

Optimization of the Anaerobic Production of Pyruvic Acid from Glucose by Recombinant *Escherichia coli* strains with Impaired Fermentation Ability via Enforced ATP Hydrolysis

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Abstract—Anaerobic production of pyruvic acid from glucose by recombinant *Escherichia coli* strains with impaired fermentation ability during respiration with nitrate as an external terminal electron acceptor was studied. During nitrate respiration in a minimal salt medium lacking ammonium ions, the core *E. coli* strain MG1655 $\Delta ackA-pta$, $\Delta poxB$, $\Delta ldhA$, $\Delta adhE$, $\Delta ptsG$, P_{lgk} , P_{lac} , $\Delta galP$, $\Delta frdAB$, $\Delta pflB$, $\Delta sdhAB$, $\Delta aceEF$ converted glucose into pyruvic acid with a yield of 1.72 mol/mol, secreting lactic acid as the only detected byproduct. The deletion of the *lldD* and *dld* genes blocked the secretion of this byproduct. The corresponding strain lacking the respiratory L- and D-lactate dehydrogenases LldD and Dld synthesized pyruvic acid from glucose with a yield of 1.76 mol/mol, consuming the available carbohydrate substrate incompletely. Enforced ATP hydrolysis due to the action of the pyruvic acid–oxaloacetic acid–malic acid–pyruvic acid or pyruvic acid–phosphoenolpyruvate–pyruvic acid futile cycles led to a drastic increase in glucose consumption by recombinants while maintaining the levels of substrate to the target product conversion. As a result, during anaerobic nitrate respiration and enforced ATP hydrolysis pyruvic acid was produced from glucose with a yield of 1.77–1.78 mol/mol with almost exhaustive consumption of the substrate by recombinants and no or minimal byproduct formation.

Keywords: *Escherichia coli*, fermentation, glucose, respiration, pyruvic acid, futile cycle

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INTRODUCTION

Pyruvic acid is both a key metabolite of living cells and an important, industrially relevant compound that can serve as a convenient precursor in the further synthesis of a wide range of high value-added substances. Organic synthesis with pyruvic acid as the starting material can produce food supplements, pharmaceuticals, and solvents, such as acetaldehyde [1, 2] and butanol [3], D/L-alanine, aromatic L-amino acids [4], 4-dihydroxy-L-phenylalanine [5], and *N*-acetyl-D-neuraminic acid [6]. In addition, pyruvic acid can be directly used in nutrition as a dietary supplement with many properties that are beneficial for human health [7].

Pyruvic acid is currently produced via petrochemical synthesis. However, the current strategies for sustainable development imply a gradual but steady replacement of chemical-industry products with the corresponding products of microbial biotechnology. Thus, it is of immediate interest to obtain microbial producers of pyruvic acid that can efficiently synthesize the target compound from cheap and readily available renewable raw materials (i.e., plant-biomass sugars). It should be noted that pyruvic acid acts in the cell as the main precursor metabolite for many bio-

chemical pathways leading to various classes of organic molecules. Thus, highly efficient pyruvic-acid producers can also serve as platform strains for the further development of specialized producers of other valuable compounds. Recent years have seen remarkable progress in the engineering of processes for the biological conversion of carbohydrates into pyruvic acid with the use of various species of industrially relevant microorganisms [8]. The best characteristics were achieved with a directly engineered *Escherichia coli* strain, which was able to synthesize pyruvic acid from glucose with a yield of 1.53 mol/mol amounting, to 77% of the theoretical maximum [9].

Glycolysis is the key biochemical pathway supplying the cell with pyruvic acid during the utilization of the relevant substrates (primarily, glucose). It is a conservative metabolic pathway in many microorganisms, including the facultatively anaerobic bacterium *E. coli*, which is traditionally used in the industrial biotechnology. It is well known that glycolysis is not transcriptionally limited and is controlled primarily by the intracellular ratios of NADH/NAD⁺ and ATP/ADP [10, 11]. Glucose utilization during glycolysis results in the formation of two pyruvic acid molecules, two

NADH molecules, and two ATP molecules. Under aerobic conditions, the NADH produced during glycolysis is reoxidized by the respiratory electron-transport chain with oxygen as the terminal electron acceptor, while pyruvic acid and ATP are involved in the anabolic biomass-formation processes. Under anaerobic conditions, a substantial portion of the glycolytically formed pyruvic acid directly or indirectly participates in fermentation reactions to maintain the intracellular redox balance, acting as an internal terminal electron acceptor for NADH oxidation.

