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Engineering *Escherichia coli* for high-level production of propionate

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Abstract Mounting environmental concerns associated with the use of petroleum-based chemical manufacturing practices has generated significant interest in the development of biological alternatives for the production of propionate. However, biological platforms for propionate production have been limited to strict anaerobes, such as *Propionibacteria* and select *Clostridia*. In this work, we demonstrated high-level heterologous production of propionate under microaerobic conditions in engineered *Escherichia coli*. Activation of the native Sleeping beauty mutase (Sbm) operon not only transformed *E. coli* to be propionogenic (i.e., propionate-producing) but also introduced an intracellular "flux competition" between the traditional C2-fermentative pathway and the novel C3-fermentative pathway. Dissimilation of the major carbon source of glycerol was identified to critically affect such "flux competition" and, therefore, propionate synthesis. As a result, the propionogenic *E. coli* was further engineered by inactivation or overexpression of various genes involved in the glycerol dissimilation pathways and their individual genetic effects on propionate production were investigated. Generally, knocking out genes involved in glycerol dissimilation (except *glpA*) can minimize levels of solventogenesis and shift more dissimilated carbon flux toward the C3-fermentative pathway. For optimal propionate production with high C3:C2-fermentative product ratios, glycerol dissimilation should be channeled through the respiratory pathway and, upon suppressed solventogenesis with minimal production of highly reduced alcohols, the alternative NADH-consuming route associated with propionate synthesis can be critical for more flexible redox balancing. With the implementation of various biochemical and genetic strategies, high propionate titers of more than 11 g/L with high yields up to 0.4 g-propionate/g-glycerol (accounting for ~50 % of dissimilated glycerol) were achieved, demonstrating the potential for industrial application. To our knowledge, this represents the most effective engineered microbial system for propionate production with titers and yields comparable to those achieved by anaerobic batch cultivation of various native propionate-producing strains of *Propionibacteria*.

Keywords *Escherichia coli* · Genetic engineering · Glycerol · Propionate · Sleeping beauty mutase

Introduction

Propionate, the anion of propanoic acid, is an important industrial chemical with major applications in the production of animal feed antibiotics, food preservatives, and herbicides, as well as minor applications in the perfume, pharmaceutical, and plastic industries [22]. Currently, industrial manufacturing of propionate is almost exclusively conducted by petrochemical processes, such as the Reppe process, whereby ethylene, carbon monoxide and steam are reacted in the presence of a metal catalyst under high temperature and pressure [39]. Environmental concerns associated with the use of unsustainable petroleum-based sources and environmentally damaging production processes have driven development of green and sustainable production platforms, including biological production. Recently, the

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U.S. Department of Energy identified propionate as one of the top 30 building block chemicals which can be derived from biomass resources, emphasizing its industrial significance and potential for large-scale biomanufacturing [40].

Current biological production of propionate is restricted to the use of native microbial producers, via the Wood–Werkman cycle in various species of *Propionibacteria* and the reductive acrylate pathway in *Clostridium propionicum*. High-level production of propionate in these microorganisms, including *P. ferudenreichii ssp shermanii*, *P. acidipropionici*, and *C. propionicum*, using glucose and glycerol as carbon sources has been demonstrated with high yields [2, 15, 20, 43, 45]. Despite these successes, production platforms on the basis of these Gram-positive anaerobes are not ideal for various reasons, including their slow growth rate, the use of costly and complex media, and the lack of available genetic tools for strain engineering. *Escherichia coli*, being the most common bacterial host for biomanufacturing but a non-native propionate producer, has recently been explored for potential propionate synthesis owing to its well-characterized physiology and genetics, simple and inexpensive cultivation methods, and the variety of existing technologies for genetic manipulation, synthetic biology, and metabolic engineering. Nevertheless, to date, engineering propionogenic (i.e., propionate-producing) *E. coli* strains has been unpopular with few reports being published. For example, the acrylate pathway of *C. propionicum* was introduced into *E. coli* for anaerobic production of propionate, but the propionate titer and yield remained relatively low [20]. Additionally, propionate was synthesized as a byproduct during cultivation of a metabolically engineered 1-propanol-producing *E. coli* strain [6].

While *E. coli* is a non-native propionate producer, it has a native pathway for extended dissimilation of succinate, i.e., the Sleeping beauty mutase (Sbm) pathway which is normally dormant but potentially relevant to the production of C3-fermentative products, i.e., 1-propanol and propionate [13] (Fig. 1). The Sleeping beauty mutase (Sbm) operon in the *E. coli* genome contains four genes whose encoding products (i.e., Sbm: methylmalonyl-CoA mutase, YgfD: an Sbm-interacting protein kinase, YgfG: methylmalonyl-CoA decarboxylase, and YgfH: propionyl-CoA/succinyl-CoA transferase) are similar to enzymes in the oxidative propionate pathway of *Propionibacteria*. The Sbm pathway is cyclical and composed of a series of biochemical conversions forming propionate as a fermentative product while regenerating the starting molecule of succinyl-CoA (Fig. 1).

Recently, we explored heterologous production of 1-propanol by developing propanogenic (i.e., 1-propanol-producing) *E. coli* strains engineered with an activated Sbm operon [34, 35]. Anaerobic cultivation of these strains favored 1-propanol synthesis as a means of consuming excess reducing equivalents, producing low levels of propionate as a byproduct. In the present study, on the basis of the activated Sbm operon, we expanded strain engineering by genetic manipulation of the glycerol dissimilation pathways and explored microaerobic cultivation strategies for high-level propionate production in *E. coli*. Given that glycerol is a waste product of biodiesel production, its use as a carbon source is both economically and environmentally attractive [9]. As a result, recent interest has been generated for biological conversion of glycerol to value-added chemicals and particularly, propionate in this study. *E. coli* has two pathways for initial glycerol dissimilation, i.e., the respiratory pathway and the fermentative pathway converging at dihydroxyacetone phosphate (DHAP) [11] (Fig. 1). In the respiratory pathway, glycerol is first phosphorylated to glycerol-3-phosphate (G3P) by an ATP-dependent glycerol kinase, encoded by *glpK*. The subsequent reaction for conversion of G3P to DHAP is mainly catalyzed by the aerobic glycerol-3-phosphate dehydrogenase, encoded by *glpD* [11]. However, the anaerobic glycerol-3-phosphate dehydrogenase, encoded by *glpABC*, is active in the absence of oxygen, though using the anaerobic enzyme for this conversion is considered a minor pathway for glycerol dissimilation [44]. Alternatively, the fermentative pathway is functional particularly under anaerobic conditions, and it includes glycerol dehydrogenase (encoded by *gldA*) and a PTS-like phosphorelay system (with various enzymes encoded by *dhaKLM*, *ptsI*, and *hpr*) for phosphorylation of dihydroxyacetone (DHA) using phosphoenolpyruvate (PEP) as a phosphate donor [12, 17]. It is generally perceived that uptake and dissimilation of the major carbon source during *E. coli* cultivation could critically affect the metabolite profile and even culture performance, such as titer/yield of recombinant proteins and target metabolites [4, 7, 25, 33, 42]. Herein, we investigated these genetic effects on Sbm-mediated production of propionate by knocking out or overexpressing various genes involved in glycerol dissimilation.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strains, plasmids, and DNA primers used in this study are listed in Table 1. To activate the Sbm operon, a strong promoter (P_{trc}) was fused with the native Sbm operon in the *E. coli* BW-ΔldhA genome using a modified λ Red-mediated recombination protocol [36]. The FRT-Cm^R-FRT cassette from pKD3 was PCR amplified using the primer set c-frt, whereas the promoter–operator region was PCR amplified using the c-ptrc primer set. The two DNA amplicons were then

