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Engineering *Escherichia coli* for efficient aerobic conversion of glucose to fumaric acid

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ABSTRACT

Escherichia coli was engineered for efficient aerobic conversion of glucose to fumaric acid. A novel design for biosynthesis of the target product through the modified TCA cycle rather than via glyoxylate shunt, implying oxaloacetate formation from pyruvate and artificial channelling of 2-ketoglutarate towards succinic acid via succinate semialdehyde formation, was implemented. The main fumarases were inactivated in the core strain MSG1.0 (Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*) by the deletion of the *fumA, fumB*, and *fumC* genes. The *Bacillus subtilis pycA* gene was expressed in the strain to ensure pyruvate to oxaloacetate conversion. The *Mycobacterium tuberculosis kgd* gene was expressed to enable succinate semialdehyde formation. The resulting strain was able to convert glucose to fumaric acid with a yield of 0.86 mol/mol, amounting to 86% of the theoretical maximum. The results demonstrated the high potential of the implemented strategy for development of efficient strains for bio-based fumaric acid production.

1. Introduction

Fumaric acid is a versatile building block chemical that can serve as a precursor of numerous valuable products, including plasticisers, alkyd and polyester resins, pharmaceuticals, food and beverage additives [1, 2]. Currently, fumaric acid is produced via cis-trans isomerisation of maleic acid, which, in turn, is obtained from petroleum-derived maleic anhydride [3]. Nevertheless, as an intermediate of the central metabolism of a variety of living organisms, fumaric acid can be produced biosynthetically from renewable carbon sources, e.g., plant biomass-derived sugars, in particular glucose, which is the most abundant. Filamentous fungi of the genus *Rhizopus*, such as *R. nigricans, R. arrhizus*, and *R. oryzae*, are naturally capable of producing fumaric acid under both aerobic and anaerobic conditions [4]. Moreover, certain progress has also been made towards improvement of the biosynthesis of the target product from carbohydrate feedstocks by corresponding species through rational metabolic and genetic engineering approaches [5, 6]. However, the morphological characteristics of *Rhizopus* represent a challenging problem for successful industrialisation of the bio-based fumaric acid production process with these fungi. The cells of *Rhizopus* species tend to form aggregates, clumps or mycelium during growth, leading to oxygen transfer limitations during large-scale fermentation [4, 7].

It is interesting to note that despite the anaerobic synthesis of fumaric acid from glucose via the reductive branch of the TCA cycle is rather attractive because of the high theoretical yield value (2 mol/mol) there are no reports on successful engineering of corresponding producing strains using microorganisms industrially more relevant than *Rhizopus* species. This is apparently caused by the fact that constructing such efficient producers would require substantial modification in the host strains of the functionality of electron transport chain, which correct operation is vital not only for aerobic, but also for facultative anaerobic microorganisms. At the same time, in recent years, such industrially relevant microorganisms, as *Saccharomyces cerevisiae* [8, 9] and *E. coli* [10–12], have been successfully engineered to produce fumaric acid from glucose under aerobic conditions. The most efficient conversion of carbohydrate substrate to the target product has been achieved with an engineered *E. coli* strain, which was capable of synthesising fumaric acid from glucose aerobically with a yield of 0.78 mol/mol [11]. However, the maximum theoretical yield value for aerobic conversion of glucose to fumaric acid is 1 mol/mol. Thus, the development of efficient *E. coli* fumaric acid-producing strains remains an important task.

In *E. coli*, fumaric acid is aerobically formed by succinate dehydrogenase from succinic acid, which is synthesised via the oxidative TCA cycle and/or in the reactions of the glyoxylate shunt (GS). The design of

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known *E. coli* strains engineered for the aerobic production of fumaric acid presumes the prevalent participation of GS in the synthesis of the target product. The main reason for this is that the expression of 2-ketoglutarate dehydrogenase in *E. coli* is rather low during aerobic growth in rich and glucose-containing media [13]. As a result, the limited carbon flux through subsequent reactions of the oxidative branch of the TCA cycle prevents efficient formation of succinic acid, the direct precursor of fumaric acid. The branching of the oxidative TCA cycle and GS occurs at the isocitrate level, and thus, the decreased activity of 2-ketoglutarate dehydrogenase does not affect the efficiency of succinic acid synthesis through GS reactions. The activation of the glyoxylate shunt in *E. coli* is generally achieved via inactivation of the transcriptional regulator IclR, which represses the expression of the *aceBAK* operon genes encoding GS enzymes, while the deletion of fumarase isozyme *fumB* and *fumAC* genes prevents fumaric to malic acid conversion [10–12, 14]. The approaches aimed at further optimisation of fumaric acid production by the engineered strains include the following: 1) the inactivation of certain pathways of mixed-acid fermentation competitively consuming pyruvate and acetyl-CoA, the key precursor in target product biosynthesis, by the deletion of *ldhA* and *ackA* genes [10, 11, 14]; and 2) the increase in intracellular availability of phosphoenolpyruvate (PEP) for carboxylation through the modification of the glucose transport and phosphorylation system with the concomitant intensification of oxaloacetate (OAA) formation upon the overexpression of PEP-carboxylating phosphoenolpyruvate carboxylase, Ppc [10–12].

In the present study, we implemented a novel design for engineering an *E. coli* strain devoid of all mixed-acid fermentation pathways and possessing a modified system of glucose transport and phosphorylation for aerobic production of fumaric acid from glucose through the oxidative TCA cycle. The design implied the ensured formation of OAA from pyruvate instead of PEP and the artificial channelling of 2-ketoglutarate towards succinic acid via intermediate succinate semialdehyde (SSA) formation, thus bypassing the 2-ketoglutarate dehydrogenase-catalysed reaction in a manner similar to that of the variant TCA cycle found in myco- [15] and cyanobacteria [16].

Initially, the *fumB* and *fumAC* genes were sequentially deleted in the previously engineered *E. coli* strain MSG1.0 (MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*) [17] to ensure the formation of fumaric acid as a secreted end-product of aerobic utilisation of glucose. Then, the carboxylation of pyruvate to OAA was enabled, resulting from expression of the *Bacillus subtilis pycA* gene, encoding pyruvate carboxylase. Finally, the *Mycobacterium tuberculosis kgd* gene coding for 2-ketoglutarate decarboxylase classified as EC 4.1.1.71, which is absent in *E. coli*, was expressed in the strain to allow the formation of SSA followed by its further oxidation to succinic acid by native succinate-semialdehyde dehydrogenases.

As a result, the engineered *E. coli* strain was able to convert glucose to fumaric acid aerobically with a yield value of 0.86 mol/mol, exceeding those reported previously for various aerobic processes.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* K-12 strain MG1655 was used as the parent for construction of all mutants described in the study.

Chromosomal modifications were performed using the lambda Red recombineering technique [18]. The primers that were used are listed in Supplementary Table 1. Linear DNA fragments for inactivation of the *fumB, fumAC, aceBAK*, and *glcB* genes were obtained by PCR using the pMW118-(λ*attL*-Cm-λ*attR*) plasmid [19] and primer pairs P1, P2; P5, P6; P9, P10, and P13, P14. The PCR products were integrated separately into the chromosome of the *E. coli* MG1655 strain carrying the pKD46 [18] helper plasmid. The deletions of the target genes in the chromosomes of the obtained strains were verified by PCR using the locus-specific primers P3, P4; P7, P8; P11, P12, and P15, P16.

Table 1
Bacterial strains and plasmids used in this study.