In *E. coli*, the key enzymes responsible for the anaerobic dissimilation of pyruvic acid include lactate dehydrogenase LdhA (EC 1.1.1.28), acetate kinase AckA (EC 2.7.2.1), phosphotransacetylase Pta (EC 2.3.1.8), bifunctional alcohol/aldehyde dehydrogenase AdhE (EC 1.1.1.1/1.2.1.3), catalyzing the formation of the main mixed-acid fermentation products, i.e., lactic acid, acetic acid, and ethanol, as well as pyruvate formate-lyase PflB (EC 2.3.1.54), which converts pyruvic acid into formic acid and acetyl-CoA. The inactivation of these enzymes is an obligatory requirement to enable efficient anaerobic production of pyruvic acid by recombinant *E. coli* strains. However, *E. coli* strains deficient in mixed-acid fermentation pathways are incapable of anaerobic growth [12]. Moreover, the residual ability of corresponding strains to reoxidize NADH is rather low, while their need for ATP is dramatically decreased. As a result, such mutants are almost incapable of utilizing glucose under anaerobic conditions [13]. At the same time, anaerobic processes are more preferable than aerobic processes for the biosynthesis of valuable products, because they have lower capital and operating costs and fewer issues related to mass transfer and contamination [14]. Since *E. coli* is a facultatively anaerobic bacterium, the anaerobic production of pyruvic acid from glucose by recombinants devoid of fermentation pathways can be achieved with a dual-phase fermentation process, comprising an aerobic phase for biomass accumulation followed by an anaerobic production phase. NAD⁺ regeneration under anoxic conditions can be ensured in this case via anaerobic respiration with an external electron acceptor, such as DMSO [15], nitrate [16], or even an electrode functioning in the anode mode [17].

Indeed, it was previously demonstrated that a directly engineered *E. coli* strain possessing a modified system of glucose transport and phosphorylation, devoid of the main mixed-acid fermentation pathways and the ability to perform fumarate-succinate interconversion and also incapable to form acetyl-CoA from the corresponding three-carbon precursor, in the presence of sodium nitrate in the medium anaerobically converts glucose into pyruvic acid with a rather high molar yield [18]. The only byproduct secreted by the strain, in addition to pyruvic acid, was lactic acid, which most likely formed resulting from the involvement of the target-product in reactions of alternative

anaerobic respiration with an internal electron acceptor [19, 20]. At the same time, despite the efficient reoxidation of the glycolytically formed NADH during anaerobic respiration with both external and internal electron acceptors the strain did not completely consume the available glucose. The key factor limiting glucose consumption by the recombinant strain under anoxic conditions appeared to be excessive intracellular ATP levels.

The goal of the work was to improve the anaerobic production of pyruvic acid from glucose by recombinant *Escherichia coli* strains with impaired fermentation ability via enforced ATP hydrolysis.

MATERIALS AND METHODS

Reagents. The restriction endonuclease *Bgl*II, T4 DNA ligase, Taq DNA polymerase (Thermo Scientific, Lithuania), and high-fidelity DNA polymerase Kapa HiFi (Roche, Switzerland) were used in the work. Polymerase chain reaction (PCR) products were purified via agarose gel electrophoresis and further isolated from gel with a QIAquick Gel Extraction Kit (Qiagen, United States). Table 1 presents the oligonucleotides used in the work (Evrogen, Russia). The culture media components, salts, and other reagents were produced by Panreac (Spain) and Sigma (United States).

Bacterial strains, plasmids, and media. Table 2 lists the bacterial strains and plasmids used in the work. The *E. coli* strain K-12 MG1655 (VKPM B-6195), the previously constructed strain *E. coli* MG1655 Δ *ackA-pta*, Δ *poxB*, Δ *ldhA*, Δ *adhE*, Δ *ptsG*, *P*₁*glk*, *P*₁*galP* [21], possessing a modified system of glucose transport and phosphorylation and lacking the main mixed-acid fermentation pathways designated as PA4, and its derivative, the strain PA4FPSA [18], with an additionally blocked fumarate–succinate interconversion and incapable of pyruvic acid conversion to acetyl-CoA, were used as the starting strains for the construction of all of the recombinants obtained in the work.

The bacteria were cultured in reach LB, SOB, SOC media and the minimal M9 medium [22], with the addition of ampicillin (100 μ g/mL) or chloramphenicol (30 μ g/mL) if necessary. The media were also supplemented with sodium acetate to enable the aerobic growth of the Δ *aceEF* PA4FPSA strain and its derivatives.

Recombinant strain construction. All chromosome modifications were performed as described previously [23].