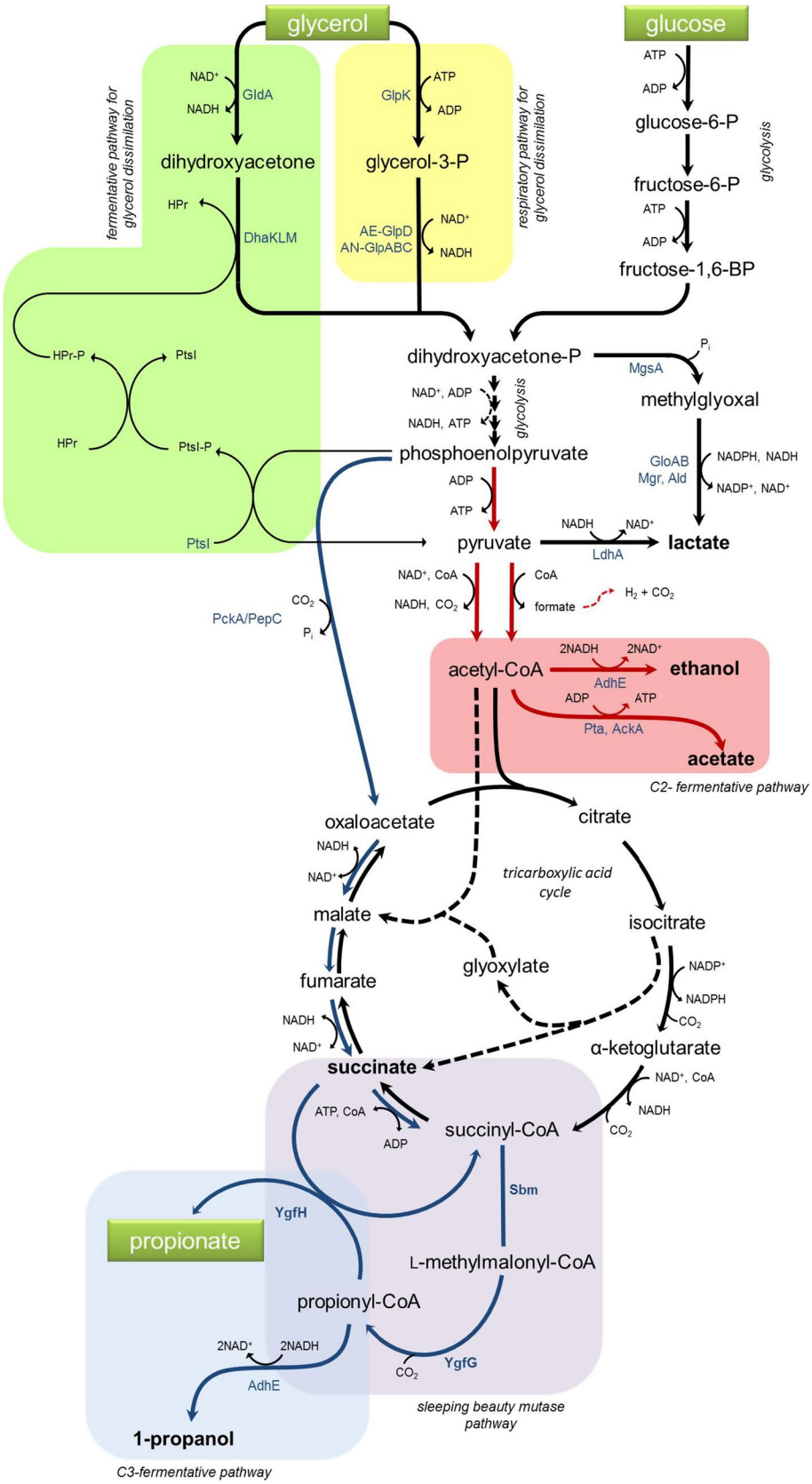

Fig. 1 The genetically engineered metabolic pathway for propionate production. Glucose and glycerol dissimilation pathways are shown under microaerobic conditions. The fermentative pathway for glycerol dissimilation is presented in a *green box* and the respiratory pathway for glycerol dissimilation is presented in a *yellow box*. The Sbm pathway is presented in a *purple box*. *Red* and *blue arrows* represent the route to the C2 and C3-fermentative products, respectively. The C2-fermentative pathway is presented in a *red box*, while the C3-fermentative pathway is presented in a *blue box*. Relevant enzymes for production of various fermentative products as well as the enzymes of the respiratory and fermentative glycerol pathways and the Sbm pathway and are in *blue*

Table 1 List of *E. coli* strains, plasmids, and primers used in this study. Notation for primers: v- verification primer, r- recombineering primer and c- cloning primer

Name	Description, relevant genotype or primer sequence (5′ → 3′)	Reference
E. coli *strains*		
DH5α	F$^-$, *endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG ϕ80d lacZΔ M15, Δ(lacZYA – argF) U169, hsdR17(rK-mK +),* λ-	Lab stock
MC4100	F-, [*araD139*]B/r, Del(*argF-lac*)169, λ–, e14-, *flhD5301*, Δ(*fruK-yeiR*)*725*(*fruA25*), *relA1*, *rpsL150*(strR), *rbsR22*, Del(*fimB-fimE*)*632*(::IS1), *deoC1*	[3]
BW25141	F-, Δ(*araD-araB*)567, Δ*lacZ4787*(::*rrnB-3*), Δ(*phoB-phoR*)*580*, λ-, *galU95*, Δ*uidA3*::pir + , *recA1*, *endA9* (del-ins)::FRT, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	[10]
BW25113	F-, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::*rrnB-3*), λ-, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	[10]
BW-ΔldhA	*ldhA* null mutant of BW25113	[10]
SbmCTRL	BW-ΔldhA, P_{trc}::*sbm* (i.e., with the FRT-P_{trc} cassette replacing the 204-bp upstream of the Sbm operon)	This study
SbmΔglpK	*glpK* null mutant of SbmCTRL	This study
SbmΔglpD	*glpD* null mutant of SbmCTRL	This study
SbmΔglpA	*glpA* null mutant of SbmCTRL	This study
SbmΔgldA	*gldA* null mutant of SbmCTRL	This study
SbmΔdhaK	*dhaK* null mutant of SbmCTRL	This study
SbmΔptsI	*ptsI* null mutant of SbmCTRL	This study
Sbm-glpK	SbmCTRL/pK-glpK	This study
Sbm-glpD	SbmCTRL/pK-glpD	This study
Sbm-glpABC	SbmCTRL/pK-glpABC	This study
Sbm-gldA	SbmCTRL/pK-gldA	This study
Sbm-dhaKLM	SbmCTRL/pK-dhaKLM	This study
Sbm-ptsI	SbmCTRL/pK-ptsI	This study
Plasmids		
pCP20	Flp$^+$, λ cI857$^+$, λ p_R Rep(pSC101 ori)^{ts}, Ap^R, Cm^R	[5]
pKD46	RepA101^{ts} ori, Ap^R, *araC*-P_{araB}::*gam-bet-exo*	[10]
pKD3	R6 K-γ ori, Ap^R, FRT-Cm^R-FRT	[10]
pK184	p15A ori, Km^R, P_{lac}::*lacZ'*	[18]
pK-glpK	Derived from pK184, P_{lac}::*glpK*	This study
pK-glpD	Derived from pK184, P_{lac}::*glpD*	This study
pK-glpABC	Derived from pK184, P_{lac}::*glpABC*	This study
pK-gldA	Derived from pK184, P_{lac}::*gldA*	This study
pK-dhaKLM	Derived from pK184, P_{lac}::*dhaKLM*	This study
pK-ptsI	Derived from pK184, P_{lac}::*ptsI*	This study
Primers		
v-ΔldhA	GATAACGGAGATCGGGAATGATTAA; GGTTTAAAAGCGTCGATGTCCAGTA	This study
v-ΔglpK	CTGATTGGTCTACTGATTGCG; TCCATATACATATCCGGCG	This study
v-ΔglpD	CGTCAATGCTATAGACCACATC; TATTATTGAAGTTTGTAATATCCTTATCAC	This study
v-ΔglpA	GATTAACAGCCTGATTCAGTGAG; CAGCTCTATTTCTGCGGTTTC	This study
v-ΔgldA	TATTACTACACTTGGCACTGCTG; ATATCTTCGTGAACCAGTTTCTG	This study
v-ΔdhaK	CATCGAGGATAAACAGCGCA; ATCTGATAAAGCTCTTCCAGTGT	This study
v-ΔptsI	GGTTCAATTCTTCCTTTAGCG; ACAGTTTGATCAGTTCTTTGATT	This study
v-frt:ptrc	GCGCTCGACTATCTGTTCGTCAGCTC; TCGACAGTTTTCTCCCGACGGCTCA	[35]
c-glpK	GATTACGAATTCGATGACTGAAAAAAAATATATCGTTGCG; TGCCTGCAGTTATTCGTCGTGTTCTT CCCACG	This study
c-glpD	CCGGGGATCCTATGGAAACCAAAGATCTGATTGTGATAG; TGCCTGCAGTTACGACGCCAGCGATAACC	This study
c-glpABC	GATTACGAATTCGATGAAAACTCGCGACTCGCA; TGCCTGCAGTTAAGCCAGCGCCTGGG	This study
c-gldA	GATTACGAATTCGATGGACCGCATTATTCAATCA; TAGAGGATCCTTATTCCCACTCTTGCAGGAAAC	This study
c-dhaKLM	GATTACGAATTCGATGAAAAAATTGATCAATGATGTGC; TGCCTGCAGTTAACCCTGACGGTTGAAACGT	This study

Table 1 continued

Name	Description, relevant genotype or primer sequence (5′ → 3′)	Reference
c-ptsI	CCGGGGATCCTATGATTTCAGGCATTTTAGCATC; TGCCTGCAGTTAGCAGATTGTTTTTTCTTCA ATGAAC	This study
c-frt	AGATTGCAGCATTACACGTCTTGAG; CCAGCTGCATTAATGAATCGGGCCATGGTCCATATGAATA TCCTCC	[35]
c-ptrc	CCGATTCATTAATGCAGCTGG; GGTCTGTTTCCTGTGTGAAATTGTTA	[35]
r-frt:ptrc	**CTCGATTATGGTCACAAAGTCCTTCGTCAGGATTAA**AGATTGCAGCATTACACGTCTTGA; **GTTGGCAAGCTGTTGCCACTCCTGCACGTTAGACAT**GGTCTGTTTCCTGTGTGAAATTGT	[35]