Strain / plasmid	Genotype	Reference
E. coli strains		
MG1655	Wild type *E. coli* K-12 strain VKPM B-6195	VKPM
MG Δ*ppc*	MG1655 Δ*ppc*	[20]
MSG1.0	MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*	[17]
MSG1.0 B	MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*fumB*	This study
MSG1.0 BAC	MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*fumB*, Δ*fumAC*	This study
MSG1.0 BACPyc	MG1655 Δ*ackA-pta*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*fumB*, Δ*fumAC, poxB*::P_L-*pycA*^{Bs}	This study
MSG1.0 BACPyc Δ*aceBAK*	MG1655 Δ*ackA-pta*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*fumB*, Δ*fumAC, poxB*::P_L-*pycA*^{Bs}, Δ*aceBAK*	This study
MSG1.0 BACPyc Δ*aceBAK* Δ*glcB*	MG1655 Δ*ackA-pta*, Δ*ldhA*, Δ*adhE*, Δ*ptsG*, P_L-*glk*, P_{tac}-*galP*, Δ*fumB*, Δ*fumAC, poxB*::P_L-*pycA*^{Bs}, Δ*aceBAK*, Δ*glcB*	This study
Plasmids		
pMW118-(λ*attL*-Cm-λ*attR*)	pSC101, *bla, cat,* λ*attL-cat-*λ*attR* cassette	[19]
pKD46	pINT-ts, *bla*, P_{araB}-λ*gam-bet-exo*	[18]
pMWts-Int/Xis	pSC101-ts, *bla*, P_R-λ*xis-int*, *c*Its857	[21]
pMW-*kgd*	pMW119 with cloned *M. tuberculosis* 2-ketoglutarate decarboxylase gene (*kgd*)	[22]

The *B. subtilis pycA* gene encoding pyruvate carboxylase was integrated into the chromosome of the MG1655-derived *E. coli* strain instead of the *poxB* gene under the control of the strong constitutive promoter P_L of phage lambda. The construction of a DNA fragment for the replacement of the coding region of the *poxB* gene by the artificial genetic element, which contains the chloramphenicol acetyltransferase (*cat*) gene flanked by λ*attR* and λ*attL*, the P_L promoter of the lambda phage, and the ribosome binding site and ORF of the *B. subtilis pycA* gene, was performed in several stages. Initially, the DNA fragment containing the *Bgl*II recognition site, P_L promoter, and *Xba*I recognition site was obtained by PCR using primers P17 and P18 and the genomic DNA of lambda phage as a template. In parallel, the DNA fragment containing the *Bgl*II recognition site, the chloramphenicol acetyltransferase (*cat*) gene flanked by λ*attR* and λ*attL*, and 36 nucleotides homologous to the 5′-terminus of the coding region of the *E. coli poxB* gene was obtained by PCR using primers P19 and P20 and plasmid pMW118-(λ*attL*-Cm-λ*attR*) [19] as a template. Additionally, a DNA fragment containing the *Xba*I recognition site, the native ribosome binding site and the ORF of the *B. subtilis pycA* gene, as well as 36 nucleotides complementary to the 3′-terminus of the coding region of the *E. coli poxB* gene, was obtained. PCR amplification was performed using primers P21 and P22 and genomic DNA of *B. subtilis* strain 168 as a template. The obtained DNA fragments were treated with restriction endonucleases *Bgl*II and *Xba*I and ligated with T4 DNA ligase. The ligation product was amplified using primers P20 and P22. The resulting DNA fragment was integrated into the chromosome of the *E. coli* MG Δ*ppc* [20] strain harbouring the pKD46 plasmid. The target clones were selected on LB medium containing 30 μg/mL chloramphenicol. The presence of the desired chromosomal modification in the obtained Cm^R integrants was verified by PCR using the locus-specific primers P23 and P24. The clones with the proven integration of the *B. subtilis pycA* gene were grown on agar M9 minimal medium containing 0.2% glucose as a sole carbon source. Individual clones capable of growing on this medium harboured the *pycA* gene, coding for functionally active pyruvate carboxylase. Then, the correspondence between the original sequence of the *B. subtilis pycA* gene and its copy integrated into the chromosome of the *E. coli* MG Δ*ppc* was confirmed by sequencing.

The modifications were first obtained individually, and then combined in the chromosomes of the target strains by P1-mediated transductions. Excision of the antibiotic resistance marker from the

chromosomes of the strains was performed using the pMWts-Int/Xis plasmid, as described previously [21]. The transformation of the strains with the pMW-*kgd* plasmid carrying the *Mycobacterium tuberculosis kgd* gene encoding 2-ketoglutarate decarboxylase [22] was performed according to a standard procedure.

E. coli cells were grown in Luria-Bertani (LB), SOB, SOC, and M9 media. Ampicillin (100 μg/mL) and/or chloramphenicol (30 μg/mL) was added as needed.

2.2. Culturing of the engineered strains for aerobic fumaric acid production

For fumaric acid production, the engineered strains were initially grown overnight in M9 medium containing 2 g/L glucose at 37 °C. Five millilitres of the overnight culture was diluted ten times with 45 mL of M9 medium containing 9 g/L (50 mM) glucose, 10 g/L yeast extract, and 2.5 g/L $NaHCO_3$. The resulting cultures were incubated aerobically in 750 mL flasks with vented plugs at 37 °C on a rotary shaker at 250 rpm for 7 or 19 h. After 2.5 h of incubation, isopropyl-β-D-thiogalactoside (IPTG) was added to the medium at a final concentration of 1 mM to induce the expression of the *kgd* gene.

Dual-phase fermentation was performed as follows. For the initial biomass accumulation, cells were grown overnight in M9 medium containing 2 g/L glucose at 37 °C. Five millilitres of the overnight culture was diluted ten times with 45 mL of M9 medium containing 9 g/L (50 mM) glucose and 10 g/L yeast extract. The resulting cultures were incubated aerobically in 750 mL flasks with vented plugs at 37 °C on a rotary shaker at 250 rpm for 5 h, and the expression of the *kgd* gene was induced by the addition of IPTG to the medium at a final concentration of 1 mM after 2.5 h of incubation. The cell suspensions were centrifuged for 15 min at ~2000 × g and 4 °C. The cell pellets were resuspended in 50 mL of M9 medium containing 9 g/L glucose and 5 g/L $NaHCO_3$ to an OD_{600} of ~11.5 (DCW ~4.6). The cultures were further incubated in 750 mL flasks with vented plugs at 37 °C on a rotary shaker at 250 rpm for 19 h.

All experiments were performed in triplicate, and the media were supplemented with 100 mg/L ampicillin to maintain plasmid stability when necessary.

2.3. Analytical techniques

Fermentation samples were freed from biomass by centrifugation for 10 min at 15,000×g, and the supernatants were used for further analysis.

The concentrations of organic acids and glucose in the culture media were measured by high-performance liquid chromatography as described previously [23]. A Waters HPLC system (Waters, USA) equipped with UV- or refractive index detector, and a Rezex ROA-Organic Acid H+ (8%) ion-exclusion column (300 × 7.8 mm, 8 μm, Phenomenex, USA) or a Spherisorb-NH2 reversed phase column (4.6 × 250 mm, 5 μm, Waters, USA) were used. The enantiomeric composition of lactic acid was determined by ligand-exchange HPLC using a Nucleosil Chiral-1 column (250 × 4.0 mm, 5 μm, Macherey-Nagel, Germany) with detection at 240 nm. An aqueous solution of copper sulphate (0.5 mM) was used as the eluent at a flow rate of 0.8 mL/min. The temperature of the column was maintained at 60 °C. Calibration of the analytical system, quality control, and enantiomer identification were performed using solutions of L-lactic acid and D,L-LACTIC acid racemic mixture. Data acquisition and processing were conducted with Waters Empower 3 software.