To inactivate the *lldD* and *dld* genes, linear DNA fragments, containing chloramphenicol resistance marker (*cat* gene), were obtained by PCR using the primer pairs P1 and P2 and P3 and P4 and the pMW118-(λ *attL*-Cm- λ *attR*) plasmid [24] as a template. The obtained DNA fragments were individually integrated into the chromosome of the *E. coli* MG1655

Table 1. Oligonucleotide primers used in the work

No.	Nucleotide sequence
P1	5'-catgattattccgcagccagcagcattatcgccgc-cgctcaagtagtataaaaaagctgaac-3'
P2	5'-ctatgccgattccctttcgccatgggagccagtc-tgaagcctgctttttataactaagtgg-3'
P3	5'-aatgtcttccatgacaacaactgataataaacctt-cgctcaagtagtataaaaaagctgaac-3'
P4	5'-ttactccacttctgcccagttttccgtttactggt-tgaagcctgctttttataactaagtgg-3'
P5	5'-gattcgatgaagatcaggctcg-3'
P6	5'-cagagtgaggagtagcatacag-3'
P7	5'-gccataagcatgatggagagag-3'
P8	5'-ccggtaaagtgatgcctgtgc-3'
P9	5'-tgcgac-agatct-ctcacctaccaacaatgccc-3'
P10	5'-atgtatatctcttc-acggccaatgctcttcc-3'
P11	5'-ccaaagcaccagcggtagcagccattgtggacat-atgtatatctcttc-acggccaatg-3'
P12	5'-ctagta-agatct-tgaagcctgctttttataactaagtgg-3'
P13	5'-tctcaaaccgttcattatcacaaaaggattgtcg-cgctcaagtagtataaaaaagctgaac-3'
P14	5'-gtcgaagagagcagatttgcgc-3'
P15	5'-cctgtctacatcattcatgccg-3'

Table 2. Strains and plasmids

Object	Genotype	Reference
Strain		
MG1655	<i>E. coli</i> wild type strain (VKPM B-6195)	VKPM
PA4	<i>E. coli</i> MSG1.0 (MG1655 $\Delta ackA$ - pta , $\Delta poxB$, $\Delta ldhA$, $\Delta adhE$, $\Delta ptsG$, P_{Lglk} , $P_{tacgalP}$)	[21]
PA4FPSA	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$	[18]
PA4FPSAL	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$, $\Delta lldD$	Present work
PA4FPSAD	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$, Δdld	"
PA4FPSALD	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$, $\Delta lldD$, Δdld	"
PA4FPSAPyc	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$, pPYC	"
PA4FPSAPps	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$, P_L -SD $_{\phi 10}$ - <i>ppsA</i>	"
PA4FPSALDPyc	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$, $\Delta lldD$, Δdld , pPYC	"
PA4FPSALDPps	<i>E. coli</i> MSG1.0 $\Delta frdAB$, $\Delta pf1B$, $\Delta sdhAB$, $\Delta aceEF$, $\Delta lldD$, Δdld , P_L -SD $_{\phi 10}$ - <i>ppsA</i>	"
Plasmid		
pMW118-($\lambda attL$ -Cm- $\lambda attR$)	pSC101, <i>bla</i> , <i>cat</i> , $\lambda attL$ - <i>cat</i> - $\lambda attR$	[24]
pKD46	pINT-ts, <i>bla</i> , P_{araB} - λgam - <i>bet</i> - <i>exo</i>	[23]
pMWts-Int/Xis	pSC101-ts, <i>bla</i> , P_R - λxis - <i>int</i> , <i>cIts857</i>	[25]
pPYC	pMW119 containing the pyruvate carboxylase gene (<i>pycA</i>) from <i>B. subtilis</i>	[33]

strain carrying the pKD46 helper plasmid. The correspondence between the assumed and experimentally obtained structures of the chromosomes of the selected strains with the individually inactivated *lldD* and *dld* genes was confirmed by PCR with pairs of the locus-specific primers P5 and P6 and P7 and P8.

The DNA fragment to replace the native regulatory region of the *ppsA* gene with the artificial genetic ele-

ment P_L -SD $_{\phi 10}$ containing the phage lambda P_L promoter and the efficient ribosome binding site of $\phi 10$ gene from the T7 phage was constructed in several stages. At the first stage, a DNA fragment containing the *Bgl*II recognition site, P_L promoter, the SD sequence of $\phi 10$ gene from T7 phage, and 36 nucleotides complementary to the 5'-end of coding region of the *ppsA* gene was obtained by PCR.

The fragment was obtained in two steps. As the first step, the DNA fragment containing the *Bgl*II recognition site, P_L promoter, and part of the SD sequence of ϕ 10 gene from T7 phage was obtained using primers P9 and P10 and genomic DNA of phage lambda as a template. The obtained PCR product served as a template for subsequent PCR with the primers P9 and P11. Primer P11 contained a region complementary to the 3'-end of the P_L promoter, SD sequence of ϕ 10 gene from T7 phage, and the first 36 nucleotides of the *ppsA* gene open reading frame. At the same time, the second step of DNA-fragment construction was performed. A DNA fragment containing the *Bgl*II recognition site, chloramphenicol resistance marker (*cat* gene), and 36 nucleotides homologous to the DNA region located immediately upstream of the coding region of the *ppsA* gene was obtained by PCR with the P12 and P13 primers and the pMW118-(λ *attL*-Cm-*attR*) plasmid as a template. The obtained DNA fragments were treated with the *Bgl*II restriction endonuclease and ligated with T4 DNA ligase. The ligation product was amplified using primers P11 and P13. The obtained PCR product was integrated into the chromosome of the *E. coli* MG1655 strain carrying the helper plasmid pKD46. The correspondence between the desired and experimentally obtained nucleotide sequence of the new regulatory element introduced upstream of the coding region of *ppsA* gene was confirmed by sequencing using primers P14 and P15.

