Strain engineering for high-level 5-aminolevulinic acid production in Escherichia coli

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Abstract

As issues surrounding depleting fossil fuels, climate change, and various other environmental impacts are becoming more prevalent, there is a growing interest in technological shifts toward a bio-based economy. Various advanced biotechnological tools have been developed to customize cell factories for the production of a wide range of complex fine chemicals from renewable feedstock. Herein, we report development of a microbial bioprocess for high-level and potentially economical production of 5-aminolevulinic acid (5-ALA), a valuable non-proteinogenic amino acid with multiple applications in medical, agricultural, and food industries, using Escherichia coli as a cell factory. We first implemented the Shemin (i.e., C4) pathway for heterologous 5-ALA biosynthesis in E. coli. To reduce, but not to abolish, the carbon flux toward essential tetrapyrrole/porphyrin biosynthesis, we applied Clustered Regularly Interspersed Short Palindromic Repeats interference (CRISPRi) to repress hemB expression, leading to extracellular 5-ALA accumulation. We then applied metabolic engineering strategies to direct more dissimilated carbon flux toward the key precursor of succinyl-CoA for enhanced 5-ALA biosynthesis. Using these engineered E. coli strains for bioreactor cultivation, we successfully demonstrated high-level 5-ALA biosynthesis solely from glycerol (~30 g l-1) under both microaerobic and aerobic conditions, achieving up to 5.95 g l-1 (36.9% yield) and 6.93 g l-1 (50.9% yield) 5-ALA, respectively. This study represents one of the most effective bio-based production of 5-ALA from a structurally unrelated carbon to date, highlighting the importance of integrated strain engineering and bioprocessing strategies to enhance bio-based production.

Introduction

Increasing concerns over climate change and fossil fuel depletion have provoked a need for sustainable production of chemicals and fuels. One approach of particular attractiveness is the use of biological cell factories, such as microorganisms, as biocatalysts to drive chemical conversions from renewable and clean carbons, so called "bio-based production". The emergence of novel biotechnological tools for strain engineering, in particular systems biology, synthetic biology, metabolic engineering, and genetic/genomic engineering, has promoted the development of hyper strains for bio-based production (Choi et al. 2019). In addition to the sustainability, bio-based production can have economical potentials over synthetic organic chemistry particularly for the production of complex chemicals in the pharmaceutical and fine chemical industries, such as amino acids, organic acids, and vitamins (Keasling 2010).

5-Aminolevulinic acid (5-ALA) is a non-proteinogenic amino acid existing in most living organisms as a metabolic intermediate toward biosynthesis of essential tetrapyrrole/porphyrin pigment compounds, such as heme (Schlicke et al. 2015) (Fig. 1). In nature, there are two major metabolic routes for 5-ALA biosynthesis, i.e., (1) C4 (also known as Shemin) pathway (mainly existing in mammals, fungi, and purple sulphur bacteria), in which succinyl-CoA and glycine are structurally fused by 5-aminolevulinate synthase (ALAS or HemA) to form 5-ALA (Kang et al. 2004), and (2) C5 pathway (existing in most bacteria, all archaea and

plants), in which glutamate is converted to 5-ALA via three enzymatic reactions of ligation, reduction, and transamination catalyzed by glutamyl-tRNA synthase (GluTS), glutamyl-tRNA reductase (GluTR), and glutamate-1-semialdehyde-2,1-aminomutase (GSAM), respectively (Woodard and Dailey 1995).

Practically, 5-ALA has broad applications in many fields, such as medicine (Inoue 2017; Juzeniene et al. 2002), agriculture (Hotta et al. 1997), and food preservation (Li et al. 2016). Hence, technologies for 5-ALA production have been developed. While 5-ALA can be chemically derived from various precursors, such as levulinic acid (MacDonald 1974), tetrahydrofurfurylamine (Kawakami et al. 1991), 5-bromo esters (Ha et al. 1994), and*N* -furfurylphthalimide (Takeya et al. 1996), these synthetic approaches are deemed uneconomical and the production processes are often complicated for implementation with low yields (Kang et al. 2017). As a result, bio-based production of 5-ALA using either multi-enzyme systems (Meng et al. 2016) or various cell factories has been explored (Sasaki et al. 2002), in particular photosynthetic microorganisms, such as*Rhodobacter sphaeroides* , *Rhodopseudomonas palustris* , and*Chlorella* sp. (Sasaki et al. 1995), *Streptomyces coelicolor*(Tran et al. 2019), *Corynebacterium glutamicum* (Zhang and Ye 2018), as well as genetically tractable *Escherichia coli*(Ding et al. 2017; Zhang et al. 2015; Zhang et al. 2019).