3. Results and discussion

3.1. Construction and evaluation of the strains for the aerobic production of fumaric acid from glucose

The *E. coli* strain MSG1.0 [17] (Table 1) was selected as a chassis to construct the strains for fumaric acid production from glucose under aerobic conditions. This strain was previously engineered to serve as a platform for the development of efficient producers of chemicals derived from the intermediates of the "PEP-OAA-pyruvate-acetyl-CoA" metabolic node. The competing pathways for acetyl-CoA and pyruvate utilisation leading to acetic acid, lactic acid, and ethanol formation were inactivated in the strain through the deletion of the *ackA, pta, poxB, ldhA*, and *adhE* genes, encoding key enzymes catalysing the respective reactions. The intracellular availability of PEP for OAA biosynthesis was increased in the strain due to PEP-independent glucose transport and phosphorylation resulting from inactivation of the *ptsG* gene, encoding the main *E. coli* glucose permease, upon enhanced expression of the *galP* and *glk* genes, which code for the H+-symporter of galactose and ATP-dependent glucokinase (Fig. 1a).

Upon the aerobic utilisation of glucose during growth in a complex nutrient medium, the core strain MSG1.0 converted the carbohydrate substrate to fumaric acid with a molar yield of only approximately 1% (Table 2), accumulating pyruvic and acetic acids as the main secreted metabolites. This indicated that the activity of the oxidative TCA cycle in the strain was rather low, resulting in glucose overflow metabolism [24, 25] upon consumption of the amino acid components of a rich medium as the main nutritional factors for cell growth. The notable secretion of acetic acid by the strain lacking phosphotransacetylase, acetate kinase and pyruvate oxidase could have resulted, in this case, from the background action of *E. coli* acyl-CoA thioesterases YciA and TesB, hydrolysing excessive acetyl-CoA that could not be completely channelled towards the TCA cycle. The carbon recovery value of 64%, which was calculated based on the amounts of detected carboxylic acids secreted by the strain, as well as the presence of succinic, fumaric and malic acids amongst the products of glucose utilisation, suggested that the entire oxidative TCA cycle was nevertheless functional in MSG1.0. Thus, to ensure the efficient formation of fumaric acid as the terminal product of aerobic utilisation of glucose by the strain MSG1.0, the TCA cycle should be interrupted at the stage of the conversion of the respective intermediate to malic acid, which is catalysed by fumarase isozymes.

E. coli possesses three major and two minor fumarases. These are well-known FumA, FumB, and FumC [26] and FumD and FumE, which have been identified just recently [27]. Fumarases A and C are enzymes that function primarily under aerobic conditions, while fumarase B is the dominant isoenzyme during anaerobiosis. Although the expression of the individual genes is regulated differently, the expression of *fumAC* and *dcuB-fumB* operons is induced by CRP-cAMP and can be upregulated in PTS-negative strains [28]. Therefore, inactivation of all three major fumarase isozymes appears to be an essential requirement to enable aerobic production of fumaric acid by the engineered *E. coli* strains.

In previous works, the *fumA* and *fumC* genes were initially deleted to prevent fumaric to malic acid conversion [10, 29]. However, these modifications did not lead to the considerable secretion of fumaric acid by the engineered strains during aerobic utilisation of glucose, and the accumulation of notable amounts of the target product was achieved only after the subsequent deletion of the *fumB* gene. Thus, an exact impact of individual deletion of *fumB* gene on biosynthetic characteristics of engineered strains remained unclear. In the current study, the *fumB* gene was deleted at first in MSG1.0 instead of *fumAC* operon in order to evaluate the effect of primary inactivation of fumarase B on the production of fumaric acid from glucose via the oxidative TCA cycle by the engineered *E. coli* strain.

The profile of metabolites produced by the resulting strain MSG1.0 B during aerobic utilisation of glucose was similar to that of the core strain MSG1.0 (Table 2, Fig. 2a). The yields of pyruvic and acetic acids secreted

