Strain engineering and bioprocessing strategies for biobased production of porphobilinogen in *Escherichia coli*

Davinder Lall, Dragan Miscevic, Mark Bruder, Adam Westbrook, Marc Aucoin, Murray Moo-Young and C. Perry Chou*

**Abstract**

Strain engineering and bioprocessing strategies were applied for biobased production of porphobilinogen (PBG) using *Escherichia coli* as the cell factory. The non-native Shemin/C4 pathway was first implemented by heterologous expression of *hemA* from *Rhodopseudomonas spheroids* to supply carbon flux from the natural tricarboxylic acid (TCA) pathways for PBG biosynthesis via succinyl-CoA. Metabolic strategies were then applied for carbon flux direction from the TCA pathways to the C4 pathway. To promote PBG stability and accumulation, Clustered Regularly Interspersed Short Palindromic Repeats interference (CRISPRi) was applied to repress *hemC* expression and, therefore, reduce carbon flowthrough toward porphyrin biosynthesis with minimal impact to cell physiology. To further enhance PBG biosynthesis and accumulation under the *hemC*-repressed genetic background, we further heterologously expressed native *E. coli hemB*. Using these engineered *E. coli* strains for bioreactor cultivation based on ~ 30 g L\(^{-1}\) glycerol, we achieved high PBG titers up to 209 mg L\(^{-1}\), representing 1.73% of the theoretical PBG yield, with improved PBG stability and accumulation. Potential biochemical, genetic, and metabolic factors limiting PBG production were systematically identified for characterization.

**Keywords:** *Escherichia coli*, Glycerol, Glyoxylate shunt, Porphobilinogen (PBG), Strain engineering, Succinyl-CoA, Tricarboxylic acid (TCA) cycle

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Introduction

Porphobilinogen (PBG) is a pyrrole-containing intermediate in the metabolic pathways for biosynthesis of essential porphyrin/tetrapyrrole compounds known as "pigments of life", including heme, cobalamin, chlorophyll, siroheme, heme d1, etc., in almost all types of biological cells (Frankenberg et al. 2003). For application purposes, PBG can act as a marker for diagnosis of diseases, such as acute intermittent porphyria (Anderson 2019) and lead (Pb) poisoning (Gibson et al. 1968). Naturally in biological systems, the precursor of PBG, i.e., 5-aminolevulinic acid (5-ALA), is synthesized via either of the two unrelated metabolic routes, i.e., the Beale/C5 pathway and the Shemin/C4 pathway (Zhang et al. 2015). Found in most bacteria (including Escherichia coli) and all archaea and plants, the C5 pathway starts with the C5-skeleton of glutamate for conducting two enzymic reactions, i.e., initial reduction of glutamyl-tRNA to glutamate-1-semialdehyde (GSA) via NADPH-dependent glutamyl-tRNA reductase (GluTR) and subsequent transamination of GSA via glutamate-1-semialdehyde-2,1-aminomutase (GSAM), to form 5-ALA (Jahn et al. 1992). On other hand, the C4 pathway, present in humans, animals, fungi and the α-group of proteobacteria, involves ALA synthase (ALAS or HemA, encoded by hemA) for molecular condensation of succinyl-CoA and glycine to form 5-ALA with the release of carbon dioxide and coenzyme A (CoA) (Nandi 1978). Subsequently, PBG is synthesized via a common reaction for molecular condensation of two 5-ALA molecules catalyzed by ALA dehydratase (ALAD or HemB, encoded by hemB) (Layer et al. 2010).

Even with relatively limited applicability up to date, technologies for PBG production have been explored. Chemical synthesis of PBG has been carried out using a variety of precursor molecules, such as diethyl 4-oxopimelate (Jones et al. 1976), 2-methoxy-4-methyl-5-nitropyridine (Frydman et al. 1965), and 2-Hydroxy-4-methyl-5-nitropyridine (Frydman et al. 1969), as well as reaction processes, such as modified synthesis via a porphobilinogen lactam (Kenner et al. 1977), MacDonald’s method (Jackson and MacDonald 1957), and ozonide cleavage reaction (Jacobi and Li 2001). However, these chemical approaches are expensive,
time-consuming, complex, and requiring harsh reaction conditions with typically low yields (Neier 2000). While purification of PBG from the urine of patients with acute porphyria is feasible, the producing capacity is knowingly limited (Westall 1952). While biosynthesis of PBG has been alternatively explored in different microbial cell factories, such as *Rhodopseudomonas spheroides* (Hatch and Lascelles 1972b), *E. coli* (Lee et al. 2013), *Chromatium vinosum* (Vogelmann et al. 1975), *Propionibacterium freudenreichii*, etc. (Piao et al. 2004), enhancing such biobased production is considered technically challenging since PBG, as a metabolic intermediate, hardly accumulates.