In this study, we explored strain engineering strategies for high-level 5-ALA production in *E. coli* . Native biosynthesis of 5-ALA in*E. coli* is achieved via the C5 pathway, which is adopted in most previous studies for 5-ALA biosynthesis using *E. coli* as a cell factory (Kang et al. 2011; Li et al. 2014; Zhang et al. 2015). However, this metabolic route is considered mechanistically complex and energetically ineffective, particularly upon high-level 5-ALA biosynthesis, as it requires multiple tightly regulated enzymes (Wang et al. 1999) and utilization of ATP/NADPH as limiting cofactors (Li et al. 1989). On the other hand, for high-level biosynthesis of a target metabolite, it is critical to ensure intracellular abundance of the corresponding precursors, i.e., succinyl-CoA/glycine for the Shemin pathway or glutamate for the C5 pathway in the 5-ALA case. This is normally achieved by proper metabolic direction of the dissimilated carbon flux in key pathways, otherwise artificial supplementation of structurally related carbons, which are often expensive, becomes necessary. Considering the above technical aspects/limitations, we chose to implement the Shemin pathway into *E. coli* for heterologous 5-ALA biosynthesis with metabolic direction of the dissimilated carbon flux toward succinyl-CoA in the tricarboxylic acid (TCA) cycle. Moreover, without supplementation of structurally related carbons, glycerol was used as the sole carbon source for cultivation of engineered *E. coli* strains due to its low cost (Ciriminna et al. 2014) and highly reduced nature, generating approximately twice the number of reducing equivalents upon its degradation compared to traditional fermentable sugars (Murarka et al. 2008; Yazdani and Gonzalez 2007).

By initializing the formation of essential porphyrin compounds, 5-ALA is among the most conserved metabolites across all biological kingdoms (Petříčková et al. 2015; Yu et al. 2015). However, 5-ALA normally acts as a metabolic intermediate toward porphyrin biosynthesis with minimal accumulation, limiting its overproduction. Given the imperative physiological role of porphyrin compounds, attempting to accumulate 5-ALA by inactivation of the immediate post-5-ALA conversion catalyzed by 5-aminolevulinate dehydratase (HemB, encoded by *hemB*) would abolish porphyrin biosynthesis (Fig. 1) and, therefore, be detrimental to the cells. Hence, instead of gene inactivation, we applied Clustered Regularly Interspersed Short Palindromic Repeats interference (CRISPRi) to repress *hemB* expression and, therefore, increase 5-ALA accumulation without imposing physiological impacts to the 5-ALA-producing cells. Using various engineered *E. coli* strains for bioreactor cultivation, we demonstrated high-level 5-ALA biosynthesis under both microaerobic and aerobic conditions with glycerol as a sole carbon source.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and deoxynucleic acids (DNAs) used in this study are listed Table 1. Genomic DNA from bacterial cells was isolated using the Blood & Tissue DNA Isolation Kit (Qiagen, Hilden, Germany). Standard recombinant DNA technologies were applied for molecular cloning (Miller 1992). *Taq* DNA polymerase was obtained from New England Biolabs (Ipswich, MA, USA). All synthesized oligonucleotides were obtained

from Integrated DNA Technologies (Coralville, IA, USA). DNA sequencing was conducted 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 *E. coli* DH5α was used as a host for molecular cloning. Note that the *ldhA* gene (encoding lactate dehydrogenase) was previously inactivated in BW25113, generating BW[?]*ldhA* (Srirangan et al. 2014).

For genetic implementation of the Shemin pathway in *E. coli* , the*hemA* gene was first amplified by polymerase chain reaction (PCR) using the primer set g-hemA and the genomic DNA of wild-type*Rhodobacter sphaeroides* DSM 158 as the template. The amplified*hemA* gene was Gibson–assembled with the PCR-linearized pK184 using the primer set g-pK-hemA to generate pK-hemA. The expression of the cloned *hemA* gene in the pK184 vector was under the control of the P_{lac} promoter.