The corresponding individual genetic modifications were further introduced into the chromosomes of the target recombinants via P1-dependent transduction [22]. The marker flanked by the phage lambda *att*-sites was removed from the chromosomes of the target strains using the pMWts-Int/Xis plasmid as described previously [25]. The strains were transformed with the plasmids according to the standard protocols.

Strain cultivation. The recombinant strains were grown overnight in M9 medium containing 2 g/L of glucose at 37°C. Overnight cultures were diluted ten times by the addition of 45 mL of M9 medium containing 10 g/L of yeast extract to 5 mL of the culture. The resulting cultures were grown in 750-mL flasks at 37°C on a rotary shaker at 250 rpm for 8 h. The cell suspensions were centrifuged for 15 min at 2000 *g* at 4°C. The cell pellets were resuspended in 15 mL of modified M9 medium lacking ammonium ions and containing 10 g/L of glucose and 50 mM of sodium nitrate to an OD_{600} of ~10. The cultures were further incubated under anaerobic conditions for 24 h in 15-mL screw-cap tubes at 37°C on the rotary shaker at 250 rpm. When strains containing the pMW-derived plasmids were grown, the media also contained 100 μ g/mL of ampicillin (OOO Sintez, Russia). To ensure the functional activity of pyruvate carboxylase in strains carrying the *Bacillus subtilis pycA* gene, NaHCO₃ was added

to the media used at the last stage of culturing to a final concentration of 10 g/L.

The cell suspensions collected for the analysis were centrifuged at 10000 *g* for 10 min. The obtained supernatants were used to determine the concentrations of secreted metabolites and residual glucose. All experiments were performed at least in triplicate.

Analytical techniques. The concentrations of the organic acids in the culture liquids freed from the biomass by centrifugation were determined via high-performance liquid chromatography (HPLC) with the Waters HPLC system (United States). The Rezex ROA-Organic Acid H⁺ (8%) ion-exclusion column (300 × 7.8 mm, 8 μ m, Phenomenex, United States) was used with detection at 210 nm. An aqueous solution of sulfuric acid (2.5 mM) was used as a mobile phase with a flow rate of 0.5 mL/min. To measure the glucose concentrations, the system was equipped with a Waters 2414 refractive index detector and a Spherisorb-NH2 column (4.6 × 250 mm, 5 μ m, Waters, United States). An acetonitrile/water mixture (vol/vol 75/25) served as a mobile phase at a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

The previously constructed *E. coli* strain PA4FPSA was used as the core pyruvic-acid producer. In this strain, the main mixed-acid fermentation pathways, which are responsible for anaerobic pyruvate dissimilation and lead to the formation of acetic and lactic acids, as well as ethanol, were inactivated via the deletion of the *ackA*, *pta*, *poxB*, *ldhA*, and *adhE* genes, which encode the enzymes catalyzing the corresponding reactions. The anaerobic respiration with fumarate as the internal electron acceptor was prevented primarily due to the deletion of the *frdAB* genes, which encode the components of fumarate reductase (EC 1.3.5.4). It is well-known that succinate dehydrogenase (EC 1.3.5.1) can functionally replace fumarate reductase in *E. coli* strains with inactivated *frdAB* genes [26]. Thus, the *sdhAB* genes, which encode the components of succinate dehydrogenase, were also deleted in this strain. The involvement of pyruvic acid, through the intermediate formation of acetyl-CoA, in the reaction of the tricarboxylic acid cycle (TCA), which is activated upon respiration, was prevented resulting from the deletion of the *pflB* and *aceEF* genes, which encode pyruvate formate-lyase and the components of the pyruvate dehydrogenase enzymatic complex (EC 1.2.4.1/2.3.1.12/1.8.1.4). In addition, the system of glucose transport and phosphorylation was modified to consume ATP for phosphorylation of the carbohydrate substrate instead of phosphoenolpyruvate (PEP) for a potential reduction of the inhibition of the initial and terminal stages of glycolysis by the ATP generated at its intermediate stages. This was achieved via inactivation of the *ptsG* gene, which encodes the glucose permease of the PEP-dependent phosphotransferase system with the overexpression of the *galP*