Underlined sequences within the primers denote restriction recognition and homology arms for recombination are in bold print. Notation for primers: *v* verification primer, *c* cloning primer, and *r* recombineering. Restriction recognition sequences are underlined and recombination homology arms are in bold print

fused together by splice overlap-extension (SOE) PCR [19] using the forward primer of the c-frt primer set and the reverse primer of the c-ptrc primer set to generate the FRT-Cm^R-FRT-P_{trc} cassette. To generate the DNA cartridge for genomic integration, the FRT-Cm^R-FRT-P_{trc} cassette was PCR amplified using the r-frt:ptrc primer set containing the 5′ and 3′ 36-bp homology arms, respectively. The homology arms were chosen so as to insert the FRT-CmR-FRT-P_{trc} cassette precisely upstream of the native and silent Sbm operon; the 5′ and 3′ homology arms correspond to nucleotides 3060611–3060646 and 3060885–3060851, respectively, from the *E. coli* MG1655 genome. To derive the plasmid-free propionogenic strain SbmCTRL, 0.1 μg of the amplified/purified DNA cassette was electro-transformed, using a Gene Pulser (BioRad Laboratories, Hercules, CA, USA) set at 1.8 kV, 25 μF, and 200 Ω, to BW-ΔldhA harboring the λ-Red recombinase expression plasmid pKD46 for DNA recombination. Expression of the λ-Red recombination enzymes and preparation of competent cells were carried out as described by Datsenko and Wanner [10]. After electroporation, cells were resuspended in 500 μL of SOC (super optimal broth with catabolite repression) medium (3.6 g/L glucose, 20 g/L tryptone, 5 g/L yeast extract, 0.6 g/L NaCl, 0.19 g/L KCI, 4.8 g/L $MgSO_4$) [14] and recuperated at 37 °C for 1 h in a rotatory shaker at 250 rpm (New Brunswick Scientific, NJ). Cells were then plated on lysogeny broth (LB) agar containing 17 μg/mL chloramphenicol and incubated at 37 °C for 16 h to select chloramphenicol-resistant recombinants. The fusion of the FRT-Cm^R-FRT-P_{trc} cassette with the Sbm operon was verified by colony PCR using the v-frt:ptrc primer set as well as DNA sequencing. Removal of the FRT-Cm^R-FRT cassette from the chromosome was achieved by passing pCP20 through the isolated mutants. Strains were cured of pCP20 and pKD46 by growth at 42 °C.

Escherichia coli BW25113 was used to provide the wild-type genetic background for propionate production. Gene knockouts (i.e., *ldhA*, *glpK*, *glpD*, *glpA*, *gldA*, *dhaK*, and *ptsI*) were introduced into BW25113 and its propionogenic derivatives by P1 phage transduction [27] using the appropriate Keio Collection strains (The Coli Genetic Stock Center, Yale University, New Haven, CT, USA) as donors [1]. To eliminate the co-transduced FRT-Km^R-FRT cassette, the mutants were transformed with pCP20 [5], a temperature-sensitive plasmid expressing a flippase (Flp) recombinase. Upon Flp-mediated excision of the Km^R cassette, a single Flp recognition site (FRT "scar site") was left behind. pCP20 was then removed by growing the cells at 42 °C. The genotypes of derived knockout strains were confirmed by whole-cell colony PCR using the appropriate "verification" primers sets listed in Table 1.

Genes of the respiratory glycerol dissimilation pathway (i.e., *glpK*, *glpD* and *glpABC*) as well as genes of the fermentative glycerol dissimilation pathway (i.e., *gldA*, *dhaKLM* and *ptsI*) were each amplified from *E. coli* BW25141 genomic DNA using the corresponding primers sets (i.e., c-glpK, c-glpD, c-glpABC, c-gldA, c-dhaKLM and c-ptsI). PCR amplifications were performed as conventional reactions using New England Biolabs LongAmp *Taq* DNA Polymerase (Ipswich, MA, USA) or Finnzymes Phusion Polymerase (Espoo, Finland) according to the manufacturers' instructions. All oligonucleotides were custom-made and purified by Integrated DNA Technologies (Coralville, IA, USA). The PCR products were digested with appropriate NEB restriction enzymes, purified and cloned into pK184 [18] for expression under the regulation of the inducible P_{lac} promoter. *E. coli* strain DH5α was used for all molecular cloning purposes. Standard recombinant DNA technologies for molecular cloning were applied [27, 32]. T4 DNA ligase and large (Klenow) fragment of DNA Polymerase I were obtained from New England Biolabs. DNA sequencing was conducted by the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada).

Media and cultivation conditions

All media components were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) except glucose, yeast extract, and tryptone which were obtained from BD Diagnostic Systems (Franklin Lakes, NJ, USA). Media were supplemented with antibiotics as required (30 μg/mL kanamycin and 12 μg/mL chloramphenicol). For propionate production, the propionogenic *E. coli* strains (stored as glycerol stocks at −80 °C) were streaked on LB agar plates with appropriate antibiotics and incubated at 37 °C for 16 h. Single colonies were picked from LB plates to inoculate 30-mL SB medium (32 g/L tryptone, 20 g/L yeast extract, and 5 g/L NaCl) with appropriate antibiotics in 125 mL conical flasks. Overnight cultures were shaken at 37 °C and 280 rpm in a rotary shaker (New Brunswick Scientific, NJ) and used as seed cultures to inoculate 200 mL SB media at 1 % (v/v) with appropriate antibiotics in 1 L conical flasks. This second seed culture was shaken at 37 °C and 280 rpm for approximately 16 h. Cells were then harvested by centrifugation at 6,000 × g and 20 °C for 15 min and resuspended in 100-mL fresh LB media. The suspended culture was used to inoculate a 1-L stirred-tank bioreactor (CelliGen 115, Eppendorf AG, Hamburg, Germany) operated anaerobically or microaerobically at 30 °C and 430 rpm. The semi-defined production medium in the bioreactor contained 30 g/L glycerol, 0.23 g/L K_2HPO_4, 0.51 g/L NH_4Cl, 49.8 mg/L $MgCl_2$, 48.1 mg/L K_2SO_4, 1.52 mg/L $FeSO_4$, 0.055 mg/L $CaCl_2$, 2.93 g/L NaCl, 0.72 g/L tricine, 10 g/L yeast extract, 10 mM $NaHCO_3$, 0.2 μM cyanocobalamin (vitamin B_{12}) and trace elements (2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2$•$4H_2O$, 0.222 mg/L $ZnSO_4$•$7H_2O$, 0.39 mg/L Na_2MoO_4•$2H_2O$, 79 μg/L $CuSO_4$•$5H_2O$, 49.4 μg/L $Co(NO_3)_2$•$6H_2O$) [29], appropriate antibiotics, and supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Anaerobic conditions were maintained by constant bubbling of nitrogen (~0.1 vvm). Microaerobic conditions were maintained by purging air into the headspace at 0.1 vvm. The pH of the production culture was maintained at 7.0 ± 0.1 with 30 % (v/v) NH_4OH and 15 % (v/v) HNO_3.

Analyses

Culture samples were appropriately diluted with saline for measuring the optical cell density (OD_{600}) using a spectrophotometer (DU520, Beckman Coulter, Fullerton, CA, USA). Replicate samples of cell-free supernatant were collected and filter sterilized for titer analysis of glycerol and the various end-fermentation metabolites using an HPLC (LC-10AT, Shimadzu, Kyoto, Japan) with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan) and a chromatographic column (Aminex HPX-87H, Bio-Rad Laboratories, CA, USA). The column temperature was maintained at 65 °C and the mobile phase was 5 mM H_2SO_4 (pH 2.0) running at 0.6 mL/min. Data acquisition and analysis were performed using the Clarity Lite Chromatographic Station (Clarity Lite, DataApex, Prague, Czech Republic).

Results

Cultivation conditions for propionate production

Given that the native *E. coli* Sbm operon is inherently silent, a functional Sbm pathway was established in the engineered strain SbmCTRL using a previously developed bacteriophage λ (λ-Red) genomic recombineering-based method [36]. Specifically, a synthetic DNA fusion containing a strong P_{trc} promoter along with a chloramphenicol-resistance *cat* cassette flanked by two FRT sites was used to replace a 204-bp superfluous region upstream of the Sbm operon. The chloramphenicol-resistance marker was excised using Flp-mediated excision and the strain was cured of all episomal plasmids for recombineering, creating the markerless and plasmid-free strain SbmCTRL.