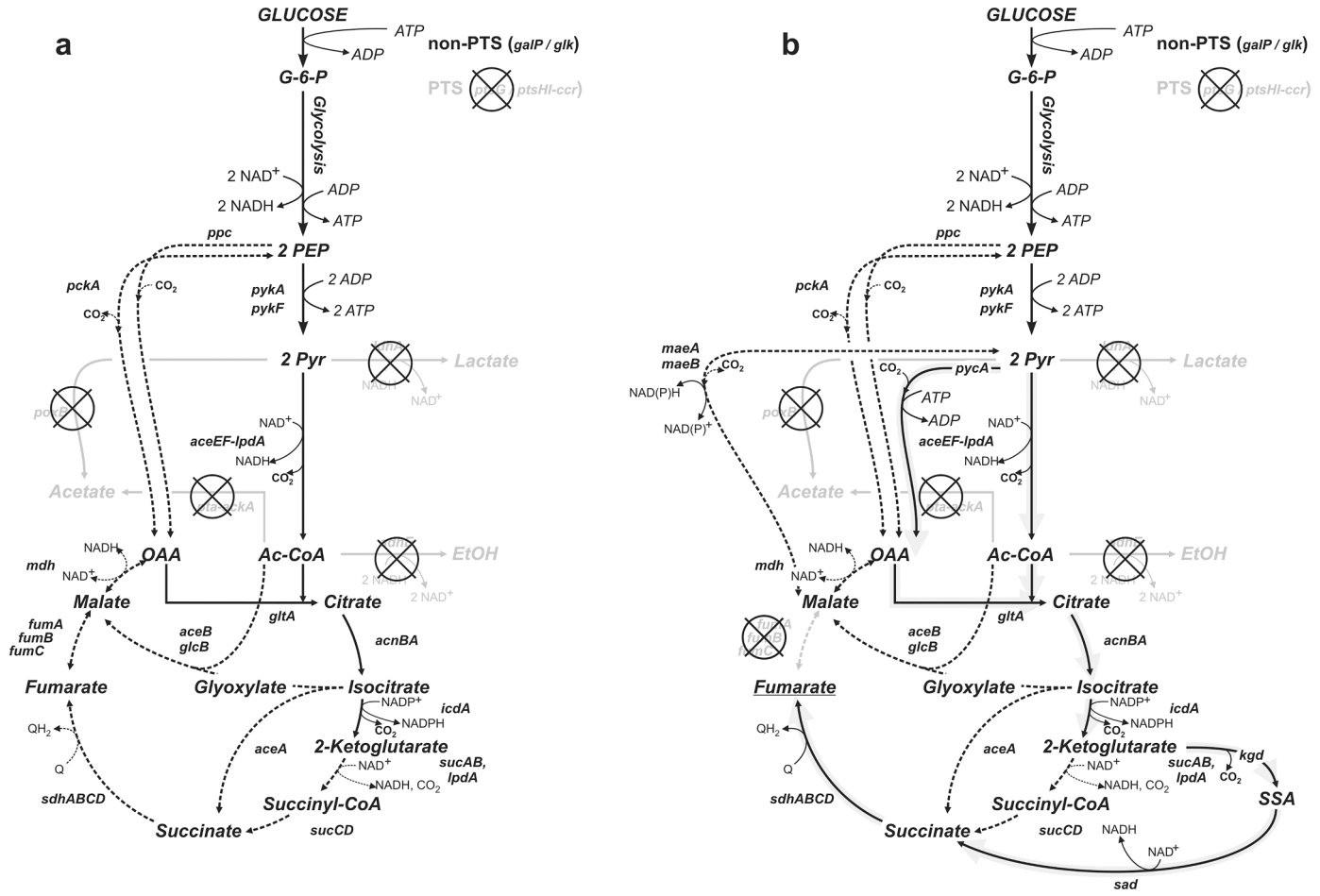

Fig. 1. Central carbon metabolism in engineered *E. coli* strains under aerobic conditions with glucose as a carbon source. (a) The core strain MSG1.0; (b) The best-performing fumaric acid-producing strain MSG1.0 BACPyc [pMW-*kgd*]. The enzymes are indicated by their gene names. Heterologous genes and enzymes are as follows: *kgd, M. tuberculosis* 2-ketoglutarate decarboxylase; *pycA, B. subtilis* pyruvate carboxylase. Abbreviations of key metabolites in the pathways are Ac-CoA, acetyl-CoA; G-6-P, glucose-6-phosphate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; SSA, succinate semialdehyde. The deleted genes are marked with crossed circles. Dotted arrows indicate reactions with decreased intensity. The reactions, as well as the genes of the respective enzymes, substrates, products, and cofactors, that are absent or inactive in the constructed strains are shown in grey. The reactions of the artificial pathway of fumaric acid biosynthesis, starting from pyruvate and including shunting of 2-ketoglutarate to succinic acid via intermediate SSA formation, which was engineered in the current study, are highlighted by wide grey arrows.

Table 2
Molar yields of metabolites produced by the MSG1.0, MSG1.0 B, MSG1.0 BAC, and MSG1.0 BACPyc strains during 7 h of aerobic glucose utilisation.

Strain	Pyruvate	Lactate	Acetate	Succinate	Malate	Fumarate	% C-recovery[a]	DCW(g/L)
MSG1.0	87.6 ± 1.5	n.d.	22.5 ± 1.0	15.6 ± 0.6	1.9 ± 0.2	1.0 ± 0.2	64	3.96 ± 0.12
MSG1.0 B	83.7 ± 1.2	n.d.	20.8 ± 0.8	10.9 ± 0.5	1.9 ± 0.2	1.7 ± 0.2	58	3.93 ± 0.10
MSG1.0 BAC	126.5 ± 1.6	n.d.	27.6 ± 0.6	6.9 ± 0.3	2.3 ± 0.2	17.6 ± 0.4	90	2.30 ± 0.04
MSG1.0 BACPyc	34.5 ± 0.2	n.d.	26.5 ± 0.7	14.9 ± 0.4	3.6 ± 0.1	42.5 ± 0.6	67	3.34 ± 0.07

Data are the means ± standard deviations of three replicates. Molar yields of the secreted metabolites are given in % per mole of consumed glucose (mol/mol, %). n.d. – not detected.

[a] – Carbon recovery was calculated as the ratio of total moles of carbon in the end-products per moles of carbon in total glucose consumed and expressed on a percentage basis.

by the strains MSG1.0 B and MSG1.0 were comparable, while the yield of succinic acid formed by the strain MSG1.0 B notably decreased compared to the parent strain (Table 2). This could indicate the relative decrease in the carbon flux through the terminal reactions of the oxidative TCA cycle that led to diminished OAA replenishment, limiting the intensity of the turnover of the cycle. The yield of fumaric acid synthesised by the strain MSG1.0 B increased only insignificantly (Table 2). Given the value of carbon recovery of 58% (Table 2), this indicated that the individual deletion of the *fumB* gene was not only insufficient to block the operation of the entire TCA cycle but also had almost no effect on the efficiency of fumaric acid oxidation via the fumarase-catalysed reaction. Therefore, the *fumAC* genes were then inactivated in strain MSG1.0 B, leading to strain MSG1.0 BAC.

The strain MSG1.0 BAC, devoid of all three major fumarase isozymes, synthesised fumaric acid from glucose aerobically with a yield that increased to 17.6% (Table 2). Concomitantly, the yield of acetic acid slightly increased and the yield of succinic acid decreased, while the yield of pyruvic acid increased 1.5-fold up to 126.5%. The value of carbon recovery reached 90%, and biomass accumulation by the strain dropped 1.7-fold. The notably increased secretion of pyruvic acid by the

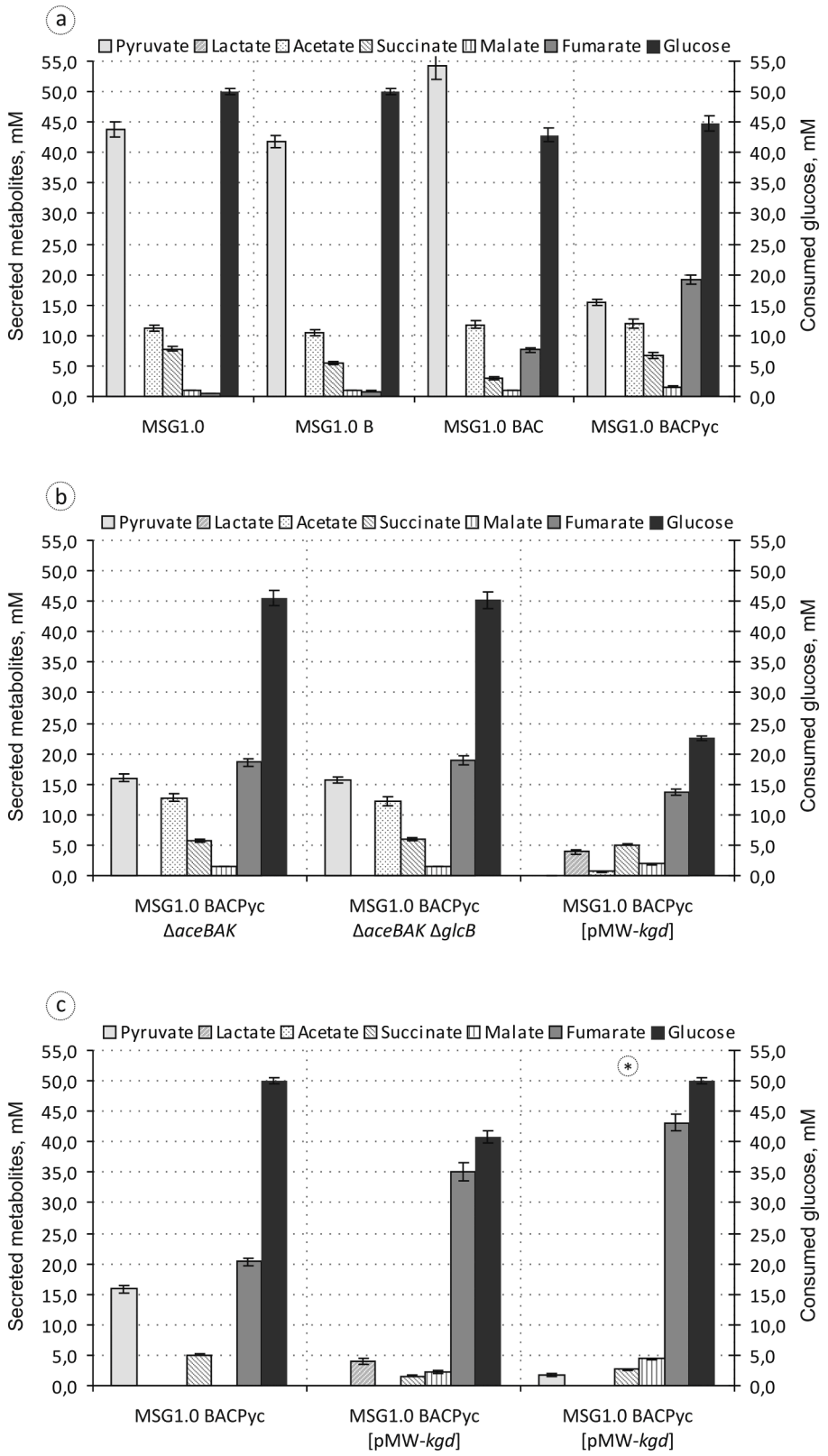

Fig. 2. Metabolite production and substrate consumption by the strains MSG1.0, MSG1.0 B, MSG1.0 BAC, and MSG1.0 BACPyc (a); MSG1.0 BACPyc Δ*aceBAK*, MSG1.0 BACPyc Δ*aceBAK* Δ*glcB*, and MSG1.0 BACPyc [pMW-*kgd*] (b); MSG1.0 BACPyc and MSG1.0 BACPyc [pMW-*kgd*] (c) during 7 h (a, b) and 19 h (c) of aerobic glucose utilisation. The parameters for the strain MSG1.0 BACPyc [pMW-*kgd*] functioning in "biocatalyst" mode are marked with asterisks.