While various cell factories have been developed for biobased production (Chen et al. 2013), bacterium *E. coli* remains the most common one. In native *E. coli*, PBG is synthesized via the C5 pathway and barely accumulates extracellularly since the produced PBG will be readily tetramerized into hydroxymethylbilane (HMB) via porphobilinogen deaminase (PBGD or HemC, encoded by *hemC*) for subsequent biosynthesis of essential porphyrins, such as heme. In this study, we chose to first implement the non-native C4 pathway into *E. coli* for PBG biosynthesis and promote PBG extracellular accumulation, from the structurally unrelated carbon of glycerol by heterologous expression of *hemA* from *R. spheroids* (Fig. 1). Recently, glycerol has been recognized as a...
promising carbon source for biobased production due to its low cost (Cirimmina et al. 2014), abundance, and high degree of reduction (Westbrook et al. 2019), resulting in high product yield compared to traditional sugars (Dhar et al. 2006). We also developed effective metabolic strategies for carbon flux direction via succinyl-CoA, a key precursor of the C4 pathway. The direction of dissimilated carbon toward succinyl-CoA is dependent on three oxygen-sensitive metabolic routes associated with the central metabolism, i.e., oxidative tricarboxylic acid (TCA) cycle, reductive TCA branch, and glyoxylate shunt (Fig. 1) (Cheng et al. 2013). Under oxygen-deprived (i.e., anaerobic) conditions, succinate (the precursor of succinyl-CoA) acts as an electron acceptor in place of oxygen and accumulates as a final product of mixed acid fermentation via the reductive TCA branch (Thakker et al. 2012). Under oxygen-rich (i.e., aerobic) conditions, succinate acts as a metabolic intermediate of the oxidative TCA cycle without accumulation, but it can also be alternatively derived via the glyoxylate shunt (Thakker et al. 2012). Here, we explored the manipulation of select genes involved in the TCA pathways and cultivation conditions to enhance carbon flux direction into the C4 pathway via succinyl-CoA.

To promote PBG accumulation, we had to limit the activity of subsequent PBG-consuming reactions toward porphyrins. Since porphyrin biosynthesis is essential for cell survival, knocking out any of these PBG-consuming reactions would be lethal (Mobius et al. 2010) (Leung et al. 2021). Hence, we applied Clustered Regularly Interspersed Short Palindromic Repeats interference (CRISPRi) (Qi et al. 2013) to repress the expression of hemC, whose encoding gene product of HemC mediates the conversion of PBG to HMB, with minimal impact to cell physiology. To further enhance PBG biosynthesis and accumulation under the hemC-repressed genetic background, we also conducted heterologous co-expression of hemA from R. spheroides and the native hemB. In summary, we demonstrated the application of integrated strain engineering and bioprocessing strategies to enhance biosynthesis and ultimate extracellular accumulation of PBG, with systematic identification of potential biochemical, genetic, and metabolic factors limiting PBG production for characterization.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 1. Isolation of Genomic DNA from bacterial cells was performed using the Blood & Tissue DNA Isolation Kit (Qiagen, Hilden, Germany). Standard recombinant DNA technologies were applied for molecular cloning (Miller 1992). Phusion and Taq DNA polymerase were obtained from New England Biolabs (Ipswich, MA, USA). All synthesized oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA, USA). DNA sequencing was performed by the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada). E. coli BW25113 was the parental strain for derivation of all engineered strains in this study and DH5a was used as an E. coli host for molecular cloning. The ldhA gene encoding lactate dehydrogenase (LDH) was previously inactivated in BW25113, generating BWΔldhA (Srirangan et al. 2014), a strain with much lower byproduct metabolite production.

Genetic implementation of the Shemin/C4 pathway in BWΔldhA was previously described (Miscevic et al. 2021). Heterologous expression of the hemA gene cloned in the pK184 vector was under the control of the Plac promoter. For heterologous co-expression of hemA and hemB in BWΔldhA, the native E. coli hemB gene was first amplified by polymerase chain reaction (PCR) using the primer set g-hemA-hemB and the genomic DNA of BWΔldhA as the template. The amplified hemB gene was Gibson—assembled with PCR-linearized pK184-hemA using the primer set g-pK-hemA-hemB to generate the plasmid pK184-hemA-hemB. Heterologous co-expression of the hemA and hemB genes cloned in the pK184 vector was also under the control of the Plac promoter.

Gene knockouts, including sdhA (encoding succinate dehydrogenase (SDH) complex flavoprotein subunit A, SdhA) and iclR (encoding transcriptional AceBAK operon repressor, IclR), were introduced into BWΔldhA by P1 phage transduction (Miller 1992) using the appropriate Keio Collection strains (The Coli Genetic Stock Center, Yale University, New Haven, CT, USA) as donors (Baba et al. 2006). For eliminating the co-transduced FRT-KnF-FRT cassette, the transductants were transformed with pCP20 (Cherepanov and Wackernagel 1995), a temperature-sensitive plasmid expressing a flippase (Flp) recombinase. After Flp-mediated excision of the KnF cassette, a single Flp recognition site (FRT “scar site”) was generated. The pCP20-containing cells were cured by incubation at 42 °C. The genotypes of derived knockout strains were confirmed by colony PCR using the appropriate verification primer sets (Additional file 1: Table S1).