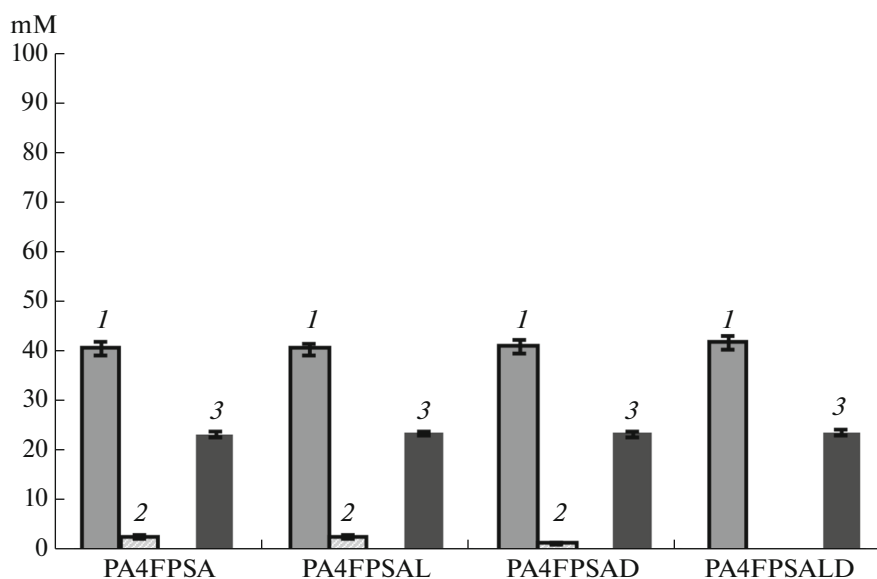


Fig. 1. Concentrations (mM) of pyruvic acid (1) and lactic acid (2) secreted by the PA4FPSA strain and its derivatives PA4FPSAL, PA4FPSAD, and PA4FPSALD during the anaerobic glucose utilization (3) upon respiration with sodium nitrate as an external electron acceptor.

and *glk* genes encoding the H⁺-galactose symporter and ATP-dependent glucokinase (EC 2.7.1.2). As a result, after aerobic growth, the PA4FPSA strain was able to convert glucose anaerobically to pyruvic acid upon respiration with nitrate as an external electron acceptor with a yield of ~1.7 mol/mol [18].

The main detected byproduct synthesized by the strain was lactic acid. The results of enantiomeric analysis of the lactic acid secreted by the strain indicated that the respiratory lactate dehydrogenases LldD and Dld (EC 1.1.5.12) could be responsible for the formation of this compound. Normally, the corresponding enzymes catalyze the quinone-dependent conversion of L-lactic acid and D-lactic acid into pyruvic acid [19]. However, under anaerobic conditions, their inverted quinol-dependent action, which resulted in the formation of the corresponding reduced product from pyruvic acid, could also contribute to the maintenance of the intracellular redox balance in the strain with impaired fermentation ability.

In addition to the LldD and Dld lactate dehydrogenases, D-alanine dehydrogenase DadA (EC 1.4.5.-) may participate in the alternative anaerobic respiration with pyruvic acid as an internal electron acceptor [20]. To exclude the potential contribution of the corresponding enzyme to the maintenance of the intracellular redox balance, the minimal M9 medium without ammonium ions was used in the present work at the final stage of culturing to characterize the anaerobic glucose utilization and pyruvic acid biosynthesis by the PA4FPSA strain and its derivatives during dual-phase fermentation.

In the presence of nitrate ions and the absence of ammonium ions in the medium, the PA4FPSA strain demonstrated characteristics similar to those described previously, i.e., it consumed about 23.6 mM of glucose and synthesized ~40.8 mM (Fig. 1) of pyruvic acid with a yield of 1.72 mol/mol (Table 3). As expected, alanine was not observed among the products of anaerobic glucose utilization secreted by the strain, and lactic acid was the only byproduct (Fig. 1). The observed synthesis of lactic acid by the strain in the presence of sodium nitrate was apparently due to the high potential (~0.19 V) of the corresponding respiratory reactions utilizing the internal electron acceptor, which was only two times lower than the corresponding value (~0.42 V) for respiration involving Nar-system nitrate reductases and exceeded the value for respiration with fumarate (~0.03 V) and even DMSO (~0.16 V) as the external electron acceptors [19].

It is well known that under the conditions of nitrate respiration the expression of at least one of the quinone-dependent lactate dehydrogenases, LldD, is induced in *E. coli* cells [27]. Thus, the *lldD* gene was initially deleted in the PA4FPSA strain to prevent the undesirable production of lactic acid. The corresponding derivative PA4FPSAL synthesized pyruvic acid from glucose with almost the same efficiency as the parental strain (Fig. 1 and Table 3), but it still secreted considerable amounts of lactic acid (Fig. 1). Moreover, despite the inactivation of the L-lactate dehydrogenase LldD, the yield of lactic acid formed by the strain during anaerobic glucose utilization did not decrease (Table 3). This could indicate that the D-lactate dehydrogenase Dld was the main enzyme respon-

Table 3. Characteristics of carbon-substrate consumption and metabolite production by the PA4FPSA, PA4FPSAL, PA4FPSAD, and PA4FPSALD strains during anaerobic glucose utilization upon respiration with sodium nitrate as an external electron acceptor*