SbmCTRL was first characterized for its ability to produce propionate and cultivation performance under various batch culture conditions is presented in Table 2 and Fig. 2. Anaerobic conditions were first tested given that propionate is a fermentative product. Furthermore, a putative FNR-binding site upstream of *sbm* in the *E. coli* chromosome was identified [31], suggesting that the expression of the Sbm operon can be positively regulated by anaerobiosis. While the activated Sbm operon was competent for propionate production, the propionate titer was limited, with ~1.23 g/L and ~0.17 g/L being produced at the end of the strict anaerobic cultivations using 30 g/L glucose or 30 g/L glycerol, respectively, as sole carbon sources.

Under anaerobic conditions, cultivation performance varies significantly with the carbon source. First, complete dissimilation of 30 g/L glucose and 30 g/L glycerol occurred at 11 h and 56 h, respectively. Both cell growth rate and biomass yield were significantly higher for anaerobic glucose culture. In addition, the two cultures showed rather different profiles in metabolite production. For the anaerobic glucose culture, the majority of carbon flux was directed toward the C2-fermentative pathway, with a C3:C2 fermentative product ratio equal to 0.15. Anaerobic cultivation using glycerol selectively favored solvent production, not only with the sum of ethanol and 1-propanol titers accounting for 87 % of dissimilated glycerol (an overall acid:solvent ratio of 0.13), but also with limited propionate production. In light of limited cultivation performance, particularly low propionate production, as well as major

Table 2 Culture performance (i.e., overall glycerol or glucose consumption and final biomass and metabolite concentrations) of SbmCTRL in a bioreactor under anaerobic and microaerobic cultivation conditions

	Glucose[a]	Glycerol[a]	Biomass[b]	Succinate[c]	Lactate[c]	Acetate[c]	Propionate[c]	Ethanol[c]	1-Propanol[c]
Anaerobic cultivation									
SbmCTRL (Glucose-fed)	30.37 ± 0.35	–	3.77	1.72 ± 0.05	0.43 ± 0.14	8.27 ± 0.11	1.23 ± 0.01	4.07 ± 0.08	0.96 ± 0.01
	(81.77 %)	–	–	(4.24 %)	(1.39 %)	(40.85 %)	(4.85 %)	(25.80 %)	(4.64 %)
SbmCTRL (Glycerol-fed)	–	27.58 ± 0.74	2.16	0.63 ± 0.04	ND	1.90 ± 0.13	0.17 ± 0.03	10.63 ± 0.19	1.93 ± 0.08
	–	(100.02 %)	–	(1.77 %)	(0.00 %)	(10.62 %)	(0.76 %)	(76.40 %)	(10.64 %)
Microaerobic cultivation									
SbmCTRL (Glucose-fed)	28.41 ± 0.89	–	3.37	1.72 ± 0.06	0.97 ± 0.12	6.34 ± 0.40	1.29 ± 0.03	1.74 ± 0.30	0.65 ± 0.06
	(62.94 %)	–	–	(4.62 %)	(3.39 %)	(33.97 %)	(5.53 %)	(11.99 %)	(3.44 %)
SbmCTRL (Glycerol-fed)	–	31.89 ± 0.91	4.20	0.26 ± 0.08	ND	3.53 ± 0.08	1.60 ± 0.10	2.06 ± 0.06	2.04 ± 0.13
	–	(47.00 %)	–	(0.63 %)	(0.00 %)	(17.28 %)	(6.32 %)	(12.93 %)	(9.83 %)

The metabolite distribution (i.e., the fraction of dissimilated glycerol to form a metabolite) is defined as the ratio of the glycerol equivalent of a metabolite (calculated based on the individual theoretical yield: 1.28 g succinate/g glycerol, 0.98 g lactate/g glycerol, 0.64 g acetate/g glycerol, 0.79 g propionate/g glycerol, 0.5 g ethanol/g glycerol, 0.65 g 1-propanol/g glycerol) to the total consumed glycerol. The glycerol efficiency toward metabolite synthesis is calculated as the sum of all metabolite fractions. Error bars represent s.d. ($n = 2$)

ND not detected

[a] Glucose or glycerol consumption (g/L); glucose or glycerol efficiency is presented in parentheses under the carbon source consumption value (%)

[b] Biomass accumulation (g-DCW/L)

[c] Metabolite concentrations (g/L); the fraction of dissimilated glycerol is presented in parentheses under each titer

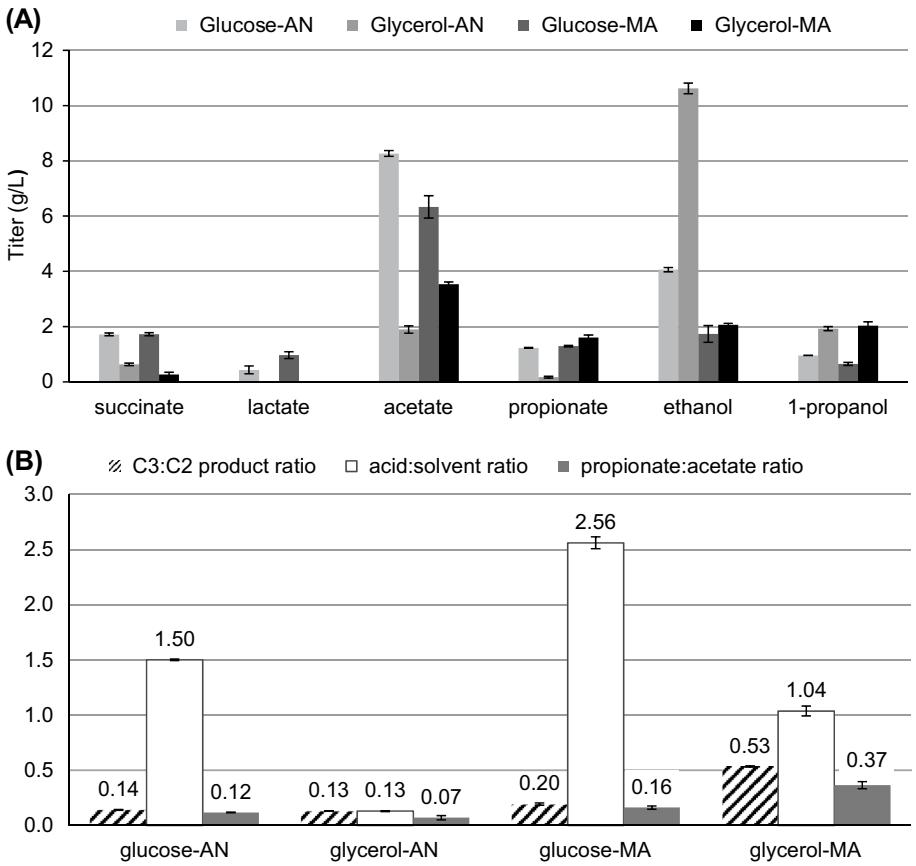

Fig. 2 **a** Major metabolites titers during batch cultivation of SbmCTRL under anaerobic (AN) or microaerobic (MA) conditions using glucose or glycerol as a carbon source and **b** ratios of C3:C2 fermentative products (i.e.: propionate + 1-propanol: acetate + ethanol), overall acid:solvent production (i.e.: propionate + acetate: 1-propanol + ethanol) as well as propionate:acetate production for each of the batch cultivation conditions; ratios are calculated from the fractions of dissimilated glycerol. *Error bars* represent s.d. ($n = 2$)

diversion of carbon flux into the C2-fermentative pathway under strict anaerobic conditions, we explored microaerobic cultivation for which air was purged into the headspace of the bioreactor at 0.1 vvm.

Switching from anaerobic to microaerobic conditions for glucose culture slightly reduced the carbon flux into C2-fermentative pathway with acetate and ethanol still being the two dominant metabolites. However, the switch had no major effect on propionate production. On the other hand, for glycerol culture, introducing microaerobic conditions significantly increased both glycerol dissimilation rate and biomass yield by ~100 % compared to the corresponding anaerobic operation. The change to a microaerobic environment also resulted in a dramatic decrease in ethanol production with a simultaneous increase in acetate production. More importantly, the propionate titer reached 1.60 g/L, representing a more than sevenfold increase compared to the anaerobic glycerol culture. However, note that introducing microaerobic conditions reduced the overall efficiency of dissimilation of carbon sources (i.e., carbon loss for both glucose and glycerol) presumably due to the formation of carbon dioxide. Overall, based on these SbmCTRL cultures, it appears that microaerobic cultivation using glycerol as the major carbon source is most suitable for propionate production as this culture showed the highest overall C3:C2 product ratio of 0.54 as well as the highest propionate:acetate ratio of 0.39 and, therefore, all subsequent cultivations were conducted under this culture condition.