Expression of the hemC was repressed by CRISPRi using various derived plasmids from pdcas9-bacteria (Addgene plasmid #44249) and pgRNA-bacteria (Addgene plasmid #44251). The web tool ChopChop (Labun et al. 2016) was used to design sgRNAs with hemC-targeting sequences based on predicted expression efficiencies ranging from approximately 20 to 70% (Additional file 1: Table S2). All synthesized
Table 1  E. coli strains and plasmids used in this study

| Name | Description or relevant genotype | Source |
|------|-----------------------------------|--------|
| E. coli host strains | | |
| DHSa | F−, endA1, glmV44, thi−1, recA1, relA1, gyrA96, deoR, nupG80d lacZΔlacZD1ΔlacZYA−argF] U169, hsdR17(kM::Tn10)lac−proAB | Lab stock |
| BW25113 | F−, Δ[araD-araI]567, ΔlacZ4787(rrnB-3), λ−, rph−1, Δ[rhaD-rhaB]568, hsdR514 | (Datsenko and Wanner 2000) |
| BWΔΔahA | BW25113 ldhA null mutant | (Sirignan et al. 2014) |
| DMH | BWΔΔahA | (Mischevic et al. 2021) |
| DMHΔsdhA | sdhA null mutant of DMH | (Mischevic et al. 2021) |
| DMHΔicLR | iclR null mutant of DMH | (Mischevic et al. 2021) |
| DMHΔicLRΔsdhA | iclR and sdhA mutants of DMH | (Mischevic et al. 2021) |
| DMH-D9ΔsdhA | DMHΔsdhA/pK-hemA/pgRNA-D9/pdcas9-bacteria | This study |
| DMH-D9ΔicLRΔsdhA | DMHΔicLRΔsdhA/pK-hemA/pgRNA-D9/pdcas9-bacteria | This study |
| DSL | BWΔΔahA/pK-hemA-hemB | This study |
| DSLΔsdhA | sdhA null mutant of DSL | This study |
| DSLΔicLR | iclR null mutant of DSL | This study |
| DSLΔicLRΔsdhA | iclR and sdhA mutants of DSL | This study |
| DSL-D9ΔsdhA | DSLΔsdhA/pK-hemA-hemB/pgRNA-D9/pdcas9-bacteria | This study |
| DSL-D1ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D1/pdcas9-bacteria | This study |
| DSL-D2ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D2/pdcas9-bacteria | This study |
| DSL-D3ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D3/pdcas9-bacteria | This study |
| DSL-D4ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D4/pdcas9-bacteria | This study |
| DSL-D5ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D5/pdcas9-bacteria | This study |
| DSL-D6ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D6/pdcas9-bacteria | This study |
| DSL-D7ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D7/pdcas9-bacteria | This study |
| DSL-D8ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D8/pdcas9-bacteria | This study |
| DSL-D9ΔicLRΔsdhA | DSLΔicLRΔsdhA/pK-hemA-hemB/pgRNA-D9/pdcas9-bacteria | This study |
| Plasmids | | |
| pCP20 | Flp+, λ cI857+, λ pR Rep(pSC101 ori, ApR, CmR | (Cherepanov and Wackernagel 1995) |
| pK184 | p15A ori, KmR, Plac: lacZ | (Jobling and Holmes 1990) |
| pdcas9-bacteria | p15A ori, Plac:Cas9 | (Qi et al. 2013) |
| pgRNA-bacteria | ColE1 origin, P23115: gRNA | (Qi et al. 2013) |
| pgRNA-D1 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D1 | This study |
| pgRNA-D2 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D2 | This study |
| pgRNA-D3 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D3 | This study |
| pgRNA-D4 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D4 | This study |
| pgRNA-D5 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D5 | This study |
| pgRNA-D6 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D6 | This study |
| pgRNA-D7 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D7 | This study |
| pgRNA-D8 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D8 | This study |
| pgRNA-D9 | Derived from pgRNA-bacteria, Pspec: hemC-gRNA-D9 | This study |
| pK-hemA | Derived from pK184, Pspec: hemA-hemB | (Mischevic et al. 2021) |
| pK-hemA-hemB | Derived from pK184, Pspec: hemA-hemB | This study |

Oligonucleotide pairs have 60 nucleotides (nt), which include 20 nt hemC-targeting sequence, 20 nt upstream and 20 nt downstream sequences of pgRNA-bacteria vector (Fig. 2). They were annealed as described previously (Pengpumkiat et al. 2016), generating double-stranded DNA fragments. These DNA fragments were then individually Gibson-assembled with the PCR-linearized pgRNA-bacteria using the primer set g-pgRNA to generate plasmids, such as pgRNA-D9 (Table 1). The hemC-repressed strains can be developed based on a triple-plasmid system (Fig. 2) containing pK184-hemA (or pK184-hemA-hemB), pdcas9-bacteria, and the gRNA-containing plasmid (such as pgRNA-D9).

**Media and bacterial cell cultivation**

All medium components were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) except yeast extract and tryptone which were obtained from BD Diagnostic Systems (Franklin Lakes, NJ, USA). E. coli strains, stored as glycerol stocks at − 80 °C, were streaked on lysogeny broth (LB; 10 g L−1 tryptone, 5 g L−1 yeast extract, and 5 g L−1 NaCl) agar plates with appropriate antibiotics.
ampicillin (100 mg L⁻¹), kanamycin (50 mg L⁻¹), and chloramphenicol (25 mg L⁻¹) and incubated at 37 °C for 14–16 h.

For shake-flask cultivations, single colonies were picked from LB plates to inoculate 30 mL of LB medium in 125-mL conical flasks. The cultures were shaken at 37 °C and 280 rpm in a rotary shaker (New Brunswick Scientific, NJ, USA) and used as seed cultures to inoculate 220 mL of LB media at 1% (v/v) in 1-L conical flasks with appropriate antibiotics. This second seed culture was shaken at 37 °C and 280 rpm until the cell density reached 0.80 OD₆₀₀. Cells were then harvested by centrifugation at 9,000 x g and 20 °C for 10 min and resuspended in 30 mL of modified M9 production medium.