Strain	Consumed glucose, mM	Metabolite yield, mol/mol		Carbon recovery, %
		Pyruvic acid	Lactic acid	
PA4FPSA	23.6 ± 0.5	1.72 ± 0.02	0.13 ± 0.01	93
PA4FPSAL	23.8 ± 0.5	1.71 ± 0.02	0.12 ± 0.01	92
PA4FPSAD	23.7 ± 0.6	1.74 ± 0.02	0.07 ± 0.01	91
PA4FPSALD	23.8 ± 0.6	1.76 ± 0.03	n/d	89

* The carbon recovery was calculated as the ratio of total moles of carbon in the secreted products per moles of carbon in total consumed glucose and expressed in percentage basis. Standard deviations for three independent experiments are given. n/d—not detected.

sible for lactic acid production by the PA4FPSA and PA4FPSAL strains. To test this hypothesis, the *lld* gene was deleted in the PA4FPSA strain. When D-lactate dehydrogenase was inactivated, the anaerobic production of pyruvic acid from glucose by the corresponding PA4FPSAD strain did not change as compared to the PA4FPSA strain, while the lactic acid secretion dropped markedly (Fig. 1 and Table 3). However, the *lld*⁻ mutant did not completely cease lactic acid production. Thus, despite the preferential production of lactic acid by D-lactate dehydrogenase in the PA4FPSA and PA4FPSAL strains, L-lactate dehydrogenase was able to compensate partially for the loss of activity of the alternative enzyme in the PA4FPSAD strain. For this reason, the genes encoding both lactate dehydrogenases were further concomitantly deleted in the PA4FPSA strain.

The PA4FPSALD strain with the inactivated respiratory lactate dehydrogenases LldD and Dld did not secrete any detectable quantities of lactic acid as a result of anaerobic glucose utilization. At the same time, it produced pyruvic acid (~42.1 mM) at a slightly higher level than the initial PA4FPSA strain did (Fig. 1), and the corresponding carboxylate was produced from glucose with a yield as high as 1.76 mol/mol (Table 3). The absence of the reduced products of glucose catabolism in the medium indicated, in turn, that respiration with exogenous nitrate as the only terminal electron acceptor was able to successfully maintain the overall intracellular redox balance in the PA4FPSALD strain.

At the same time, PA4FPSALD did not consume all of the available glucose (55.5 mM), as was also the case with its precursors (Fig. 1 and Table 3), although the Nar and Nrf nitrate and nitrite respiration systems can reoxidize 250 mM NADH with 50 mM of NaNO₃ added to the medium [19], which correspond to the number of reduced equivalents formed during glycolytic utilization of 125 mM glucose. Thus even upon efficient reoxidation of glycolytically formed NADH via anaerobic respiration with external electron acceptor, an excessive intracellular ATP levels remained, as in the case of the PA4FPSA strain, the key factor limiting glu-

cose consumption under anoxic conditions by its promising, directly engineered derivative, PA4FPSALD.

Several approaches to the intensification of the glycolytic carbon flux in recombinant *E. coli* strains based on the decreasing of intracellular ATP levels have been described to date. The main approach is based on manipulation of components of the (F₁F₀) H⁺-ATP synthase complex in order to prevent oxidative phosphorylation and/or enhance ATP hydrolysis. Overexpression of the genes encoding the components of the cytoplasmic F₁ subunit comprising the catalytic site should promote ATP hydrolysis. Conversely, inactivation of the genes encoding the components of the membrane-bound F₀ subunit, which forms the proton channel, will block ATP production via oxidative phosphorylation with a concomitant increase in ATPase activity. These two strategies have both been successfully implemented to improve the biosynthetic performance of recombinant *E. coli* strains engineered to produce pyruvic acid from glucose under aerobic conditions [9, 28]. A rather promising, alternative approach implies enforced ATP hydrolysis in the cell due to the action of futile cycles [29].

It was previously demonstrated that the expression of the heterologous pyruvate carboxylase (EC 6.4.1.1) in *E. coli* strains deficient in main mixed-acid fermentation pathways provoked the emergence of an artificial futile cycle pyruvic acid—oxaloacetic acid (OAA)—malic acid—pyruvic acid during the anaerobic utilization of glucose [30]. In this cycle, OAA produced from pyruvic acid by ATP-dependent pyruvate carboxylase was converted into malic acid through the NADH-consuming initial reaction of the reductive branch of the TCA cycle, which is catalyzed by the malate dehydrogenase Mdh (EC 1.1.1.37) and then, was decarboxylated to pyruvic acid by the NAD⁺-dependent malic enzyme MaeA (EC 1.1.1.39), which is constitutively expressed in *E. coli*, resulting in the hydrolysis of a single ATP molecule in the absence of NADH generation. Inactivation of this cycle promoted the anaerobic production of four-carbon dicarboxylic acids by the corresponding recombinant strains [31], whereas its activation improved pyruvic acid production [18, 30].