strain, as well as decreased formation of succinic acid, along with the increased production of fumaric acid (Table 2, Fig. 2a), indicated the reduction in the intensity of the TCA cycle due to its interruption at the stage of malic acid formation. The decline in the replenishment of OAA via the TCA cycle limited the availability of the corresponding intermediate for both the initiation of new rounds of the cycle and the reactions of transamination and biosynthesis of aspartate-family amino acids. The latter, in turn, led to restrictions in biomass formation by the strain, since during the growth of *E. coli* in rich or complex LB-like media, available amino acids are completely consumed at the early stages of growth [30, 31] and then need to be synthesised *de novo* by the cell to maintain further proliferation. The reason for pronounced excretion of the excessive pyruvic acid was presumably insufficient activity of anaplerotic pathways in the strain for the efficient formation of OAA from the glycolytic PEP and their inability to supply the TCA cycle with a sufficient amount of one of the necessary precursors. 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, which uses pyruvic acid as a substrate [32]. Phosphoenolpyruvate carboxylase and pyruvate carboxylase irreversibly carboxylate corresponding substrates to form OAA, while the reaction catalysed by phosphoenolpyruvate carboxykinase is reversible. *E. coli* cells possess both PEP carboxylating enzymes but lack pyruvate carboxylase activity. Consequently, during the utilisation of glycolytic substrates, phosphoenolpyruvate carboxylase serves in *E. coli* as the main anaplerotic enzyme [32]. Since phosphoenolpyruvate carboxylase competes for the common precursor metabolite PEP, with glycolytic pyruvate kinases PykA and PykF synthesising pyruvic acid, which is required for further acetyl-CoA formation, the presence of heterologous pyruvate carboxylase activity could significantly increase the flexibility and efficiency of anaplerotic processes in the *E. coli* strain engineered for the production of fumaric acid via the oxidative TCA cycle. Although pyruvate carboxylase, in contrast to phosphoenolpyruvate carboxylase, consumes ATP for the substrate carboxylation, the formation of OAA from the glycolytic precursors by these enzymes is energetically equivalent, since pyruvate kinases generate ATP during PEP to pyruvic acid conversion. In this regard, it should be noted that in PTS-negative strains namely pyruvate kinases are primary responsible for the formation of pyruvic acid from PEP during utilisation of glucose instead of PEP-dependent system of glucose transport and phosphorylation. Taking into account these considerations, and given that phosphoenolpyruvate carboxylase is known to be inhibited by fumarate [33], we decided to introduce heterologous pyruvate carboxylase in the engineered strain to ensure efficient formation of OAA instead of the overexpression of the native phosphoenolpyruvate carboxylase that was performed in previous studies. Therefore, the *Bacillus subtilis pycA* gene, encoding pyruvate carboxylase, was integrated into the chromosome of the strain MSG1.0 BAC under the control of promoted P_L of the phage lambda.

The resulting strain MSG1.0 BACPyc synthesised fumaric acid as the main product of aerobic utilisation of glucose (Fig. 2a) with a molar yield of 42.5% (Table 2). The yield of pyruvic acid dropped more than 3 times to 34.5% (Table 2). The biomass accumulation by the strain markedly improved and was comparable with that of the strain MSG1.0 B and even the core strain MSG1.0. This was apparently caused by the increased intracellular OAA availability. The enhanced formation of OAA also led to the intensification of the truncated TCA cycle, as indicated by the relatively increased secretion of all four-carbon intermediates of the cycle (Fig. 2a). Nevertheless, although the strain MSG1.0 BACPyc efficiently converted glucose to the target product, and the continued secretion of pyruvic, acetic, succinic and even malic acids, as well as decreased value of carbon recovery, indicated the emergence of the effect of glutamate overflow [30], the results suggested the necessity of further improvement of the engineered fumaric acid-producing strain.

3.2. Improvement of the engineered fumaric acid-producing strain

The residual production of pyruvic and acetic acids by the strain MSG1.0 BACPyc was obviously caused by glucose overflow metabolism, as the TCA cycle in the strain still did not keep pace with glycolysis. The production of succinic and malic acids could probably have resulted from not only the action of the truncated TCA cycle but also GS reactions. The key enzymes catalysing reactions of GS, isocitrate lyase and malate synthase, are encoded in *E. coli* by the *aceA* and *aceB* genes, constituting the *aceBAK* operon with the *aceK* gene, which encodes the bifunctional kinase/phosphatase of isocitrate dehydrogenase. The latter enzyme regulates the activity of isocitrate dehydrogenase, redistributing the carbon flux between the oxidative TCA cycle and GS [34]. Upon phosphorylation and thus inactivation of isocitrate dehydrogenase, the enzyme substrate isocitrate is channelled through GS reactions. The dephosphorylated active form of isocitrate dehydrogenase possesses a stronger affinity for isocitrate than isocitrate lyase, causing the prevalent involvement of isocitrate in subsequent reactions of the oxidative TCA cycle. The expression of the *aceBAK* operon is subjected to complex transcriptional regulation involving many factors, such as IclR, FadR, FruR, ArcAB, and CRP-cAMP [35, 36]. The primary regulator, IclR, represses the expression of the *aceBAK* operon under glucose-abundant conditions, while the global regulator CRP-cAMP activates the expression of the corresponding genes upon glucose limitation [36]. Nevertheless, in the PTS-negative strains, CRP-cAMP may activate transcription irrespective of the presence of glucose in the medium, competing with the repressor IclR. Therefore, GS could potentially contribute to the synthesis of four-carbon dicarboxylates in the engineered fumaric acid-producing strain. To verify this assumption, the *aceBAK* genes were deleted in the strain MSG1.0 BACPyc leading to the strain MSG1.0 BACPyc Δ*aceBAK*.

During aerobic utilisation of glucose, the strain MSG1.0 BACPyc Δ*aceBAK* formed a metabolite profile nearly identical to that of the parent strain MSG1.0 BACPyc (Fig. 2b, Fig. 2a), and the yields of the respective products were almost unchanged (Table 3, Table 2). Moreover, the strain still secreted malic acid. Indeed, in addition to malate synthase A, which is encoded by the *aceB* gene, *E. coli* possesses malate synthase G, which is encoded by the *glcB* gene. The expression of the corresponding *glc* operon is known to be indirectly activated by CRP-cAMP [37] and is upregulated in the presence of acetic acid [38]. As a result of glucose overflow metabolism, the strains MSG1.0 BACPyc Δ*aceBAK* secreted not only pyruvic but also acetic acid due to the basal activity of thioesterases, as mentioned above. The presence of acetic acid in the medium could induce the expression of the *glc* operon, causing the formation of malic acid by malate synthase G. Thus, to preclude potential formation of malic acid via the malate synthase G-catalysed reaction, the *glcB* gene was deleted in the MSG1.0 BACPyc Δ*aceBAK*.