Knockouts of the genes, including *sdhA* (encoding succinate dehydrogenase complex flavoprotein subunit A, SdhA) and *iclR*(encoding transcriptional AceBAK operon repressor, IclR), were introduced into BW[?]*ldhA* 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). To eliminate the co-transduced FRT-Kn^R-FRT cassette, the transductants were transformed with pCP20 (Cherepanov and Wackernagel 1995), a temperature sensitive plasmid expressing a flippase (Flp) recombinase. Upon Flp-mediated excision of the Kn^R cassette, a single Flp recognition site (FRT "scar site") was generated. Plasmid pCP20 was then cured by growing cells at 42°C. The genotypes of derived knockout strains were confirmed by colony PCR using the appropriate verification primer sets listed in Table 1.

The expression of *hemB* was repressed by CRISPRi using various derived plasmids from pdcas9-bacteria (Addgene plasmid # 44249) and pgRNA-bacteria (Addgene plasmid # 44251). All synthesized oligonucleotide pairs have 60 nucleotides (nt), which includes 20 nt*hemB* -targeting sequence, 20 nt upstream and 20 nt downstream sequences of pgRNA-bacteria vector (Fig. 2). They were ordered from Integrated DNA Technologies (Coralville, IA, USA) and annealed as described previously (Pengpumkiat et al. 2016), generating four double-stranded DNA fragments of*hemB* -gRNA-L1, *hemB* -gRNA-L2, *hemB* -gRNA-L3, and*hemB* -gRNA-L4 (Table 1). All these four DNA fragments were individually Gibson-assembled with the PCR-linearized pgRNA-bacteria using the primer set g-pgRNA to generate pgRNA-L1, pgRNA-L2, pgRNA-L3, and pgRNA-L4, respectively (Table 1). The four *hemB* -repressed strains, i.e., DMH-L1, DMH-L2, DMH-L3, and DMH-L4, were developed by creating a triple-plasmid system (Fig. 2) containing pK-hemA, pdcas9-bacteria, and the gRNA-containing plasmid (i.e., pgRNA-L1, pgRNA-L2, pgRNA-L3, or pgRNA-L4). For the control strain DMH-CT, the original pgRNA-bacteria plasmid without any *hemB* -targeting sequence was used as the third plasmid.

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 and incubated at 37°C for 14-16 hours.

For shake-flask cultivations, single colonies were picked from LB plates to inoculate 30 ml 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 LB media at 1% (v/v) in 1 L conical flasks. This second seed culture was shaken at 37°C and 280 rpm until a cell density of 0.80 OD_{600} was reached. Cells were then harvested by centrifugation at 9,000 ×g and 20°C for 10 minutes and resuspended in 30 ml modified M9 production media. The suspended culture was transferred into a 125 ml screwed cap plastic production flasks and incubated at 37°C at 280 rpm in a rotary shaker. Unless otherwise specified, the modified M9 production medium contained 20 g l^{-1} glycerol, 5 g l^{-1}yeast extract, 10 mM $NaHCO_3$, 1 mM $MgCl_2$,5^{th} dilution of M9 salts mix (33.9 g l^{-1} Na_2HPO_4, 15 g l^{-1} KH_2PO_4, 5 g l^{-1} NH_4Cl, 2.5 g l^{-1}NaCl), $1{,}000^{th}$ dilution of Trace Metal Mix A5 (2.86 g l^{-1} H_3BO_3, 1.81 g l^{-1} $MnCl_2$*$4H_2O$, 0.222 g l^{-1} $ZnSO_4$*$7H_2O$, 0.39 g $l^{-1}$$Na_2MoO_4$*$2H_2O$, 79 µg l^{-1} $CuSO_4$*$5H_2O$, 49.4 µg $l^{-1}$$Co(NO_3)_2$*$6H_2O$), and was supplemented

with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). All shake-flask cultivation experiments were performed in triplicate.