Inactivation of the respiratory pathway for glycerol dissimilation

In light of the effectiveness of glycerol as a carbon source for propionate production, we aimed to re-engineer the propionogenic strain SbmCTRL by targeting glycerol metabolism in an attempt to identify the link between glycerol dissimilation and propionate production. Six genes associated with glycerol dissimilation, either via the respiratory or fermentative pathway, were manipulated by gene knockout or episomal overexpression to observe their individual effects on cultivation performance, particularly cell growth, relative levels of acidogenesis and solventogenesis, metabolite profile, and propionate production.

The respiratory pathway of glycerol dissimilation includes glycerol kinase, encoded by *glpK,* and two glycerol-3-phosphate dehydrogenases, encoded by *glpD* (aerobic) and *glpABC* (anaerobic), respectively (Fig. 1). This ATP-dependent glycerol dissimilation pathway starts with aerobic phosphorylation of glycerol to G3P, followed by the oxidation of G3P to DHAP. While the oxidation step is primarily carried out by aerobic GlpD, the minor pathway via anaerobic GlpABC can be functional when oxygen is unavailable. Microaerobic cultivations of three single-knockout mutants, i.e., Sbm-ΔglpK, Sbm-ΔglpD, and Sbm-ΔglpA, using glycerol as the major carbon source were conducted and the results are summarized in Table 3 and Fig. 3. While the overall glycerol dissimilation rate for Sbm-ΔglpK and Sbm-ΔglpD was slightly slower than that of the control strain SbmCTRL (i.e., taking more than 30 h to consume 30 g/L glycerol for the two mutants and 26 h for SbmCTRL), implying slightly defective glycerol dissimilation associated with these mutations under microaerobic conditions, the biomass yield for these two mutants was significantly higher than that of SbmCTRL. However, the Sbm-ΔglpA mutant had approximately the same glycerol dissimilation rate and biomass yield as those of SbmCTRL.

The various single-gene knockouts associated with the respiratory pathway of glycerol dissimilation also resulted in major changes in metabolite production. In comparison to SbmCTRL, solventogeneis was significantly inhibited for Sbm-ΔglpD and was even completely abolished for Sbm-ΔglpK. This was not the case for Sbm-ΔglpA, which had a solventogenesis level similar to that of SbmCTRL. On the other hand, acidogenesis was significantly enhanced particularly for Sbm-ΔglpK and Sbm-ΔglpD, and their propionate titers (i.e., 6.67 and 5.22 g/L, respectively) were more than threefold that of SbmCTRL (i.e., 1.60 g/L). However, the propionate titer for Sbm-ΔglpA (i.e., 1.97 g/L) was only slightly increased compared to SbmCTRL though acetate titer was significantly increased. Apart from a higher level of acidogenesis, the overall cultivation performance of Sbm-ΔglpA was more or less the same as that of SbmCTRL.

Inactivation of the fermentative pathway for glycerol dissimilation

Alternatively, glycerol dehydrogenase and dihydroxyacetone kinase (encoded by *gldA* and *dhaKLM*, respectively) comprise the fermentative pathway of glycerol dissimilation in *E. coli* (Fig. 1). Active during anaerobic conditions, this pathway mediates the conversion of glycerol to DHA which is subsequently phosphorylated to DHAP using a PTS-like phosphorelay system [12]. Microaerobic cultivations of three single-knockout mutants, i.e., Sbm-ΔgldA, Sbm-ΔdhaK, and Sbm-ΔptsI, using glycerol as the major carbon source were conducted and the results are summarized in Table 3 and Fig. 4. While the overall glycerol dissimilation rate was slightly reduced by these knockouts (i.e., taking more than 30 h to consume 30 g/L glycerol for the three mutants and 26 h for SbmCTRL), the biomass yield for all three mutants had a ~50 % increase compared to SbmCTRL. Similar to the respiratory-pathway knockout mutants, minimal solventogenesis and high acidogenesis were

Table 3 Culture performance (i.e., overall glycerol consumption and final biomass and metabolite concentrations) of glycerol pathway mutant strains (respiratory pathway mutants: Sbm-ΔglpK, Sbm-ΔglpD, and Sbm-ΔglpA; fermentative pathway mutants: Sbm-ΔgldA, Sbm-ΔdhaK and Sbm-ΔptsI)

	Glycerol[a]	Biomass[b]	Succinate[c]	Lactate[c]	Acetate[c]	Propionate[c]	Ethanol[c]	1-Propanol[c]
Parent propionogenic strain								
SbmCTRL	31.89 ± 0.91	4.20	0.26 ± 0.08	ND	3.53 ± 0.08	1.60 ± 0.10	2.06 ± 0.06	2.04 ± 0.13
	(47.00 %)	–	(0.63 %)	(0.00 %)	(17.28 %)	(6.32 %)	(12.93 %)	(9.83 %)
Respiratory pathway mutants								
SbmΔglpK	31.35 ± 0.92	8.41	ND	ND	8.62 ± 0.52	6.67 ± 0.09	ND	ND
	(69.78 %)	–	(0.00 %)	(0.00 %)	(42.95 %)	(26.83 %)	(0.00 %)	(0.00 %)
SbmΔglpD	30.14 ± 0.12	6.70	0.49 ± 0.10	ND	7.88 ± 0.16	5.22 ± 0.04	1.03 ± 0.10	0.98 ± 0.04
	(75.73 %)	–	(1.27 %)	(0.00 %)	(40.80 %)	(21.82 %)	(6.84 %)	(5.00 %)
SbmΔglpA	27.21 ± 0.83	3.98	0.52 ± 0.04	ND	6.07 ± 0.09	2.29 ± 0.07	1.97 ± 0.04	1.86 ± 0.23
	(71.88 %)	–	(1.49 %)	(0.00 %)	(34.78 %)	(10.62 %)	(14.48 %)	(10.50 %)
Fermentative pathway mutants								
SbmΔgldA	31.74 ± 0.66	6.29	0.05 ± 0.06	ND	5.01 ± 0.07	11.39 ± 0.39	0.12 ± 0.03	0.21 ± 0.06
	(71.76 %)	–	(0.11 %)	(0.00 %)	(24.63 %)	(45.23 %)	(0.76 %)	(1.02 %)
SbmΔdhaK	30.03 ± 0.05	6.56	0.48 ± 0.09	ND	4.44 ± 0.16	11.83 ± 0.09	ND	ND
	(73.94 %)	–	(1.24 %)	(0.00 %)	(23.04 %)	(49.66 %)	(0.00 %)	(0.00 %)
SbmΔptsI	29.50 ± 0.79	6.56	0.78 ± 0.03	ND	4.61 ± 0.05	8.74 ± 0.08	ND	ND
	(63.77 %)	–	(2.07 %)	(0.00 %)	(24.36 %)	(37.35 %)	(0.00 %)	(0.00 %)

The metabolite distribution (i.e., the fraction of dissimilated glycerol to form a metabolite) is calculated as in Table 2. Error bars represent s.d. ($n = 2$)

ND not detected

[a] Glycerol consumption (g/L); glycerol efficiency is presented in parentheses under the carbon source consumption value (%)

[b] Biomass accumulation (g-DCW/L)

[c] Metabolite concentrations (g/L); the fraction of dissimilated glycerol is presented in parentheses under each titer

observed for the fermentative-pathway knockout mutants. More importantly, the high-level acidogenesis preferentially favored propionate production over acetate, leading to a high propionate titer of more than 11 g/L for Sbm-ΔgldA and Sbm-ΔdhaK, accounting for up to 50 % of dissimilated glycerol and representing a sevenfold increase over the propionate titer of the microaerobic SbmCTRL culture. Inactivation of the alternative phosphorelay system in Sbm-ΔptsI also significantly increased the propionate titer to 8.74 g/L. Comparison of the C3:C2 fermentative product ratio as well as the propionate:acetate ratio between SbmCTRL and the fermentative pathway mutants suggests the effectiveness of inactivating the fermentative pathway of glycerol dissimilation for enhancing propionate production.

Overexpression of the respiratory pathway genes for glycerol dissimilation

Microaerobic cultivations of strains episomally overexpressing the genes encoding each of the enzymes in the respiratory pathway of glycerol dissimilation, i.e., Sbm-glpK, Sbm-glpD and Sbm-glpABC, were conducted and their culture performance is presented in Table 4 and Fig. 5. In comparison to SbmCTRL, overexpression of *glpD* and *glpABC* had a minor effect on the overall rate of glycerol dissimilation, whereas *glpK* overexpression slightly decreased the overall glycerol dissimilation rate. While the biomass yield of Sbm-glpD was similar to SbmCTRL, Sbm-glpK and Sbm-glpABC had significantly lower and higher biomass yields, respectively.

Metabolite profiles of Sbm-glpK, Sbm-glpD and Sbm-glpABC varied considerably. Acidogenesis was generally enhanced in all three strains, accounting for more than 30 % of dissimilated glycerol. Note that Sbm-glpK, though with an Δ*ldhA* genetic background, had an unusually high lactate titer potentially associated with its low glycerol dissimilation rate, biomass yield, and even propionate titer. Overexpression of *glpD* and *glpABC* resulted in improved propionate production potentially due to enhanced acidogenesis and less carbon loss (i.e., higher glycerol efficiency).