The formation of secreted metabolites by the strain MSG1.0 BACPyc Δ*aceBAK* Δ*glcB* during aerobic utilisation of glucose was not affected by the inactivation of all enzymes catalysing GS reactions and was similar to those demonstrated by both parent strains, MSG1.0 BACPyc Δ*aceBAK* and MSG1.0 BACPyc (Fig. 2b, Fig. 2a; Table 3, Table 2). Consequently, GS had no or little impact on the biosynthesis of four-carbon dicarboxylates by the strain MSG1.0 BACPyc engineered for aerobic production of fumaric acid from glucose, and the residual formation of malic acid by the tested strains could have been caused by the basal activity of the minor fumarases E and D. However, the respective enzymes were shown to play insignificant roles in aerobic metabolism of *E. coli* [39] and, then, the contribution of the malic enzymes to the formation of malic acid by the MSG1.0-derived strains could also not be excluded. In *E. coli*, malic enzymes catalyse the reversible interconversion of pyruvic and malic acids, and excessive formation of pyruvic acid upon the intensive intracellular generation of CO_2 by pyruvate dehydrogenase and the oxidative branch of the TCA cycle could shift the respective reaction towards reductive pyruvate carboxylation [40]. The exact reason for malic acid secretion by strains lacking the main fumarase isozymes and

Table 3
Molar yields of metabolites produced by the MSG1.0 BACPyc Δ*aceBAK*, MSG1.0 BACPyc Δ*aceBAK* Δ*glcB*, and MSG1.0 BACPyc [pMW-*kgd*] strains during 7 h of aerobic glucose utilisation.

Strain	Pyruvate	Lactate	Acetate	Succinate	Malate	Fumarate	% C-recovery[a]	DCW(g/L)
MSG1.0 BACPyc Δ*aceBAK*	34.4 ± 0.3	n.d.	28.1 ± 0.7	12.4 ± 0.3	3.2 ± 0.1	40.8 ± 0.4	65	3.38 ± 0.07
MSG1.0 BACPyc Δ*aceBAK* Δ*glcB*	34.8 ± 0.2	n.d.	27.0 ± 0.8	13.1 ± 0.4	3.2 ± 0.1	41.9 ± 0.5	65	3.36 ± 0.08
MSG1.0 BACPyc [pMW-*kgd*][b]	n.d.	17.2 ± 1.2	3.0 ± 0.4	22.5 ± 0.5	8.6 ± 0.3	61.2 ± 1.1	71	1.89 ± 0.03

Data are the means ± standard deviations of three replicates. Molar yields of the secreted metabolites are given in % per mole of consumed glucose (mol/mol, %). n.d. – not detected.
[a] – Carbon recovery was calculated as the ratio of total moles of carbon in the end-products per moles of carbon in total glucose consumed and expressed on a percentage basis.
[b] – Upon induction of *kgd* gene expression by IPTG. The yields of metabolites formed by the strain MSG1.0 BACPyc [pMW-*kgd*] without induction did not differ from those of the parent strain MSG1.0 BACPyc.

GS enzymes needs to be further investigated.

Malic acid was the minor product of aerobic utilisation of glucose by the strain MSG1.0 BACPyc, and pyruvic and acetic acids were secreted as the main byproducts. As mentioned above, this was due to persistent glucose overflow metabolism caused by incomplete coordination between glycolysis and the TCA cycle. The key reason for this was the limitation of carbon flux through the cycle caused by the insufficiency of the 2-ketoglutarate dehydrogenase-catalysed reaction. *E. coli* 2-ketoglutarate dehydrogenase, as well as pyruvate dehydrogenase, is a complicated enzymatic complex assembled from multiple copies of three protein components [41], one of which, dihydrolipoamide dehydrogenase, is not only common to both enzymatic complexes but also participates in the functioning of the glycine cleavage system [41]. Moreover, 2-ketoglutarate dehydrogenase from *E. coli* was shown to be a hybrid comprising other pyruvate dehydrogenase components, in particular E1 encoded by the *aceE* gene [42]. Consequently, a deficiency in the 2-ketoglutarate dehydrogenase activity can hardly be overcome by direct overexpression of the *sucAB* genes encoding the components of the corresponding enzymatic complex.

However, 2-ketoglutarate dehydrogenase and subsequent succinyl-CoA synthetase reactions can be bypassed, resulting from the artificial shunting of 2-ketoglutarate to succinic acid analogous to that of the variant TCA cycle of myco- and cyanobacteria [15, 16]. These bacteria lack 2-ketoglutarate dehydrogenase activity, and the respective TCA cycle variant ensures the closing of the cycle resulting from the sequential action of 2-ketoglutarate decarboxylase, EC 4.1.1.71, catalysing the decarboxylation of 2-ketoglutarate with SSA formation, and succinate-semialdehyde dehydrogenase oxidising SSA to succinic acid. *E. coli* cells naturally possess both NAD^+-dependent succinate-semialdehyde dehydrogenase, Sad, and $NADP^+$-dependent succinate semialdehyde dehydrogenase, GabD, but lack the activity of 2-ketoglutarate decarboxylase classified as EC 4.1.1.71. Thus, the presence of heterologous 2-ketoglutarate decarboxylase in engineered *E. coli* strains is required to enable the operation of the corresponding bypass.

The expression of the *sad* gene encoding NAD^+-dependent succinate-semialdehyde dehydrogenase, which catalyses the oxidation of SSA to succinic acid, markedly increases in *E. coli* in the presence of the appropriate substrate, and the physiological role of the enzyme is to protect the cell from the toxic effect of this reactive aldehyde [43]. Consequently, the intracellular formation of SSA resulting from 2-ketoglutarate decarboxylation by the heterologous enzyme could serve as a signal for an adaptive response leading to an increase in the level of native cellular SSA-neutralising succinate-semialdehyde dehydrogenase activity. Therefore, to allow the channelling of 2-ketoglutarate to succinic acid via intermediate SSA formation, the *Mycobacterium tuberculosis kgd* gene, encoding 2-ketoglutarate decarboxylase, was expressed in the fumaric acid-producing strain MSG1.0 BACPyc (Fig. 1b).

Upon the expression of the 2-ketoglutarate decarboxylase gene, the strain MSG1.0 BACPyc [pMW-*kgd*] aerobically synthesised fumaric acid from glucose with a yield that increased to ~61% (Table 3). The secretion of pyruvic acid by the strain ceased, and succinic and lactic acids accumulated as the main byproducts of glucose utilisation (Fig. 2b, Table 3). At the same time, the consumption of glucose and the biomass accumulation dropped approximately two times compared to the parent strain. The dramatic decrease in biomass accumulation by strain MSG1.0 BACPyc [pMW-*kgd*] expressing 2-ketoglutarate decarboxylase was most likely caused by the decline in the intracellular availability of succinyl-CoA for the biosynthesis of the methionine, lysine and the cell wall components that occurred due to the preferred and efficient diversion of 2-ketoglutarate towards the formation of SSA instead of succinyl-CoA. It was recently shown that bypassing the succinyl-CoA synthetase-catalysed reaction inhibits the growth of *E. coli* strains engineered for 2-ketoglutarate-dependent hydroxylation of proline coupled with the formation of succinic acid [44, 45]. The retardation in biomass formation by the engineered fumaric acid-producing strain expressing 2-ketoglutarate decarboxylase led, in turn, to a decrease in carbohydrate substrate consumption.