For bioreactor cultivation, single colonies were picked from LB plates to inoculate 30 mL of SB medium (32 g L⁻¹ tryptone, 20 g L⁻¹ yeast extract, and 5 g L⁻¹ NaCl) in 125 mL conical flasks. The overnight cultures were shaken at 37 °C and 280 rpm in a rotary shaker (New Brunswick Scientific, NJ, USA) and used as seed cultures to inoculate 220 mL of SB media at 1% (v/v) in 1-L conical flasks with appropriate antibiotics. This second seed culture was shaken at 37 °C and 280 rpm for 14–16 h. Cells were then harvested by centrifugation at 9,000 x g and 20 °C for 10 min and resuspended in 50 mL fresh LB media. The suspended culture was used to inoculate a 1-L stirred tank bioreactor (containing two Rushton radial flow disks as impellers) (CelliGen 115, Eppendorf AG, Hamburg, Germany) at 37 °C and 430 rpm. The semi-defined production medium in the batch bioreactor contained 30 g L⁻¹ glycerol, 5 g L⁻¹ yeast extract, 10 mM NaHCO₃, 1 mM MgCl₂, 200 mL L⁻¹ of M9 salts mix (33.9 g L⁻¹ Na₂HPO₄, 15 g L⁻¹ KH₂PO₄, 5 g L⁻¹ NH₄Cl, 2.5 g L⁻¹ NaCl), 1 mL L⁻¹ dilution of Trace Metal Mix A5 (2.86 g L⁻¹ H₂BO₃, 1.81 g L⁻¹ MnCl₂•4H₂O, 0.222 g L⁻¹ ZnSO₄•7H₂O, 0.39 g L⁻¹ Na₂MoO₄•2H₂O, 79 µg L⁻¹ CuSO₄•5H₂O, 49.4 µg L⁻¹ Co(NO₃)₂•6H₂O), and was supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

For bioreactor cultivation, single colonies were picked from LB plates to inoculate 30 mL of super broth (SB) medium (32 g L⁻¹ tryptone, 20 g L⁻¹ yeast extract, and 5 g L⁻¹ NaCl) in 125 mL conical flasks. The overnight cultures were shaken at 37 °C and 280 rpm in a rotary shaker (New Brunswick Scientific, NJ, USA) and used as seed cultures to inoculate 220 mL of SB media at 1% (v/v) in 1-L conical flasks with appropriate antibiotics. This second seed culture was shaken at 37 °C and 280 rpm for 14–16 h. Cells were then harvested by centrifugation at 9,000 x g and 20 °C for 10 min and resuspended in 50 mL fresh LB media. The suspended culture was used to inoculate a 1-L stirred tank bioreactor (containing two Rushton radial flow disks as impellers) (CelliGen 115, Eppendorf AG, Hamburg, Germany) at 37 °C and 430 rpm. The semi-defined production medium in the batch bioreactor contained 30 g L⁻¹ glycerol, 0.23 g L⁻¹ K₂HPO₄, 0.51 g L⁻¹ NH₄Cl, 49.8 mg L⁻¹ MgCl₂, 48.1 mg L⁻¹ K₂SO₄, 1.52 mg L⁻¹ FeSO₄, 0.055 mg L⁻¹ CaCl₂, 2.93 g L⁻¹...
NaCl, 0.72 g L$^{-1}$ tricine, 10 g L$^{-1}$ yeast extract, 10 mM NaHCO$_3$, and 1 mL L$^{-1}$ trace elements (2.86 g L$^{-1}$ H$_2$BO$_3$, 1.81 g L$^{-1}$ MnCl$_2$, 4H$_2$O, 0.222 g L$^{-1}$ ZnSO$_4$•7H$_2$O, 0.39 g L$^{-1}$ Na$_2$MoO$_4$•2H$_2$O, 79 µg L$^{-1}$ CuSO$_4$•5H$_2$O, 49.4 µg L$^{-1}$ Co(NO$_3$)$_2$•6H$_2$O) (Neidhardt et al. 1974), and was supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Aerobic and microaerobic conditions were maintained by purging the headspace of the bioreactor with air into the bulk culture at 1 vvm and into the headspace at 0.2 vvm. The quantification of extracellular metabolites and glycerol was conducted using a high-performance liquid chromatography (HPLC) (LC-10AT, Shimadzu, Kyoto, Japan) with a refractive index detector (RID; RID-10A, Shimadzu, Kyoto, Japan) and a chromatographic column (Aminex HPX-87H, Bio-Rad Laboratories, CA, USA). The HPLC column temperature was maintained at 35 °C and the mobile phase was 5 mM H$_2$SO$_4$ (pH 2) running at 0.6 mL min$^{-1}$. The RID signal was acquired and processed by a data processing unit (Clarity Lite, DataApex, Prague, Czech Republic).