Overexpression of the fermentative pathway genes for glycerol dissimilation

Alternatively, microaerobic cultivations of strains episomally overexpressing the genes encoding each of the

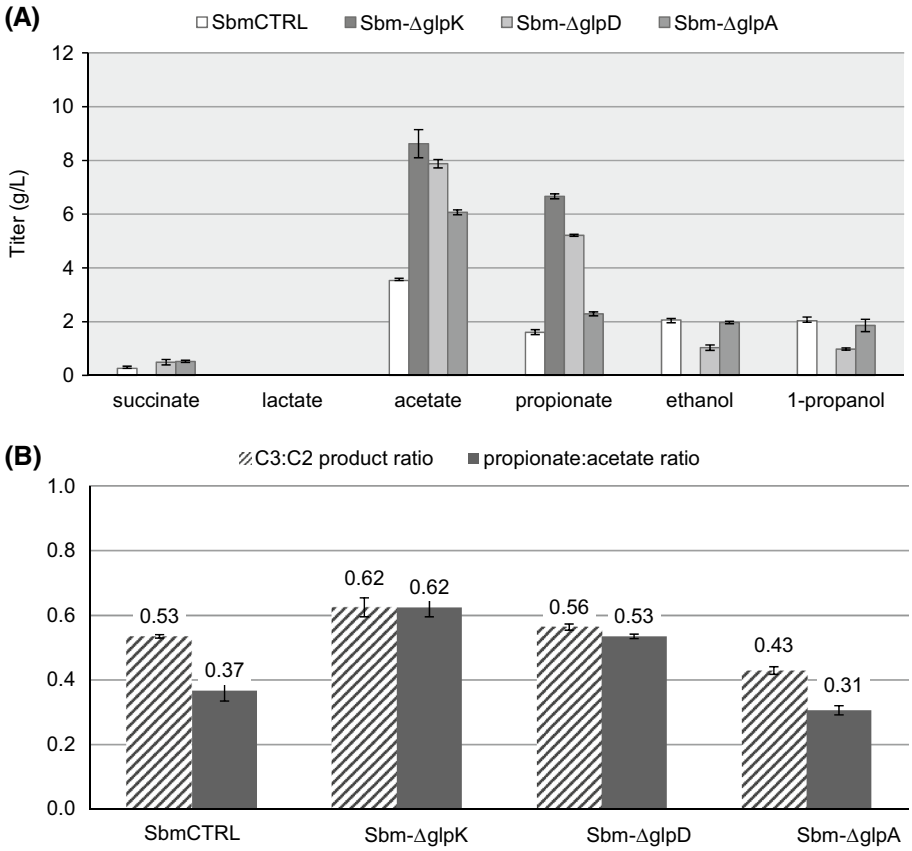

Fig. 3 **a** Major metabolites titers during microaerobic batch cultivation of respiratory pathway mutants (Sbm-ΔglpK, Sbm-ΔglpD and Sbm-ΔglpA) using glycerol as a carbon source and **b** ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation; ratios are calculated from the fractions of dissimilated glycerol. *Error bars* represent s.d. ($n = 2$)

enzymes in the fermentative pathway of glycerol dissimilation, i.e., Sbm-gldA, Sbm-dhaKLM and Sbm-ptsI, were conducted and their culture performance is presented in Table 4 and Fig. 6. The biomass yields of the three strains were similar to that of SbmCTRL. Compared to SbmCTRL, the glycerol dissimilation rates of Sbm-gldA and Sbm-dhaKLM were slightly reduced (i.e., taking more than 30 h to consume 30 g/L glycerol for the two mutants and 26 h for SbmCTRL), whereas that of Sbm-ptsI was significantly slowed (i.e., taking more than 45 h to consume 30 g/L glycerol).

Overexpression of *gldA* resulted in a substantial shift in the metabolite profile, compared to SbmCTRL, with high acetate and succinate titers accompanied by the loss of propionate and 1-propanol synthesis. The results imply that *gldA* overexpression completely inactivated the Sbm pathway by stalling the dissimilated carbon flux at the succinate node (Fig. 1). On the other hand, overexpression of *dhaKLM* or *ptsI* resulted in enhanced solventogenesis with a propionate titer either similar to (for Sbm-dhaKLM) or lower than (for Sbm-pstI) that of SbmCTRL. These results suggest that carbon flux should be preferentially channeled through the respiratory pathway, but not the fermentative pathway, of glycerol dissimilation for effective propionate production.

Discussion

While propionate biosynthesis has primarily been conducted by anaerobic cultivation of Gram-positive native producers, such as *Propionibacteria* and *Clostridia* [2, 15, 43, 45], or by anaerobic *E. coli* cultivation but with limited propionate titers [6, 20], we herein present a novel approach through activation of the endogenous Sbm operon of *E. coli*. Further strain engineering resulted in high-level propionate production, with titers comparable to those achieved by anaerobic batch cultivation of *Propionibacteria* [2, 8, 15, 30]. Though glucose can be rapidly dissimilated by propionogenic *E. coli*, it was deemed an unsuitable carbon source primarily due to overflow metabolism of acetate [24, 38]. Excretion of acetate may lead to transcriptional repression of respiratory genes of the TCA cycle [38] and potentially limit carbon flux into the C3-fermentative pathway. Under anaerobic conditions, expression of the Sbm operon appears to be more active when glycerol is used as the major carbon source rather than glucose, resulting in less succinate accumulation and more diversion of dissimilated carbon flux into the Sbm pathway [35]. Nevertheless, glycerol can be a recalcitrant carbon source particularly under strict anaerobic conditions for *E. coli* cultivation. Also, because of its higher degree of reductance,

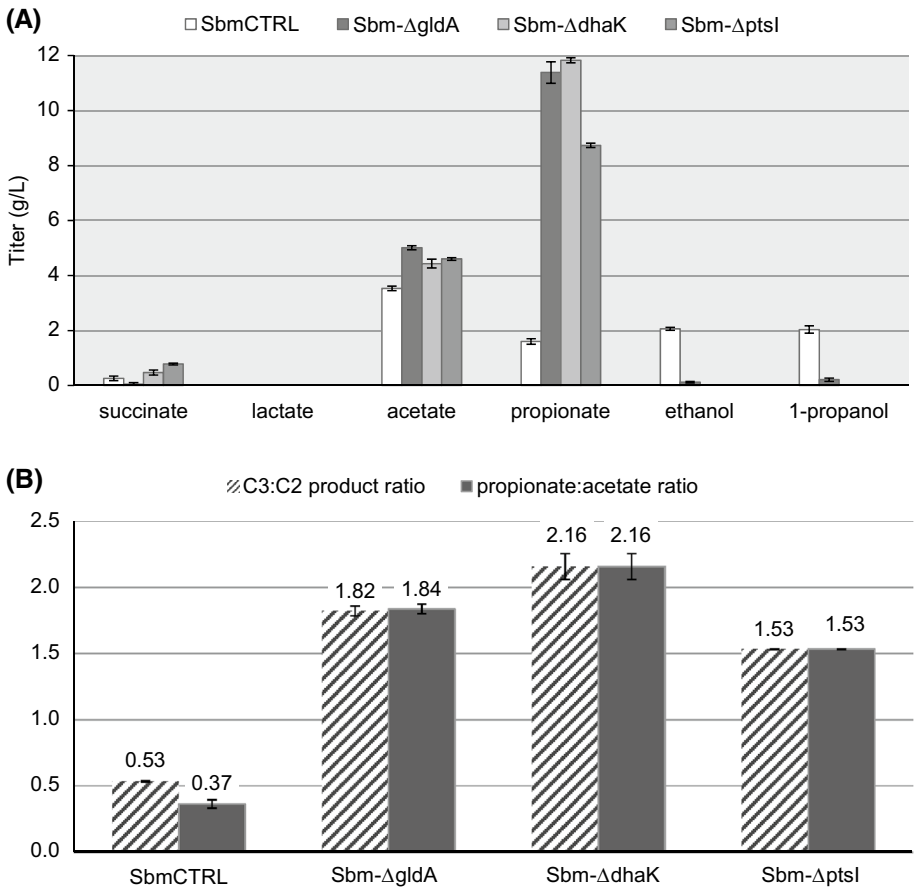

Fig. 4 **a** Major metabolites titers during microaerobic batch cultivation of fermentative pathway mutants (Sbm-ΔgldA, Sbm-ΔdhaK and Sbm-ΔptsI) using glycerol as a carbon source, and **b** ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation; ratios are calculated from the fractions of dissimilated glycerol. *Error bars* represent s.d. ($n = 2$)

solventogenesis often dominates during anaerobic cultivations on glycerol and, as a result, propionate production is impacted. Microaerobic conditions can offer low levels of oxygen as an electron acceptor, leading to improved cell growth and even biomass yield, particularly when glycerol is used as the major carbon source. Compared to 1-propanol which normally requires strict anaerobiosis for effective production, propionate is a less reduced C3-fermentative product which can be effectively produced under microaerobic conditions. While cell growth improved and glycerol dissimilation was more effective under microaerobic conditions for SbmCTRL, acetate was to some extent overproduced, potentially due to carbon flux overflow and stalling of intracellular NADH turnover [41] and, therefore, propionate production was limited. Since AdhE is normally repressed in the presence of oxygen, the persistent solventogenesis during microaerobic cultivation of SbmCTRL with glycerol was likely associated with the increase in the NADH/NAD^+ ratio [21].