On the other hand, the value of carbon recovery was slightly higher than that of the strain MSG1.0 BACPyc (Table 3, Table 2), and the profile of metabolites secreted by the strain MSG1.0 BACPyc [pMW-*kgd*] shifted towards the preferred formation of four-carbon intermediates of the TCA cycle instead of three- and two-carbon glycolytic and derived end-products, i.e., acetic and pyruvic acids (Fig. 2b). As a result, the proportion of four-carbon dicarboxylates amongst the products of aerobic utilisation of glucose formed by the strain MSG1.0 BACPyc [pMW-*kgd*] was 82% versus 50% in the case of the strain MSG1.0 BACPyc. This indicated the markedly intensified carbon flux through the truncated TCA cycle in the strain MSG1.0 BACPyc [pMW-*kgd*] upon the diminished contribution of overflow metabolism to the formation of the products of glucose utilisation. Indeed, when accounting for the amounts of CO_2 generated during the synthesis of succinic, fumaric and malic acids through the reactions of the oxidative TCA cycle in the total sum of the products formed by the strains, the value of carbon recovery reached nearly 100% in the case of the strain MSG1.0 BACPyc [pMW-*kgd*] and only 87% in the case of the strain MSG1.0 BACPyc. Although potential secretion of glutamic acid by the recombinants was not directly evaluated in the current study, the above observations allowed to suppose that the known effect of at least glutamate overflow [30], presumably causing incomplete recovery of the carbon to the detected carboxylic acids secreted by the strain MSG1.0 BACPyc, was successfully overcome in the strain MSG1.0 BACPyc [pMW-*kgd*] due to the efficient channelling of 2-ketoglutarate towards the synthesis of succinic acid followed by the further formation of the target product, fumaric acid, via the reactions of the modified TCA cycle.

Nevertheless, succinic acid synthesised in the strain resulting from the action of artificial 2-ketoglutarate decarboxylase-mediated bypass was incompletely converted to fumaric acid, and the yield of the corresponding precursor of the target product synthesised by the strain

MSG1.0 BACPyc [pMW-*kgd*] increased compared to the parent strain. This could be explained by either the insufficiency of the catalytic activity of succinate dehydrogenase or the overload of the respiratory electron transfer chain. The notable secretion of lactic acid by the strain supported the latter assumption. Indeed, in the absence of the main fermentative lactate dehydrogenase, LdhA, the synthesis of lactic acid from pyruvic acid was apparently caused in the strain by the action of alternative respiratory D- and L-lactate dehydrogenases Dld and LldD [46], which are potentially capable to directly oxidise quinol without auxiliary action of terminal oxidases, cytochromes *bd* and *bo* [46]. Although these dehydrogenases normally catalyse lactate to pyruvate conversion, an excessive intracellular generation of reducing power could shift the equilibrium of the corresponding redox reactions, according to Le Chatelier's principle, towards the formation of more reduced product (i.e. lactic acid). Indeed, analysis of the enantiomeric composition of lactic acid synthesised by the strain showed that both D- and L-stereoisomers of the respective compound were formed in a ratio of 70:30. On the other hand, it cannot be completely excluded that secretion of lactic acid by the strain MSG1.0 BACPyc [pMW-*kgd*] was caused by the action of methylglyoxal bypass. However, this seems unlikely because this pathway is activated in *E. coli* under phosphate starvation [47], whereas phosphate concentrations of 0.3 mM strongly inhibit methylglyoxal synthase [48], a key enzyme of the corresponding biochemical pathway, and the strain MSG1.0 BACPyc [pMW-*kgd*] was cultured in the medium containing ~70 mM phosphate. Moreover, although this pathway could be induced under the stress conditions even upon phosphate abundance, it was previously shown that concomitant deletion of *ldhA* gene and *mgsA* gene, encoding methylglyoxal synthase, in *E. coli* strains engineered for malic acid production from glucose did not prevent lactic acid secretion [49]. At the same time, lactic acid accumulation by the corresponding LdhA and MgsA deficient strains was increased upon supplementation with pyruvate and decreased after the deletion of pyruvate kinase genes *pykA* or *pykF*, suggesting that the formation of lactic acid by the recombinants lacking main lactate dehydrogenase was primarily caused by the action of alternative enzymes capable of directly utilising pyruvate as a substrate. Consequently, the aerobic secretion of lactic acid by the strain MSG1.0 BACPyc [pMW-*kgd*] presumably indicated an increased intracellular quinol pool and decreased availability of respective quinones for succinate dehydrogenase, which limited the conversion of succinic acid to fumaric acid.

It should be noted that *E. coli* can utilise succinic acid as a carbon source under aerobic conditions. Thus, the problem of the byproduction of succinic acid by the engineered fumaric acid-producing strain can be overcome, in particular, by prolonging the fermentation process. In this case, succinic acid that accumulates in the medium will be gradually re-consumed by the strain and converted to the target product via the succinate dehydrogenase-catalysed residual reaction of the truncated oxidative TCA cycle.

3.3. Evaluation of the biosynthetic potential of the engineered fumaric acid-producing strain

As mentioned above, the induction of the expression of heterologous 2-ketoglutarate decarboxylase in the engineered producing strain considerably increased the yield of fumaric acid while negatively affecting cellular growth, presumably due to bypassing the reaction catalysed by succinyl-CoA synthetase. The impaired biomass formation led to a decrease in substrate consumption, limiting the accumulation of the target product. However, the succinyl-CoA synthetase-catalysed reaction was not completely blocked in the strain, since the *sucAB* genes were kept intact in MSG1.0 BACPyc [pMW-*kgd*] and the observed biosynthesis of fumaric acids suggested the successful expression of the *sdhABCD-sucABCD* operon in the cells. Therefore, the growth of the strain could potentially continue, even after the induction of *kgd* gene expression, and the strain could continue the consumption of carbohydrate substrate. Prolonging the process of aerobic cultivation of the strain in the presence of glucose could favour the accumulation of greater amounts of fumaric acid in the medium. Alternatively, dual-phase fermentation can be considered another approach to increase the titre of fumaric acid synthesised by the engineered strain. The process comprises a growth phase for biomass accumulation followed by a production phase. An increased amount of biomass could be obtained at the first stage, and then, during the second stage, which is uncoupled from growth, the producing strain could perform as a whole-cell biocatalyst converting substrate to the target product with high efficiency. Therefore, the biosynthetic potential of the engineered fumaric acid-producing strain MSG1.0 BACPyc [pMW-*kgd*] was evaluated under both modes.

Initially, the effect of prolonged cultivation on aerobic fumaric acid production from glucose by the strain MSG1.0 BACPyc [pMW-*kgd*] was assessed in comparison to its influence on the biosynthetic performance of the parent strain MSG1.0 BACPyc. Upon prolongation of the cultivation period to 19 h, the strain MSG1.0 BACPyc accumulated 1.35 times more biomass, suggesting that the growth of the strain continued after 7 h of incubation in complex medium (Table 4, Table 2). Concomitantly, the strain synthesised fumaric acid from glucose with a yield similar to that demonstrated in 7 h of cultivation (Table 4, Table 2), while the yields of pyruvic and succinic acids slightly decreased and acetic and malic acids were absent amongst the end-products of glucose utilisation formed by the strain (Table 4, Fig. 2c). This observation, along with the decreased carbon recovery, implied that acetic and malic acids were re-assimilated from the medium by the strain after glucose exhaustion in accordance with the well-known effect of the acetate switch [50]. While acetic acids were apparently utilised by acetyl-CoA synthetase Asc to form acetyl-CoA, malic acid was converted to OAA, and both of these metabolites replenished the pool of intermediates required for cellular growth.