PBG titer in the cell-free medium was measured using a regular Ehrlich’s reagent and PBG was colorimetrically quantified by taking an absorbance reading at 555 nm (Mauzerall and Granick 1956). The percentage yield of PBG was defined as the mole ratio of the produced PBG to the theoretically maximal PBG produced based on the consumed glycerol with a molar ratio of one-to-six (i.e., one-mole PBG is derived from six-mole glycerol). Note that one-mole succinyl-CoA (derived from two-mole glycerol) and one-mole glycine (derived from one-mole glycerol) generate one-mole 5-ALA, whereas two-mole 5-ALA forms one-mole PBG. The bulk level of porphyrin compounds in the cell-free medium was estimated using a spectrophotometer at two specific wavelengths, i.e., 405 nm (measuring Soret band) and 495 nm (measuring Q-band). Note that all bioreactor cultivation results shown in this study were, respectively, obtained from a single batch run, with most of cultivation batches being duplicated or even triplicated to ensure their data reproducibility.

Real-time quantitative reverse transcription PCR (qRT-PCR)
For RNA extraction, E. coli cells were cultivated in 30 mL liquid LB medium at 37 °C and harvested in the exponential growth phase. Total RNA isolation was done using the High Pure RNA Isolation Kit (Roche Diagnostics, Basel, Switzerland) as per manufacturer’s instructions and stored at −80 °C for later analysis. Complementary DNAs (cDNAs) were synthesized from 100 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, MA). Sequence-specific primers for hemC cDNA (i.e., q-hemC) and internal control rrsA (encoding ribosomal RNA 16S) cDNA (i.e., q-rrsA) were used for real-time PCR amplification in 25 µL reaction mixture. qRT-PCR was carried out using the Power SYBR® Green PCR Master Mix (ThermoFisher Scientific; MA) in an Applied Biosystems StepOnePlus™ System as per the manufacturer’s instructions. All quantification experiments were performed in duplicate.

Statistical analysis
All experimental data in this study were collected in duplicate for statistical analysis. In addition, data comparison was statistically analyzed with an unpaired two-tail Student’s t-test based on 95% confidence level to ensure its statistical significance (Additional file 1: Table S4). Hence, P<0.05 was used as a standard criterion of statistical significance when comparing the means of experimental data, such as PBG titer.

Results
Carbon flux direction from the TCA pathways to the Shemin/C4 pathway
The Shemin/C4 pathway was implemented in E. coli via heterologous expression of hemA from R. sphaeroides in BWΔIdhA (Miskevic et al. 2021). The resulting control strain, DMH, was cultivated under aerobic conditions in a batch bioreactor with ~30 g L$^{-1}$ of glycerol as the carbon source. The supply of excess oxygen supported cell growth with effective glycerol consumption, resulting in 120 mg L$^{-1}$ of the peak PBG titer (1.31% yield) with substantial acetate formation (69.3% yield) (Fig. 3). Extending the cultivation, based on the remaining glycerol, and produced acetate, resulted in reduction of PBG titer to 75.1 mg L$^{-1}$ (0.65% yield) with increased porphyrin formation. While the formation of other byproduct metabolites, such as ethanol, succinate, and formate, was minimal, the results suggest the need for metabolic strategies to reduce carbon flux drainage toward acetogenesis and porphyrin biosynthesis for enhanced PBG accumulation. Note that bioreactor characterization was used in this study since...
Shake-flask cultivation resulted in minimal PBG biosynthesis and accumulation (data not shown). PBG biosynthesis via the C4 pathway utilizes succinyl-CoA as a key precursor (with the other being glycine) to produce 5-ALA as an intermediate before subsequent conversion to PBG. The intracellular succinyl-CoA supply is affected by three oxygen-sensitive metabolic routes associated with the central metabolism, i.e., oxidative TCA cycle, reductive TCA branch, and glyoxylate shunt (Fig. 1). Due to more effective cell growth and glycerol consumption, we first characterized our engineered strains under aerobic conditions. To direct more carbon flux toward the succinyl-CoA node, we inactivate the oxidative TCA cycle by knocking out the \( sdhA \) gene, resulting in the mutant strain DMH\( \Delta sdhA \), with an improved peak PBG titer of 154 mg L\(^{-1} \) (1.41% yield) and 115 mg L\(^{-1} \) (1.01% yield) at the end of bioreactor cultivation (Fig. 3). On the other hand, we also deregulated glyoxylate shunt by knocking out the \( iclR \) gene, resulting in the mutant strain DMH\( \Delta iclR \) in which more carbon flux could be directed toward the succinyl-CoA node via glyoxylate shunt with reduced decarboxylation through bypassing the oxidative TCA cycle. Aerobic bioreactor cultivation of DMH\( \Delta iclR \) also showed improved peak PBG titer of 130 mg L\(^{-1} \) (1.13% yield) and 80.7 mg L\(^{-1} \) (0.69% yield) at the end of the cultivation (Fig. 3). Both single-mutant strains of DMH\( \Delta iclR \) and DMH\( \Delta sdhA \) displayed effective cell growth and glycerol consumption, with reduced acetate production (32.1% and 38.0% yield, respectively) compared to control strain DMH.

