase activity is absent in this bacterium. Consequently, the presence of heterologous pyruvate carboxylase activity increases the flexibility and efficiency of anaplerotic processes in recombinant *E. coli* strains [14, 15]. However, it was shown that the heterologous expression of the pyruvate carboxylase gene in *E. coli* strains engineered for the anaerobic conversion of glucose into four-carbon dicarboxylic acids provoked the appearance of a pyruvate-OAA-malate-pyruvate futile cycle, caused by the decarboxylating activity of cellular malic enzymes (EC 1.1.1.39/40) [16].

It is obvious that the functioning of the revealed futile cycle, wastefully consuming pyruvic and oxaloacetic acid, is competitive with the biosynthesis of the target dicarboxylates through the reductive branch of the TCA cycle, and may affect the efficiency of their anaerobic production from glucose.

The goal of the work was to study the effect of the inactivation of malic enzymes on the anaerobic production of four-carbon dicarboxylic acids by recombinant *Escherichia coli* strains expressing pyruvate carboxylase.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

Table 1 presents the strains, plasmids, and primers used in the work.

The *E. coli* strain K-12 MG1655 (VKPM B-6195) and the previously constructed *E. coli* strain MSG1.0 Δ*frdAB* Δ*pflB* [17] (designated as FP), possessing 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 parent strains for the consrtuction of all strains obtained in the work. Bacteria were cultured in rich media LB, SOB, and SOC, as well as in minimal

M9 medium [18] with the addition of 100 μg/mL ampicillin (Synthesis, Russia) or 30 μg/mL chloramphenicol (Sigma, United States) if necessary.

Reagents

Taq-DNA polymerase (Thermo Scientific, Lithuania) was used in the work. Oligonucleotide primers (Table 1) were synthesized by Evrogen (Russia). Nutrient media components, salts, and other reagents were from Panreac (Spain) and Sigma (United States). PCR products were purified by agarose gel electrophoresis and isolated with a QIAquick Gel Extraction Kit (Qiagen, United States).

Construction of Strains and Plasmids

All chromosomal modifications were performed with a modified [19] method developed by Datsenko and Wanner [20]. Linear DNA fragments for inactivation of the *maeA* and *maeB* genes containing the chloramphenicol resistance marker (*cat* gene) were obtained by PCR with two primer pairs (P1, P2 and P3, P4) and pMW118-(*λattL*-Cm-*λattR*) plasmid [19] as a template (see Table 1). The resulting DNA fragments were individually integrated into the chromosome of *E. coli* strain MG1655, carrying the pKD46 helper plasmid [20]. The correspondence between the expected and experimentally obtained chromosome structures of the selected strains with individually inactivated *maeA* and *maeB* genes was confirmed by PCR with pairs of locus-specific primers: P5, P6 and P7, P8.

Strains FP Δ*maeA*, FP Δ*maeB*, and FP Δ*maeA*Δ *maeB* were obtained by the introduction of the individual modifications into the chromosome of the FP strain via P1-dependent transduction [18]. Removal of the marker, flanked by *att*-sites of lambda phage, from the chromosome of target strains was carried out using pMWts-Int/Xis plasmid as described previously [21]. Transformation of the strains with the pPYC plasmid [22] was carried out according to the standard procedure.

Cultivation of Strains for the Biosynthesis of Dicarboxylic Acids

The strains FP [pPYC], FP Δ*maeA* [pPYC], FP Δ*maeB* [pPYC], and FP Δ*maeA* Δ*maeB* [pPYC] were grown overnight in M9 medium containing 2 g/L glucose at 37°C. Five milliliters of the overnight culture was diluted ten times with 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 at 37°C on a rotary shaker at 250 rpm for 8 h and then 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 and 10 g/L of $NaHCO_3$. Subsequently, the cultures were incubated in 15 mL tubes closed with screw caps on a rotary shaker (250 rpm) for 24 h at 37°C. All media additionally contained 100 μg/mL ampicillin.

The cell suspensions were then centrifugated at 10000 *g* for 10 min, and the concentrations of secreted metabolites and residual glucose were determined in the resulting supernatants. All experiments were repeated at least three times, and the results of the repeated experiments varied in a range not exceeding 10%.

Analytical Methods

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

RESULTS AND DISCUSSION

The FP strain was used as the chasis to study the effect of the inactivation of malic enzymes on the anaerobic production of four-carbon dicarboxylic acids by recombinant *Escherichia coli* strains expressing pyruvate carboxylase (see Table 1). This strain was previously engineered for the anaerobic production of malic, fumaric, and succinic acids from glucose through the reductive branch of the TCA cycle. The main pathways of mixed-acid fermentation in the strain were inactivated by the deletion of the *ackA*, *pta*, *poxB*, *ldhA*, and *adhE* genes, which encode key *E. coli* enzymes responsible for the formation of acetic acid, lactic acid, and ethanol.