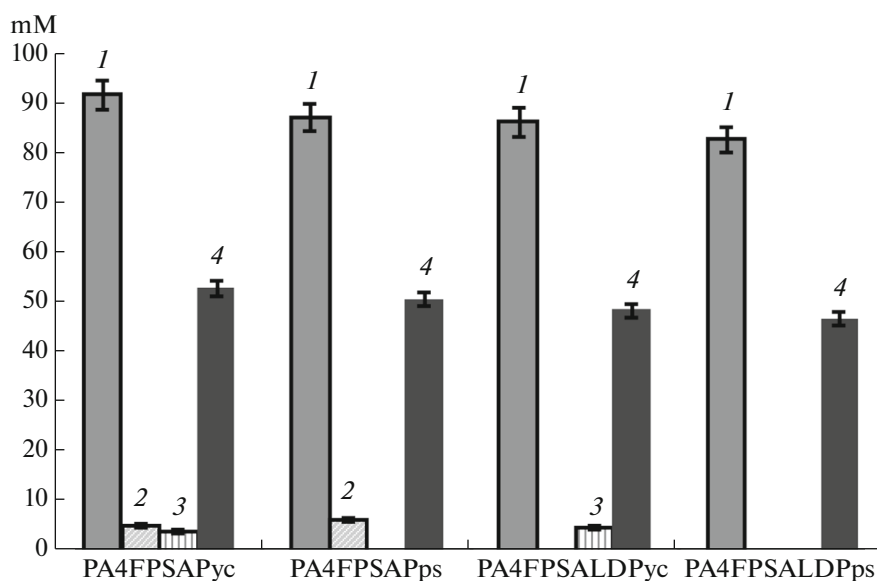


Fig. 2. Concentrations (mM) of pyruvic (1), lactic (2), and malic (3) acids secreted by the PA4FPSAPyc, PA4FPSAPps, PA4FPSALDPyc, and PA4FPSALDPps strains during the anaerobic glucose utilization (4) upon respiration with sodium nitrate as an external electron acceptor and enforced ATP hydrolysis resulted from the action of the futile cycles pyruvic acid–OAA–malic acid–pyruvic acid (PA4FPSAPyc and PA4FPSALDPyc strains) and pyruvic acid–PEP–pyruvic acid (PA4FPSAPps and PA4FPSALDPps strains).

The pyruvic acid–PEP–pyruvic acid futile cycle may serve as an alternative to the pyruvic acid–OAA–malic acid–pyruvic acid cycle, which functions exclusively under anaerobic conditions when the reductive branch of the TCA cycle is active. Given the presence of glucose in the medium, the pyruvic acid–PEP–pyruvic acid futile cycle may be active both under aerobic and anaerobic conditions. In this cycle, pyruvate kinase (EC 2.7.1.40) catalyzes the ADP-dependent dephosphorylation of PEP to pyruvic acid with the formation of a single ATP molecule, while phosphoenolpyruvate synthase (EC 2.7.9.2) phosphorylates pyruvic acid, using ATP as a cofactor and breaking it into AMP and inorganic phosphate. AMP regeneration to ATP by adenylate kinase (EC 2.7.4.3) requires two ATP molecules. The potential of this cycle to improve the production of the target compound from glucose under anoxic conditions by decreasing the intracellular ATP pool was recently demonstrated with a lactic acid producing *E. coli* strain as an example [32].

The potential of the pyruvic acid–OAA–malic acid–pyruvic acid and pyruvic acid–PEP–pyruvic acid futile cycles to improve anaerobic glucose consumption by the engineered recombinant pyruvic acid producers was tested by enabling their functionality in both the core PA4FPSA strain and its derivative PA4FPSALD strain, which lacks the respiratory lactate dehydrogenases. In order to ensure the activity of pyruvic acid–OAA–malic acid–pyruvic acid futile cycle, the pPYC plasmid [33], expressing *B. subtilis pycA* gene, was introduced in the strains resulting in the corresponding derivative strains PA4FPSAPyc and

PA4FPSALDPyc. The functional activity of the pyruvic acid–PEP–pyruvic acid futile cycle was ensured in the PA4FPSAPps and PA4FPSALDPps strains by the constitutive expression of the *ppsA* gene, which encodes phosphoenolpyruvate synthase, under the control of the phage lambda P_L promoter.

The anaerobic consumption of glucose by the obtained PA4FPSAPyc, PA4FPSALDPyc, PA4FPSAPps, and PA4FPSALDPps strains increased dramatically as compared to their precursor strains (Figs. 1 and 2) with pyruvic acid yields remained at the same high level (Table 4). This indicated the positive effect of enforced ATP hydrolysis resulting from the action of each of the futile cycles on the intensity of carbon flux through the cascade of glycolytic reactions, leading to the target production of pyruvic acid as the main product of carbon substrate utilization.