Next, we derived the double-mutant strain DMH\( \Delta iclR \Delta sdhA \) such that the carbon flux from the deregulated glyoxylate shunt could be further directed toward the succinyl-CoA node via the reductive TCA branch for enhanced biosynthesis of PBG and porphyrins while minimizing decarboxylation. Aerobic bioreactor cultivation of DMH\( \Delta iclR \Delta sdhA \) produced 87.3 mg L\(^{-1} \) (0.66% yield) at the end of cultivation (Fig. 4). Moreover, we observed significantly reduced acetate formation with 35.9% yield, compared to the control strain DMH. These results indicate successful carbon flux direction from the TCA pathways to the C4 pathway in DMH\( \Delta iclR \Delta sdhA \). However, the directed carbon flux appeared to proceed toward porphyrin formation rather than PBG accumulation in these engineered strains, as indicated by subsequent reduction in PBG titer after reaching a peak value. While blocking the conversion of PBG to HMB by knocking out \( hemC \) appears to be a feasible way to promote PBG accumulation, such gene knockout is lethal due to physiological requirement of essential porphyrins.
Repression of hemC expression for PBG biosynthesis and accumulation

Since hemC is essential for heme biosynthesis, gene knockdown to repress hemC expression was explored to promote PBG accumulation with minimal impact on cell physiology and PBG biosynthesis. Hence, CRISPRi was applied using hemC-targeting gRNAs with distinct expression efficiencies (predicted by CHOPCHOP). Upon first screening of a selection of gRNAs targeting different areas of hemC (Fig. 2; Additional file 1: Table S2) based on bioreactor cultivation, hemC-gRNA-D9 appeared to show effective hemC repression with enhanced PBG biosynthesis and accumulation. The hemC-repression effect was further verified by qRT-PCR (Additional file 1: Figs. S1 and S2). Hence, the resulting hemC-repressed strains based on the use of hemC-gRNA-D9 were selected for complete bioreactor characterization. Under aerobic bioreactor conditions, cell growth and glycerol utilization for DMH-D9ΔiclRΔsdhA were minimally affected compared to the control strain DMHΔiclRΔsdhA, suggesting that the need of essential porphyrins for cell survival was properly met in the presence of hemC repression. Importantly, we observed more effective biosynthesis and accumulation of PBG, achieving a peak/final titer of 140 mg L\(^{-1}\) (1.22% yield) at the end of the cultivation (Fig. 4). Note that the Soret peak and Q-band absorbance values of the cell-free medium for the culture sample of DMH-D9ΔiclRΔsdhA was reduced to some extent, suggesting successful hemC repression with reduced porphyrin formation.

Increasing hemB expression to enhance PBG biosynthesis and accumulation

To further enhance PBG biosynthesis and accumulation, we cloned the native hemB gene from E. coli for heterologous expression along with hemA from R. sphaeroides, resulting in another control strain DSL. While aerobic bioreactor cultivation of DSL led to a much higher peak PBG titer compared to DMH, the PBG titer reduced rapidly upon extended cultivation to 65.7 mg L\(^{-1}\) (0.52%
yield) (Fig. 5), a level similar to DMH. Porphyrin bio-
synthesis in DSL appeared to be higher than DMH, as
evidenced by higher Soret peak and Q-band absorbance
values of the cell-free medium for the culture sample.
Also note that cell growth and glycerol consumption
remained effective for DSL compared to DMH.

Similar to DMH, the metabolic limitations associated
with excessive carbon flux drainage toward acetogenesis
and porphyrin formation in DSL should be addressed. We
derived single-mutant strains of DSLΔsdhA and
DSLΔiclR with the sdhA and iclR gene knockouts,
respectively. While these single-mutant strains did not
improve PBG biosynthesis significantly upon aerobic
bioreactor cultivation, they showed metabolic effects
similar to the corresponding DMH single-mutant strains
(Fig. 5). We further derived the double-mutant strain
DSLΔiclRSdhA, which showed significantly enhanced
PBG biosynthesis compared to the DSL control and sin-
gle-mutant strains upon aerobic bioreactor cultivation,
i.e., a PBG titer of 104 mg L⁻¹ (0.81% yield) at the end
of the cultivation (Fig. 6). Moreover, reduced acetogen-
esis was observed in DSLΔiclRSdhA with effective glyc-
erol utilization and cell growth, suggesting successful
carbon flux direction towards the succinyl-CoA node for

PBG and porphyrin biosynthesis under this new genetic
background.

Furthermore, hemC-gRNA-D9 was used to repress
hemC expression in the double-mutant strain
DSLΔiclRSdhA, resulting in DSL-D9ΔiclRSdhA. Aero-
bic bioreactor cultivation of DSL-D9ΔiclRSdhA showed
much improved PBG biosynthesis and accumulation,
i.e., a PBG titer at 209 mg L⁻¹ (1.73% yield) at the end
of the cultivation, though glycerol consumption and cell
growth were slightly affected. Note that the final PBG
yield for DSL-D9ΔiclRSdhA was 2.14-fold that for the
control DSLΔiclRSdhA, suggesting the effectiveness of
hemC repression toward enhanced PBG biosynthesis and
accumulation.