The intracellular availability of PEP, the substrate of cellular OAA-synthesizing anaplerotic enzymes, was increased due to PEP-independent glucose transport and phosphorylation. The formation of the full range of four-carbon intermediates of the TCA cycle, malic, fumaric, and succinic acids, was ensured resulting from the deletion of *frdAB* genes, which encode the catalytic components of the fumarate reductase complex (EC 1.3.5.4). Anaerobic biosynthesis of the respective metabolites in FP strain and its derivatives suggested, therefore, the channelling of OAA through a series of residual fermentation reactions, including, in particular, the conversions 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) [16, 17]. At the same time, the contribu-

Table 2. Characteristics of fermentative metabolism in constructed strains during anaerobic glucose utilization

Strain	The portion of four-carbon metabolites (%) among	
	secreted fermentation products	detected reduced fermentation products
FP [pPYC]	57.4	78.1
FP Δ*maeA* [pPYC]	65.7	84.8
FP Δ*maeB* [pPYC]	61.0	81.1
FP Δ*maeA* Δ*maeB* [pPYC]	77.6	91.5

tion of GS reactions to the biosynthesis of malic and succinic acids in the strain was decreased due to inactivation of the *pflB* gene, which encodes pyruvate formate lyase (EC 2.3.1.54), the main enzyme providing the *E. coli* cell with acetyl-CoA under anaerobiosis.

During anaerobic utilization of glucose, the FP [pPYC] strain expressing pyruvate carboxylase of *B. subtilis* synthesized malic, fumaric, and succinic acids with the molar yields of about 0.11, 0.02, and 0.76 mol/mol, respectively (Fig. 1).

At the same time, the portion of four-carbon metabolites among fermentation products formed by the strain was only 57.4% (Table 2), and pyruvic acid was secreted as the main by-product (see the Fig. 1).

Considerable secretion of pyruvic acid by the strain was resulted from the functioning of the previously revealed pyruvate-OAA-malate-pyruvate futile cycle mediated by the action of cellular malic enzymes [16]. *E. coli* possesses two malic enzymes, NADH-dependent MaeA (EC 1.1.1.39) and NADPH-dependent MaeB (EC 1.1.1.40) [23]. The NADH-dependent MaeA could presumably served as the main malic enzyme responsible for the functioning of the futile cycle. Indeed, the basal activity of the NADH-dependent malic enzyme in *E. coli* is relatively high [24]. Moreover, besides the reports on the positive or neutral effect of *maeA* gene overexpression on the anaerobic production of succinic acid by *E. coli* strains [25], an increased biosynthesis of intermediates of the reductive branch of the TCA cycle caused by the inactivation of the corresponding gene has also been demonstrated [26, 27]. Thus, the gene encoding the NADH-dependent malic enzyme was initially inactivated in the FP [pPYC] strain.

During the anaerobic glucose utilization, the resulting strain FP Δ*maeA* [pPYC] synthesized malic acid with a yield of ~0.2 mol/mol, increased mainly at the expense of pyruvate and lactate secretion (see Fig. 1). The yields of fumaric and succinic acids were also slightly increased, and the total fraction of the four-carbon dicarboxylates formed by the strain reached 65.7% of all fermentation products (see Table 2). Thus, the inactivation of the NADH-dependent malic enzyme reliably decreased the activity of the pyruvate-OAA-malate-pyruvate futile cycle in the

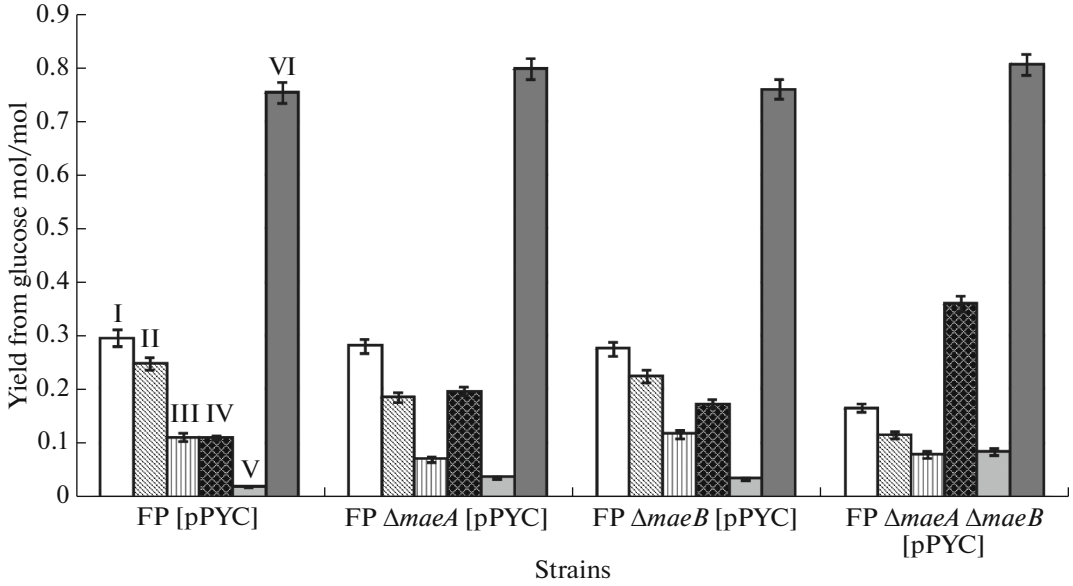

Fig. 1. Molar yield of metabolites synthesized by the studied strains during anaerobic glucose utilization: I, pyruvate; II, lactate; III, acetate; IV, malate; V, fumarate; VI, succinate.