Nevertheless, the effect of the introduction of the respective futile cycles was somewhat different for the different strains. In particular, the PA4FPSAPyc and PA4FPSALDPyc strains with the active futile cycle of pyruvic acid–OAA–malic acid–pyruvic acid secreted malic acid as one of the products of glucose utilization (Fig. 2). On the one hand, this indicated the functionality of this particular futile cycle in the recombinant strains, but, on the other hand, it pointed to the insufficient activity of the malic enzymes in the cells. At the same time, the carbon recovery, which characterizes the completeness of substrate conversion into the detected products, increased in the case of the PA4FPSAPyc and PA4FPSALDPyc strains as compared to the parental strains, while

Table 4. Characteristics of carbon-substrate consumption and metabolite production by the PA4FPSAPyc, PA4FPSAPps, PA4FPSALDPyc, and PA4FPSALDPps strains during anaerobic glucose utilization upon respiration with sodium nitrate as an external electron acceptor and enforced ATP hydrolysis*

Strain	Consumed glucose, mM	Metabolite yield, mol/mol			Carbon recovery, %
		Pyruvic acid	Lactic acid	Malic acid	
PA4FPSAPyc	53.1 ± 1.6	1.73 ± 0.02	0.10 ± 0.01	0.08 ± 0.01	97
PA4FPSAPps	51.0 ± 1.5	1.72 ± 0.02	0.13 ± 0.01	n/d	92
PA4FPSALDPyc	48.8 ± 1.5	1.78 ± 0.03	n/d	0.10 ± 0.01	96
PA4FPSALDPps	47.1 ± 1.4	1.77 ± 0.03	n/d	n/d	88

* The carbon recovery was calculated as the ratio of total moles of carbon in the secreted products per moles of carbon in total consumed glucose and expressed in percentage basis. Standard deviations for three independent experiments are given. n/d—not detected.

remained almost unchanged for the PA4FPSAPps and PA4FPSALDPps strains (Tables 3 and 4).

Since the functioning of the pyruvic acid—PEP—pyruvic acid futile cycle did not result in the formation of the new glucose-utilization products, it could indicate that some intermediates of glycolysis were involved in the side reactions of substrate consumption, which resulted in unproductive carbon loss in the corresponding recombinant strains. With the absence of pyruvate dehydrogenase in the studied strains and, consequently, the inactivity of the oxidative branch of the TCA cycle even under the respirative conditions, the partially activated oxidative branch of the pentose phosphate pathway (PPP) could be responsible for the carbon loss due to the release of CO₂ in the reaction catalyzed by 6-phosphogluconate dehydrogenase (EC 1.1.1.44), and then for the decrease in carbon recovery values. The activity of the pyruvic acid—OAA—malic acid—pyruvic acid futile cycle decreased the contribution of the oxidative branch of PPP to the nonproductive carbon loss by the recombinant strains, whereas the enforced pyruvic acid—PEP interconversion had almost no effect on the intensity of the corresponding process. At the same time, the PA4FPSALDPps strain, which hydrolyzed excessive ATP via the pyruvic acid—PEP—pyruvic acid futile cycle, produced pyruvic acid with an increased yield (1.77 mol/mol), secreted no detectable amounts of any byproducts, and consumed the available glucose almost completely, in contrast to the PA4FPSALDPyc strain, which reduced the intracellular pool of the corresponding cofactor as a result of the activity of the pyruvic acid—OAA—malic acid—pyruvic acid futile cycle. Conversely, the high carbon recovery (96%, Table 4) demonstrated by the PA4FPSALDPyc strain upon the anaerobic glucose utilization under nitrate-respiration conditions suggested that the yield of pyruvic acid achieved by the strain (1.78 mol/mol, Table 4) could be further elevated by an increase in the activity of the malic enzymes in the cell. However, the pyruvic acid—OAA—malic acid—pyruvic acid futile cycle, which ensures enforced ATP hydrolysis in the PA4FPSALDPyc strain, is inactive in the absence of the available CO₂ source in

the medium, while the addition of bicarbonate to the medium will increase the total cost of the target compound synthesis.

CONCLUSIONS

Thus, the potential for the industrial use of each of the approaches based on enforced ATP hydrolysis achieved due to the action of artificially created futile cycles to obtain efficient anaerobic production of pyruvic acid from glucose by recombinant *E. coli* strains should be evaluated considering both the potential benefits and the possible increase in the operating costs.

The results of the present study clearly demonstrate the potential of enforced ATP hydrolysis, due to the use of ATP-consuming futile cycles, to improve the biosynthetic characteristics of recombinant *E. coli* strains directly engineered for the anaerobic production of pyruvic acid from glucose during anaerobic respiration with an external electron acceptor.

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COMPLIANCE WITH ETHICAL STANDARDS

No experimentation involving animals or human was performed by any of the authors.

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