Based on the hypothesis of carbon flux overflow, we explored potential slowing of glycerol dissimilation under microaerobic conditions by inactivating various genes involved in the glycerol dissimilation pathway. Note that all single-knockout mutants investigated in this study, except Sbm-ΔglpD, cannot be cultivated anaerobically using glycerol as the major carbon source (data not shown). Though Sbm-ΔglpD can be cultivated anaerobically, its glycerol dissimilation rate was much lower than that of SbmCTRL, as dissimilation of 30 g/L glycerol took 65 h and 30 h for Sbm-ΔglpD and SbmCTRL, respectively. The result suggests the coordinated and synergistic roles of all enzymes involved in both respiratory and fermentative pathways for glycerol dissimilation under anaerobic conditions, including GlpD which is identified as an aerobic glycerol-3-phosphate dehydrogenase [11, 28, 44]. Nevertheless, such defective glycerol dissimilation under anaerobic conditions associated with various gene knockouts can be prevented by adopting microaerobic cultivation, suggesting that the respiratory and fermentative pathways of glycerol dissimilation are complimentary under microaerobic conditions [11].

Note that, given a slightly reduced overall glycerol dissimilation rate, all single-knockout mutants except Sbm-ΔglpA had a biomass yield at least 50 % higher than that of SbmCTRL and the increased biomass yield occurred consistently with high-level acidogenesis (accounting for 60–73 % of dissimilated glycerol) and suppressed solventogenesis, resulting in enhanced propionate production.

Table 4 Culture performance (i.e., overall glycerol consumption and final biomass and metabolite concentrations) of strains overexpressing the genes of glycerol dissimilation (respiratory pathway overexpression strains: Sbm-glpK, Sbm-glpD, and Sbm-glpA; fermentative pathway overexpression strains: Sbm-gldA, Sbm-dhaK and Sbm-ptsI)

	Glycerol[a]	Biomass[b]	Succinate[c]	Lactate[c]	Acetate[c]	Propionate[c]	Ethanol[c]	1-Propanol[c]
Parent propionogenic strain								
SbmCTRL	31.89 ± 0.91	4.20	0.26 ± 0.08	ND	3.53 ± 0.08	1.60 ± 0.10	2.06 ± 0.06	2.04 ± 0.13
	(47.00 %)	–	(0.63 %)	(0.00 %)	(17.28 %)	(6.32 %)	(12.93 %)	(9.83 %)
Respiratory pathway overexpression strains								
Sbm-glpK	30.08 ± 1.45	1.98	1.09 ± 0.08	6.38 ± 0.28	6.22 ± 0.18	0.87 ± 0.34	2.87 ± 0.49	0.56 ± 0.06
	(82.52 %)	–	(2.83 %)	(21.72 %)	(32.33 %)	(3.62 %)	(19.19 %)	(2.83 %)
Sbm-glpD	29.12 ± 0.16	4.58	0.51 ± 0.05	ND	7.10 ± 0.47	4.45 ± 0.28	1.49 ± 0.01	1.44 ± 0.62
	(76.46 %)	–	(1.35 %)	(0.00 %)	(38.01 %)	(19.26 %)	(10.24 %)	(7.60 %)
Sbm-glpABC	30.79 ± 1.01	8.01	0.26 ± 0.06	ND	8.41 ± 0.26	5.67 ± 0.15	0.29 ± 0.11	0.51 ± 0.16
	(70.90 %)	–	(0.66 %)	(0.00 %)	(42.59 %)	(23.21 %)	(1.90 %)	(2.54 %)
Fermentative pathway overexpression strains								
Sbm-gldA	30.87 ± 0.59	4.72	4.07 ± 0.08	ND	9.24 ± 1.02	ND	2.35 ± 0.11	ND
	(72.15 %)	–	(10.30 %)	(0.00 %)	(46.65 %)	(0.00 %)	(15.19 %)	(0.00 %)
Sbm-dhaKLM	30.25 ± 0.08	4.93	0.63 ± 0.13	ND	4.61 ± 0.25	1.61 ± 0.11	4.80 ± 0.21	2.03 ± 0.04
	(74.12 %)	–	(1.63 %)	(0.00 %)	(23.75 %)	(6.71 %)	(31.71 %)	(10.33 %)
Sbm-ptsI	26.09 ± 0.73	3.52	0.12 ± 0.02	ND	2.59 ± 0.33	0.40 ± 0.07	6.29 ± 0.24	1.73 ± 0.01
	(76.14 %)	–	(0.35 %)	(0.00 %)	(15.44 %)	(1.93 %)	(48.22 %)	(10.21 %)

The metabolite distribution (i.e., the fraction of dissimilated glycerol to form a metabolite) is calculated as in Table 2. Error bars represent s.d. ($n = 2$)

ND not detected

[a] Glycerol consumption (g/L); glycerol efficiency is presented in parentheses under the carbon source consumption value (%)

[b] Biomass accumulation (g-DCW/L)

[c] Metabolite concentrations (g/L); the fraction of dissimilated glycerol is presented in parentheses under each titer

With the introduction of these knockouts, redox constraints are relaxed as carbon flow is uncoupled from the necessity to maintain redox balance through synthesis of highly reduced metabolites (i.e., alcohols). Therefore, the oxidized pathways are activated, allowing for enhanced synthesis of organic acids [11]. The slower glycerol dissimilation rate of these mutants in comparison to that of SbmCTRL and their suppressed solventogenesis might imply a reduced NADH/NAD^+ ratio and carbon flux overflow resulting from these gene knockouts. On the other hand, the similar overall cultivation performance for SbmCTRL and Sbm-ΔglpA (in terms of glycerol dissimilation rate, biomass yield, and metabolite production) suggests that under microaerobic cultivation conditions, the biological role of GlpABC is slight. The genes encoding the anaerobic and aerobic glycerol-3-phosphate dehydrogenase (*glpABC and glpD*, respectively) are not on the same operon in the *E. coli* chromosome and, as such, are under different regulation [23]. Specifically, *glpABC* expression is induced by the anaerobic regulator FNR which is deactivated in the presence of oxygen and, as a result, the anaerobic respiratory pathway plays a minor role in glycerol dissimilation under microaerobic conditions.

Knocking out genes involved in glycerol dissimilation (except *glpA*) can potentially shift more dissimilated carbon flux toward the C3-fermentative pathway, resulting in an increased propionate:acetate ratio (i.e., 0.62 for Sbm-ΔglpK, 0.53 for Sbm-ΔglpD, 1.84 for Sbm-ΔgldA, 2.16 for Sbm-ΔdhaK, 1.53 for Sbm-ΔptsI) compared to the ratio of 0.37 for SbmCTRL (Figs. 3b, 4b). Furthermore, genes involved in the fermentative pathway of glycerol dissimilation appear to be the best targets for knockout not only due to minimized solventogenesis but also propionate was preferentially produced over acetate under high-level acidogenesis conditions. The results suggest not only the effectiveness of this gene manipulation strategy but also the importance of the respiratory pathway of glycerol dissimilation for enhancing propionate production under microaerobic conditions. The observed high propionate titers upon inactivation of the fermentative pathway can be associated with the release of the pathways' dependence on PEP as a phosphate donor for DHAP synthesis, increasing the PEP pool for its subsequent conversion to oxaloacetate (OAA) and then propionate (Fig. 1). Zhang et al. [44] previously reported that the *ptsI* knockout enhanced succinate production during anaerobic fermentation of