The growth of the strain MSG1.0 BACPyc [pMW-*kgd*] also continued during 19 h of cultivation, despite the induced expression of heterologous 2-ketoglutarate decarboxylase. Moreover, the biomass accumulation by the strain rose 2.3 times and reached a value analogous to that of the strain MSG1.0 BACPyc (Table 4, Table 3). Concomitantly, the consumption of glucose and the production of fumaric acid by the strain significantly improved. In contrast to MSG1.0 BACPyc, the strain MSG1.0 BACPyc [pMW-*kgd*] synthesised fumaric acid from glucose in 19 h of aerobic cultivation with a yield that increased to ~86.1%, primarily at the expense of the decreased accumulation of succinic acid (Table 4, Table 3; Fig. 2c, Fig. 2b). It should be noted that the strain MSG1.0 BACPyc [pMW-*kgd*] accumulated less succinic acid during 19 h of cultivation (1.6 mM) than during 7 h (5.1 mM) (Fig. 2c, Fig. 2b). This indicated that the strain gradually re-consumed succinic acid during cultivation and reaffirmed the assumption that the problem with the byproduction of the corresponding dicarboxylate by the fumaric acid-producing strain can be overcome by prolongation of the fermentation process. The yield of lactic acid synthesised by the strain also decreased, suggesting that this reduced product formed at the early growth stages due to insufficient activity of the electron-transport chain could also be further re-assimilated if the culture respired longer. Interestingly, upon prolonged growth, the strain re-assimilated succinic and lactic acids even retaining incomplete glucose consumption. This was caused by the absence of the effect of glucose catabolite repression in PTS-negative *E. coli* strains.

In biocatalyst mode, the strain MSG1.0 BACPyc [pMW-*kgd*], expressing 2-ketoglutarate decarboxylase, consumed all available glucose (50 mM) in 19 h, converting it to fumaric acid with a yield of ~86.3%, analogous to that achieved upon prolonged growth (Table 4). The strain secreted no lactic acid but accumulated slightly more succinic and malic acids compared to the growing culture (Fig. 2c, Table 4). The carbon recovery, which was calculated based on the amounts of products secreted by the strain in the culture medium, constituted 69% (Table 4), while the value, calculated by taking into account the amounts of CO_2 generated during the synthesis of intermediates of the

Table 4
Molar yields of metabolites produced by the MSG1.0 BACPyc and MSG1.0 BACPyc [pMW-*kgd*] strains during 19 h of aerobic glucose utilisation.

Strain	Pyruvate	Lactate	Acetate	Succinate	Malate	Fumarate	% C-recovery[a]	DCW(g/L)
MSG1.0 BACPyc	32.2 ± 0.7	n.d.	n.d.	10.3 ± 0.3	n.d.	40.9 ± 0.9	50	4.50 ± 0.14
MSG1.0 BACPyc [pMW-*kgd*][b]	n.d.	9.9 ± 0.8	n.d.	3.9 ± 0.3	5.3 ± 0.2	86.1 ± 1.5	69	4.42 ± 0.12
MSG1.0 BACPyc [pMW-*kgd*][c]	3.3 ± 0.4	n.d.	n.d.	5.3 ± 0.4	8.7 ± 0.3	86.3 ± 1.8	69	4.61 ± 0.17

Data are the means ± standard deviations of three replicates. Molar yields of the secreted metabolites are given in % per mole of consumed glucose (mol/mol, %). n.d. – not detected.

[a] – Carbon recovery was calculated as the ratio of total moles of carbon in the end-products per moles of carbon in total glucose consumed and expressed on a percentage basis.

[b] – Upon induction of *kgd* gene expression by IPTG. The yields of metabolites formed by the strain MSG1.0 BACPyc [pMW-*kgd*] without induction did not differ from those of the parent strain MSG1.0 BACPyc.

[c] – Upon functioning in "biocatalyst" mode.

oxidative TCA cycle in the total sum of products formed by the strain, was almost 100%, indicating that no carbon source utilised in the productive stage was used for biomass formation. The overall yield from glucose of TCA cycle-derived dicarboxylates, fumaric acid and its direct precursor succinic acid, constituted ~91.6%, indicating that the final yield of the target compound, fumaric acid, synthesised by the strain might potentially reach this high value. Thus, the strain MSG1.0 BACPyc [pMW-*kgd*] could be considered a prospective whole-cell biocatalyst efficiently converting glucose to fumaric acid. Moreover, its biosynthetic characteristics may be further improved.

Indeed, the accumulation of malic acid by the strain, as mentioned above, could be explained by the basal activity of the minor fumarases E and D or even malic enzymes. The formation of this unwanted byproduct of glucose utilisation by the engineered strain may apparently be prevented by the inactivation of the genes *fumE, fumD, maeA* and *maeB*. At the same time, the direct conversion of excreted succinic acid to fumaric acid may be intensified due to overexpression of the *sdhABCD* genes encoding components of succinate dehydrogenase.

It should be noted that the specific productivity of fumaric acid demonstrated by the strain MSG1.0 BACPyc [pMW-*kgd*] functioning in biocatalyst mode constituted 0.061 g/g DCW/h, which was slightly lower than the best performance (0.096 g/g DCW/h) reported previously for growth-coupled production of fumaric acid by engineered *E. coli* [11]. It is known that *E. coli*, like the mitochondria of eukaryotic organisms, are subjected to respiratory control [51] when ATP is not produced through oxidative phosphorylation until enough ADP is available; in other words, electrons are not transferred from the donor molecules to the terminal electron acceptor unless ATP needs to be synthesised. However, the demand of nongrowing cultures for ATP is decreased, thus limiting the relative intracellular ADP availability. As a result, excessive intracellular levels of ATP that formed glycolytically or via oxidative phosphorylation not only inhibited glycolysis [52], limiting or even completely cancelling carbohydrate substrate consumption, but also restricted the functionality of the respiratory electron transport chain and associated succinate dehydrogenase, as well as other enzymes generating reducing power. This problem can be solved, in particular, via the conditional or direct enforcement of ATP hydrolysis [53].

4. Conclusion

We metabolically engineered *E. coli* to efficiently convert glucose to fumaric acid aerobically due to artificial shunting of 2-ketoglutarate to SSA and then to succinic acid with a yield reaching 0.86 mol/mol, amounting to 86% of the theoretical maximum and exceeding the best value, 0.78 mol/mol, reported previously for an engineered *E. coli* strain. While the current work represents a proof-of-concept metabolic engineering study, the implemented strategy enabling efficient conversion of substrate to target product by the directly engineered *E. coli* strain utilising an artificial biochemical pathway analogous to the variant TCA cycle of myco- and cyanobacteria provides an opportunity for the further development of highly efficient strains and processes for the bio-based production of fumaric acid from renewable resources.

Supplementary material

Supplementary Table 1. Primers used in the study.

CRediT authorship contribution statement

Alexandra Yu. Skorokhodova: Conceptualization, Investigation, Data curation, Methodology, Validation, Project administration, Writing – original draft, Writing – review & editing. **Andrey Yu. Gulevich:** Conceptualization, Investigation, Data curation, Methodology, Validation, Project administration, Writing – original draft, Writing – review & editing, Visualization. **Vladimir G. Debabov:** Supervision, Writing – review & editing.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00703.

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