FP Δ*maeA* [pPYC] strain, leading to relatively enhanced synthesis of the target four-carbon dicarboxylic acids. However, the distribution of the metabolites synthesized by the strain indicated that the glycolytic carbon flux was not completely channeled toward the reductive branch of the TCA cycle. At the same time, continued notable production of lactic acid indicated the inability of the strain to efficiently reoxidize reducing equivalents exclusively via the NADH-dependent reactions of malic, fumaric, and succinic acid formation. The secretion of lactic acid by strains lacking fermentative lactate dehydrogenase LdhA (EC 1.1.1.28) indicated activation of the respiratory lactate dehydrogenases Dld (EC 1.1.5.12) and/or LldD to compensate for the insufficiency of fermentation processes for the maintenance of the intracellular redox balance. Nevertheless, the contribution of respiratory enzymes to the formation of reduced products of anaerobic glucose utilization by the FP Δ*maeA* [pPYC] strain decreased compared to the parent FP [pPYC] strain, and the portion of the four-carbon metabolites among these compounds reached 84.8% (Table 2).

Accordingly, it could not be excluded that NADPH-dependent malic enzyme contributed to the functioning of pyruvate-OAA-malate-pyruvate futile cycle to match the requirements for the maintenance of intracellular NADH/NADPH balance during anaerobiosis. To verify this assumption, the gene encoding this enzyme was also inactivated in the core producing strain.

The profile of metabolites synthesized by the resulting strain FP Δ*maeB* [pPYC] during anaerobic glucose utilization was similar to that of the FP Δ*maeA* [pPYC] strain (Fig. 1).

The molar yield of malic, fumaric, and succinic acids was slightly lower than that of the FP Δ*maeA* [pPYC] strain, exceeding, nevertheless, the value demonstrated by the parent FP [pPYC] strain and indicating a negative impact of NADPH-dependent malic enzyme activity on the anaerobic formation of four-carbon intermediates of the TCA cycle.

Thus, both genes encoding malic enzymes were inactivated in the core strain. The production of pyruvic and lactic acids synthesized by the FP Δ*maeA* Δ*maeB* [pPYC] strain during anaerobic glucose utilization sharply decreased, not only when compared to the core strain FP [pPYC], but also with control strains FP Δ*maeA* [pPYC] and FP Δ*maeB* [pPYC] (see Fig. 1). The secretion of malic acid by the strain concomitantly increased, and the molar yield of this dicarboxylate from glucose reached ~0.36 mol/mol. The yield of fumaric acid raised up to two times (Fig. 1). As a result, though the level of succinic acid synthesis was almost unchanged, the portion of the target four-carbon dicarboxylates among the fermentation products formed by the FP Δ*maeA* Δ*maeB* [pPYC] strain increased to 77.6%, while their portion among the reduced metabolites reached 91.5% (Table 2). This result indicates that the abolishment of the functioning of the of pyruvate-OAA-malate-pyruvate futile cycle allowed the FP Δ*maeA* Δ*maeB* [pPYC] strain to efficiently reoxidize the glycolically formed NADH in the reactions of the reductive branch of the TCA cycle upon the enhanced formation of the required precursor (OAA) by the combined action of both PEP- and pyruvate-carboxylating anaplerotic enzymes. Since the respective fermentation process is directly coupled with the biosynthesis of malic, fumaric, and succinic acids, the opening of the futile cycle eventually led to increased anaerobic synthesis of the target dicarboxylates by the constructed strain.

Residual secretion of pyruvic, lactic, and acetic acids by the strain was apparently caused the suboptimal distribution of carbon fluxes in the metabolic node PEP-pyruvic acid-acetyl-CoA. Efficient coordination between the reactions constituting the corresponding metabolic node, favorable for OAA formation, can be further achieved by the overexpression of the *ppc* and/or *pckA* genes, which code for phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase, and by diminishing of the pyruvate to acetyl-CoA conversion by pyruvate dehydrogenase.

CONCLUSIONS

The results of this study indicate that the inactivation of malic enzymes promotes the synthesis of four-carbon dicarboxylic acids through the reductive branch of the TCA cycle in engineered *E. coli* strains upon overexpression of OAA-forming pyruvate carboxylase, and it could be considered an obligatory requirement for enabling an efficient redox balanced anaerobic production of target compounds from glucose.

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