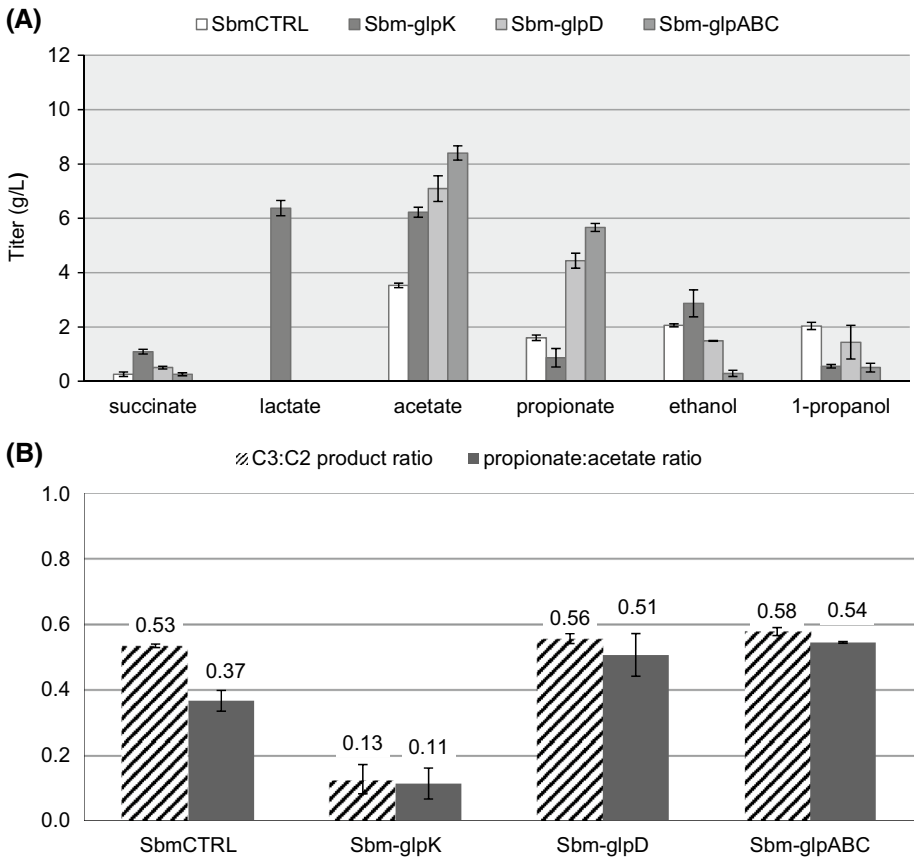

Fig. 5 **a** Major metabolites titers during microaerobic batch cultivation of respiratory pathway overexpression strains (Sbm-glpK, Sbm-glpD and Sbm-glpABC) using glycerol as a carbon source and **b** ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation; ratios are calculated from the fractions of dissimilated glycerol. *Error bars* represent s.d. ($n = 2$)

glycerol, crediting the knockout effect on conserving PEP. Conversely, when *gldA* and *dhaK* were episomally overexpressed, a decrease in succinate production was observed, presumably due to a reduced PEP pool [26].

Alternatively, the effects of overexpression of genes involved in glycerol dissimilation on propionate production were also explored. Generally, overexpression of genes involved in respiratory pathway of glycerol dissimilation (i.e., *glpD* and *glpABC*), but not fermentative pathway, enhanced propionate production. The results are consistent with the above knockout results suggesting that the dissimilated carbon flux should be channeled through the respiratory pathway for effective propionate production under microaerobic conditions. Interestingly, overexpression of *glpK* impaired cell growth and propionate production with an unusual lactate accumulation even though the *ldhA* gene of the propionogenic strain was inactivated. The redirection of carbon flux toward lactate can potentially result from activation the methylglyoxal pathway, a bypass of glycolysis at the DHAP node (Fig. 1). Generally associated with a loss of regulation of carbon uptake resulting in an increased DHAP pool, the physiological role of this pathway is to replenish intracellular inorganic phosphate when its concentration is low, as conversion of DHAP to methylglyoxal releases an inorganic phosphate [16]. However, methylglyoxal is toxic and, therefore, its synthesis must be balanced by detoxification through its subsequent enzymatic conversion to lactate [37]. In Sbm-glpK, this pathway was potentially activated as an effective means for ATP generation to sustain the heightened ATP-dependent phosphorylation of glycerol to G3P.

Similar to the previous observation [26], overexpression of genes involved in the fermentative pathway of glycerol dissimilation may cause a decrease in the PEP pool, since phosphorylation of DHA to DHAP is dependent on PEP as the phosphate donor, resulting in less flux into the TCA cycle and Sbm pathway for propionate production. In particular, overexpression of glycerol dehydrogenase (*gldA*) caused a severe hindrance of the Sbm pathway with a high succinate accumulation and no production of propionate and 1-propanol.

Conclusions

In response to the recent focus on sustainable production processes, there has been significant interest directed toward the development of effective biological production systems. We herein demonstrated a biological process of using engineered *E. coli* for high-level propionate production. With the

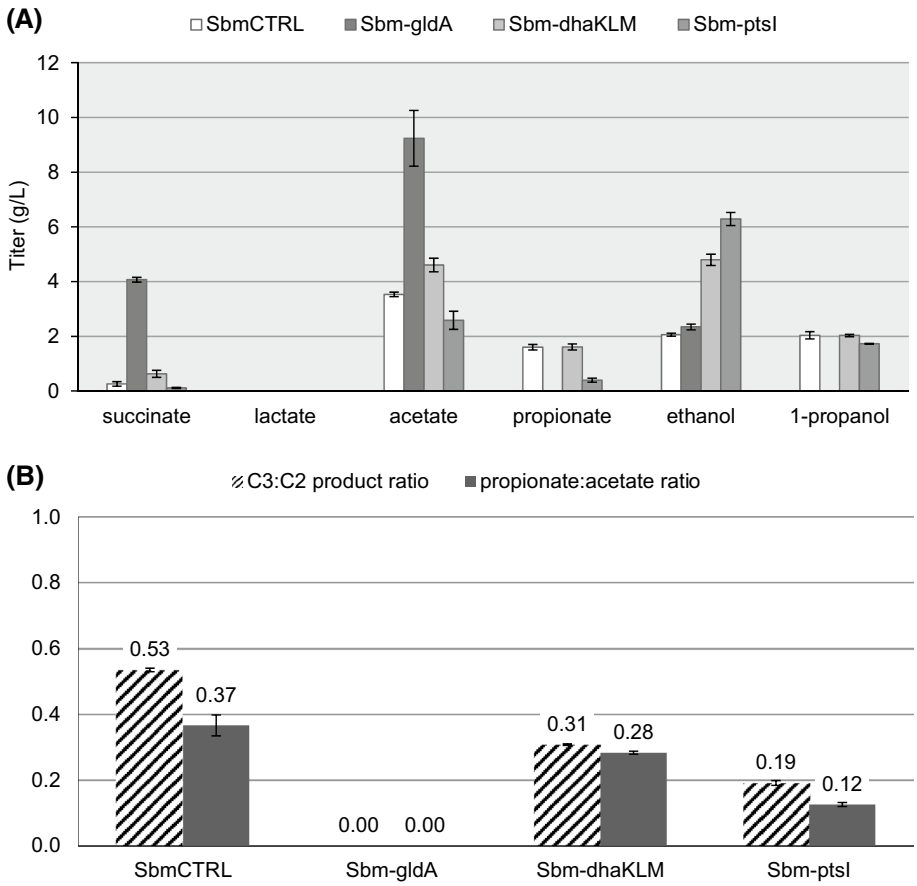

Fig. 6 **a** Major metabolites titers during microaerobic batch cultivation of fermentative pathway overexpression strains (Sbm-gldA, Sbm-dhaKLM and Sbm-ptsI) using glycerol as a carbon source and **b** ratios of C3:C2 fermentative products, overall acid:solvent production as well as propionate:acetate production for each cultivation; ratios are calculated from the fractions of dissimilated glycerol. *Error bars* represent s.d. ($n = 2$)

implementation of various biochemical and genetic strategies, high propionate titers of more than 11 g/L with high yields up to 0.4 g-propionate/g-glycerol were achieved. To our knowledge, this represents the most effective non-native engineered microbial system for propionate production with titers and yields comparable to those achieved by anaerobic batch cultivation of native producers. The use of glycerol as a carbon source is not only economically and environmentally attractive but also effective for microaerobic *E. coli* cultivation for propionate production. For optimal propionate production with high C3:C2 fermentative product ratio, glycerol dissimilation should be channeled through the respiratory pathway and the biochemical grounds associated with this preferential carbon flux channeling warrant in-depth exploration. Knocking out genes involved in glycerol dissimilation (except *glpA*) can minimize solventogenesis and shift more dissimilated carbon flux toward the C3-fermentative pathway. Upon suppressed solventogenesis with minimal production of highly reduced alcohols, the alternative NADH-consuming route associated with propionate synthesis can be critical for more flexible redox balancing. Further strategies for strain engineering should be developed toward reducing acetate production, though knocking out genes involved in the fermentative pathway has been shown effective in increasing the propionate:acetate ratio. The derivation of various propionogenic *E. coli* strains in this study has also offered a unique opportunity for investigating "flux competition" behaviors between the C2- and C3-fermentative pathways under different genetic backgrounds and cultivation conditions. Lastly, the enhanced propionate production reported herein suggests the existence of high-level intracellular non-native propionyl-CoA, which serves as a key precursor for novel biosynthesis of several value-added chemicals and we are currently exploring these topics.

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