For bioreactor cultivations, single colonies were picked from LB plates to inoculate 30 mL 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 SB media at 1% (v/v) in 1 L conical flasks. This second seed culture was shaken at 37°C and 280 rpm for 14-16 hours. Cells were then harvested by centrifugation at 9,000×g and 20°C for 10 minutes 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} K_2HPO_4, 0.51 g l^{-1} NH_4Cl, 49.8 mg l^{-1} $MgCl_2$, 48.1 mg l^{-1} K_2SO_4, 1.52 mg l^{-1} $FeSO_4$, 0.055 mg l^{-1} $CaCl_2$, 2.93 g l^{-1} NaCl, 0.72 g l^{-1} tricine, 10 g l^{-1} yeast extract, 10 mM $NaHCO_3$, and 1,000th dilution (i.e., 1 ml l^{-1}) trace elements (2.86 g l^{-1} H_3BO_3, 1.81 g l^{-1} $MnCl_2$* $4H_2O$, 0.222 g l^{-1} $ZnSO_4$* $7H_2O$, 0.39 g l^{-1} Na_2MoO_4* $2H_2O$, 79 μg l^{-1} $CuSO_4$* $5H_2O$, 49.4 μg $l^{-1}Co(NO_3)_2$* $6H_2O$) (Neidhardt et al. 1974), and was supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Microaerobic and semiaerobic conditions were maintained by purging air into the headspace and bulk culture, respectively, at 0.1 vvm, designated as aeration level I (AL-I) and II (AL-II). Aerobic conditions were maintained by purging air into the bulk culture at 1 vvm (AL-III). The pH of the production culture was maintained at 7.0 ± 0.1 with 30% (v/v) NH_4OH and 15% (v/v) H_3PO_4.

Analysis

Culture samples were appropriately diluted with 0.15 M saline solution for measuring cell density in OD_{600} using a spectrophotometer (DU520, Beckman Coulter, Fullerton, CA). Cell-free medium was prepared by centrifugation of the culture sample at 9,000×g for 5 minutes, followed by filter sterilization using a 0.2 μM syringe filter. Extracellular metabolites and glycerol were quantified using 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_2SO_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).

The 5-ALA titer in the cell-free medium was measured using a modified Ehrlich's reagent (Mauzerall and Granick 1956). The percentage yield of 5-ALA was defined as the mole (or mass) ratio of the produced 5-ALA to the theoretically maximal 5-ALA produced based on the consumed glycerol with a molar ratio of one-to-three. Note that one-mole succinyl-CoA (derived from two-mole glycerol) and one-mole glycine (derived from one-mole glycerol) are required to generate one-mole 5-ALA. The bulk level of secreted porphyrin compounds in the cell-free medium was estimated using a spectrophotometer at two specific wavelengths, i.e., OD_{405} (measuring Soret band) and OD_{495} (measuring Q-band).

Real-time quantitative reverse transcription PCR (qRT-PCR)

Cells used for RNA extraction were cultivated in 30 mL liquid LB medium at 37°C and harvested in the exponential growth phase. Total RNA of*E. coli* was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics, Basel, Switzerland) according to manufacturer's instructions. Complementary DNAs (cDNAs) were synthesized using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, MA). Sequence specific primers were used for reverse transcription of*hemB* mRNA (i.e., q-hemB) and internal control *rrsA*(encoding ribosomal RNA 16S) mRNA (i.e., q-rrsA) (Table 1), at a final concentration of 1 μM. 100 ng of the total RNA was used in a 20 μL reaction mixture. Real-time 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 experiments were performed in duplicate.

Results

Repression of **hemB** ***expression for extracellular 5-ALA accumulation***

We first implemented the Shemin pathway by episomal expression of*hemA* from *R. sphaeroides* in BW[?]*ldhA* , deriving DMH. The effects of the implemented Shemin pathway were observed by comparing the two cultures of BW[?]*ldhA* and DMH in screw-cap shake-flasks containing 20 g l^{-1} glycerol as the sole carbon source. While BW[?]*ldhA* generated no detectable levels of 5-ALA or porphyrin, DMH produced 0.44 g l^{-1} 5-ALA (5.22% yield) with significant porphyrin biosynthesis (Fig. 3), suggesting that the Shemin pathway was active in DMH. In particular, the dark red color of the DMH culture (Fig. 3) suggests that most glycerol was converted to porphyrin pigments with limited 5-ALA accumulation.