**Strain engineering for PBG biosynthesis under microaerobic conditions**

Using engineered strains with the single sdhA mutation,
we also explored PBG biosynthesis under oxygen-lim-
ited (i.e., microaerobic) conditions. Due to the inacti-
vated oxidative TCA cycle with a regulated glyoxylate
shunt, cell growth and glycerol utilization under micro-
aerobic conditions for these control and mutant strains
were ineffective compared to aerobic cultivation. In

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**Fig. 5** Bioreactor cultivation of DSL, DSLΔsdhA, and DSLΔiclR for PBG biosynthesis under aerobic conditions. Time profiles of cell density (OD₆₀₀),
glycerol consumption and metabolite production profiles, acetate and PBG percentage yields, and extracellular accumulation of porphyrins
(represented by the absorbance readings of the Soret peak (A₄₀₅) and Q-band (A₄₉₅)) are shown. The percentage yields of acetate/PBG and
absorbance readings of porphyrin compounds are calculated/measured based on the consumed glycerol at end of cultivation. (I) DSL, (II)
DSLΔsdhA, (III) DSLΔiclR. All values are reported as means ± SD (n = 2).
general, PBG biosynthesis under microaerobic conditions was also ineffective compared to aerobic cultivation. For the control strain DMH, the final PBG titer for microaerobic bioreactor cultivation was lower than that for aerobic cultivation, only reaching 48.7 mg L\(^{-1}\) (0.41% yield) (Fig. 7), with poor glycerol utilization and cell growth. Interestingly, porphyrin biosynthesis under microaerobic conditions appeared to be more effective, as evidenced by higher Soret peak and Q-band absorbance values, than aerobic cultivation. Compared to the control strain DMO, PBG biosynthesis under microaerobic conditions for the single-mutant strain DMH\(^{\Delta sdhA}\), in which only the reductive TCA branch was functional, was slightly improved, reaching a final PBG titer of 55.9 mg L\(^{-1}\) (0.46% yield), with similar acetogenesis, cell growth, glycerol utilization, and porphyrin formation (Fig. 7). We then evaluated the effects of hemC repression in DMH-D9\(^{\Delta sdhA}\) under microaerobic conditions and observed slightly better PBG biosynthesis, achieving a final PBG titer of 62.5 mg L\(^{-1}\) (0.53% yield), with reduced porphyrin formation (Fig. 7). Note that the peak PBG titers for DMH and DMH\(^{\Delta sdhA}\) cultivations were comparatively higher than that for DMH-D9\(^{\Delta sdhA}\), implying PBG was rather unstable under such genetic backgrounds. Similar genetic and metabolic effects under microaerobic conditions described above in DMH single-mutant strains were also observed in the corresponding DSL single-mutant strains with higher hemB gene dosages. The final PBG titers for microaerobic bioreactor cultivation were 57.9, 67.2, and 83.8 mg L\(^{-1}\) for DSL, DSL\(^{\Delta sdhA}\), and DSL-D9\(^{\Delta sdhA}\), respectively (Fig. 8). Note that the PBG yield for DSL-D9\(^{\Delta sdhA}\) was only 1.16-fold that for DSL\(^{\Delta sdhA}\), suggesting that the effect of hemC repression on PBG biosynthesis and accumulation was insignificant under microaerobic conditions.
Discussion

As an intermediate in the metabolic pathway for essential porphyrin biosynthesis, PBG barely accumulates and, therefore, can be hardly detected in the extracellular medium upon cultivation of wild-type *E. coli*. In this study, we employed genetic and metabolic strategies for strain engineering of *E. coli* to enhance PBG biosynthesis for extracellular accumulation. First, the Shemin/C4 pathway was genetically implemented in *E. coli* by heterologous expression of *hemA* from *R. sphaeroides* to mediate molecular fusion of succinyl-CoA and glycine to form the key precursor 5-ALA for biosynthesis of PBG and porphyrins. Second, metabolic strategies were applied to direct carbon flux from the TCA pathways to the C4 pathway via the succinyl-CoA node. Third, the metabolic flux within the C4 pathway was further boosted by heterologous co-expression of *hemA* from *R. sphaeroides* and the native *E. coli* *hemB*. Finally, CRISPRi was applied to repress *hemC* expression to promote PBG accumulation with minimal impact to cell physiology and viability. PBG biosynthesis and accumulation in various engineered *E. coli* strains were characterized using bioreactor cultivation under different oxygenic (i.e., aerobic and microaerobic) conditions. Note that inclusion of episomal plasmids for heterologous expression of genes and implementing CRISPRi strategy in various engineered strains require constant use of antibiotic selection during cultivation, subsequently increasing the overall production cost.

Compared to the native *E. coli* in which porphyrin biosynthesis was primarily mediated via the C5 pathway, implementation of the heterologous C4 pathway significantly enhanced porphyrin biosynthesis based on visualization of high red-pigmentation upon bacterial cultivation (Additional file 1: Table S3). Nevertheless, PBG titer remained low with significant carbon spill toward acetogenesis, as shown in the control strain DMH cultivated under aerobic conditions. Since succinyl-CoA serves as a key precursor of the C4 pathway for biosynthesis of PBG and porphyrins, metabolic strategies were developed to increase this precursor supply. In *E. coli*, succinyl-CoA can be derived via three oxygen-dependent TCA pathways: (i) reductive TCA branch; (ii) oxidative TCA cycle, and (iii) glyoxylate shunt (Fig. 1) (Cheng et al. 2013). In this study, we explored two metabolic routes for carbon flux direction toward succinyl-CoA within the TCA pathways, i.e., (i) deregulated glyoxylate shunt and reductive TCA branch via the double mutation of *iclR* and *sdhA* under aerobic conditions, and (ii) reductive
TCA branch via the single mutation of sdhA under microaerobic conditions. Hence, the effects of individual single mutations and double mutation of iclR and sdhA on PBG biosynthesis were investigated.

Under aerobic conditions, biosynthesis of PBG and porphyrins was enhanced in DMH∆iclR∆sdhA compared to the control DMH, suggesting that carbon flux was successfully directed toward succinyl-CoA and then into the C4 pathway. Also, note that acetogenesis was reduced upon involving glyoxylate shunt (which can bypass decarboxylation associated with the oxidative TCA cycle) for carbon flux direction, improving biosynthesis yields for PBG and porphyrins. Nevertheless, a general trend of the time course of PBG titer remained unchanged, i.e., the PBG titer reached a peak value and then declined toward the end of the cultivation. Such PBG instability, potentially caused by unregulated subsequent reactions toward porphyrins, was alleviated by repression of hemC expression via CRISPRi in DMH-D9∆iclR∆sdhA. PBG (and porphyrin) biosynthesis was further enhanced by heterologous co-expression of hemA from R. spheroides and the native E. coli hemB and, most importantly, all the above metabolic and hemC-repression strategies were still functional under this new genetic background, as shown in all corresponding DSL strains. Note that ALA dehydratase (i.e., HemB, encoded by hemB) is subject to feedback inhibition by its downstream metabolite of protoporphyrinogen IX (PPIX) (Zhang et al. 2015), potentially limiting the PBG yield. Repression of hemC expression could potential reduce PPIX formation and its feedback inhibition on hemB expression, and subsequently increase PBG formation. The effects of heterologous expression of hemB could be clearly observed by much higher peak and final PBG titers between the corresponding DMH and DSL strains. The effects of amplification of various genes in the porphyrin biosynthetic pathway on porphyrin formation were documented (Lee et al. 2013). Note that, under aerobic culture conditions, the PBG yield of DSL-D9∆iclR∆sdhA with all implemented metabolic and genetic strategies was 2.66-fold that of the control DMH.

Under microaerobic conditions, succinyl-CoA was derived primarily via the reductive TCA branch (Shin et al. 2007) and, therefore, the oxidative TCA cycle had to be inactivated, such as mutating sdhA in DMH∆sdhA, to support functional operation of the central metabolism.
While PBG can be produced under microaerobic conditions, bioreactor cultivation suffered poor cell growth and glycerol utilization with significant acetogenesis and PBG instability. Interestingly, porphyrin biosynthesis appeared to be more effective under microaerobic conditions (as evidenced by higher absorbance values for Soret peak and Q-band) than aerobic cultivation though PBG biosynthesis showed the opposite. Compared to aerobic cultivation, significant amounts of formate were observed for PBG-producing strains cultivated under microaerobic conditions, presumably due to the induced activity of pyruvate formate lyase (PFL) under oxygen-limited conditions instead of pyruvate dehydrogenase (PDH) which is mostly active in oxygen-rich environments (Durnin et al. 2009). Adverse effects arising from accumulated formate and acetate on culture performance were reported (Kirkpatrick et al. 2001). Nevertheless, the strain engineering strategies developed for aerobic cultivation, specifically heterologous hemB expression and repression of hemC expression, were still applicable to microaerobic cultivation though the improving effects were less significant than those under aerobic conditions. Under microaerobic culture conditions, the PBG yield of DSL-D9∆sdhA with all implemented strain engineering strategies was 1.73-fold that of the control DMH.

This study has several advantages over other reported PBG biosynthesis studies in variety of microbial systems. We utilized glycerol as cheap feedstock for direct PBG biosynthesis, compared to the process of PBG preparation from 5-ALA by pretreated cells of Chromatium vinosum (Vogelmann et al. 1975). We attained a PBG concentration of 0.182 mmol/g-DCW in E. coli without extraneous supplementation of succinate and glycine. We obtained maximum PBG concentration of 924 µM compared to 72 µM from Propionibacterium freudenreichii (Piao et al. 2004) or 200 µM from Rhodopseudomonas spheroids (Hatch and Lascelles 1972b).

Conclusions
In this study, we demonstrated that implementation of the non-native C4 pathway in E. coli was effective to supply carbon flux from the natural TCA pathways for PBG biosynthesis via succinyl-CoA. Metabolic engineering and bioprocessing strategies were further applied for effective carbon flux direction from the TCA pathways to the C4 pathway for enhanced PBG biosynthesis. To promote PBG accumulation, CRISPRi was successfully applied to repress hemC expression with minimal impact to cell physiology. The heterologous expression of the native E. coli hemB further enhanced overall PBG biosynthesis which was limited by fusion of two 5-ALA molecules catalyzed by HemB. Overall, we enhanced PBG formation and accumulation in engineered E. coli by utilizing a cheap carbon source for direct biosynthesis without precursor supplementation. In addition, potential biochemical, genetic, and metabolic factors limiting PBG production were characterized.

Supplementary Information
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Authors’ contributions
DL and DM conceived the study. DL formulated research plan, coordinated research team, carried out experiments, performed result interpretation and data analysis, and drafted the manuscript. DM, MB, AW, and MA provided technical assistance on experimentation. MM-Y and CPC conceived, planned, supervised, and managed the study as well as helped to draft the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Most of data generated or analyzed during this study are included in this published article and its Additional file 1. Additional file 1 data can be made available from the corresponding author upon reasonable request.
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