We then aimed to reduce the carbon flux toward porphyrin biosynthesis in DMH by repressing the expression of *hemB* (Fig. 1), leading to a limited conversion of two 5-ALA molecules into porphobiliongen (PBG) and extracellular 5-ALA accumulation. We applied CRISPRi by designing four*hemB* -targeting gRNAs with different relative expression efficiencies, generating four corresponding strains of DMH-L1, DMH-L2, DMH-L3, and DMH-L4 (Fig. 3). Note that DMH-CT is the control strain without a *hemB* -specific gRNA for CRISPRi. While cell growth and glycerol consumption were somewhat affected by the presence of the triple-plasmid system, all *hemB* -repressed strains had significant extracellular 5-ALA accumulation compared to the control strain DMH-CT upon shake-flask cultivation. The minimal physiological impacts suggest that the *hemB* -repressed strains still had sufficient biosynthesis of essential porphyrins. In particular, DMH-L1 and DMH-L4 had both titers and yields up to 4-5 fold higher than the control strain DMH-CT (Fig. 3). This significant increase in 5-ALA accumulation in DMH-L1 (1.26 g l^{-1}) and DMH-L4 (1.61 g l^{-1}) occurred simultaneous with considerably reduced levels of the relative*hemB* expression at 67% and 40%, respectively (Fig. 2). Additional supporting evidence for the repressed *hemB* expression was reflected by the decrease in porphyrin biosynthesis in all four*hemB* -repressed strains as the degrees of pigmentation and the corresponding absorbances (in 405 and 495 nm) of the cell-free media were significantly lower than that of the control strain DMH-CT (Fig. 3). Given the lowest relative *hemB* expression and the most superior extracellular 5-ALA accumulation in DMH-L4 among all*hemB* -repressed strains, we used this strain for all subsequent experiments.

Effects of oxygenic condition on 5-ALA biosynthesis

5-ALA biosynthesis via the Shemin pathway requires succinyl-CoA as one of the two key precursors. Several metabolic pathways are involved in succinyl-CoA formation in *E. coli* , i.e., reductive TCA branch, oxidative TCA cycle, and glyoxylate shunt (Fig. 1). These metabolic pathways, along with cell growth and acetogenesis, can be sensitive to the oxygenic condition, which critically directs the dissimilated carbon flux toward succinyl-CoA for 5-ALA biosyntheses. To investigate such oxygenic effects, batch cultivation of the control strain DMH in a bioreactor was subject to three different levels of aeration, i.e., AL-I (microaerobic), AL-II (semiaerobic), and AL-III (aerobic) (Fig. 4). While cell growth and glycerol consumption were favored by oxygen exposure, 5-ALA biosynthesis was more effective under lower aeration levels, i.e., 0.53 g l^{-1} (3.40% yield) under AL-I, 0.39 g l^{-1} (2.44% yield) under AL-II, and 0.31 g l^{-1} (2.08% yield) under AL-III, presumably because that more carbon was directed toward the succinyl-CoA node under microaerobic conditions. In addition, the reduced 5-ALA biosynthesis under aerobic conditions occurred with less porphyrin formation, reflected by a significantly less pigmentation of the culture medium. Note that, in addition to low levels of 5-ALA biosynthesis in DMH under all investigated culture conditions, acetate was the major side metabolite with high yields up to 75.9%, implying a significant carbon spill at the acetyl-CoA node. The results suggest that 5-ALA biosynthesis in DMH was favored by lower oxygenic levels and potentially limited by acetogenesis.

Metabolic engineering to enhance 5-ALA biosynthesis under microaerobic conditions

To alleviate growth limitation and acetogenesis in DMH under AL-I, we derived a single-knockout mutant DMH[?]*sdhA* , in which the oxidative TCA cycle was inactivated (Fig. 5-I). In addition to reduced acetogenesis, DMH[?]*sdhA* produced 0.94 g l^{-1}5-ALA with 6.61% yield. With an increased carbon flux toward porphyrin biosynthesis, pigmentation of the DMH[?]*sdhA* culture medium was significantly enhanced. On

the other hand, while glycerol consumption and cell growth were significantly retarded upon cultivation of the*hemB* -repressed strain DMH-L4 under AL-I, 5-ALA biosynthesis was drastically improved with much reduced acetogenesis, achieving 4.73 g l^{-1} 5-ALA with 32.0% yield (Fig. 5-II). The improved 5-ALA biosynthesis was also evidenced by significant reduction in pigmentation of the culture medium compared to DMH. Notably, compared to DMH[?]*sdhA* or DMH-L4, 5-ALA biosynthesis was further improved upon cultivation of DMH-L4[?]*sdhA* , in which the *sdhA* mutation and*hemB* -repression was simultaneously introduced, under AL-I, achieving 5.95 g l^{-1} 5-ALA with 36.9% yield (Fig. 5-III). These results suggest that the dissimilated carbon flux was directed toward the succinyl-CoA node for 5-ALA biosynthesis primarily via the reductive TCA branch under microaerobic conditions, and such carbon flux direction was rather effective upon simultaneous disruption of the oxidative TCA cycle and *hemB* repression. Finally, compared to DMH, higher levels of succinate, formate, and ethanol were observed upon cultivation of all engineered strains, i.e. DMH[?]*sdhA* , DMH-L4, and DMH-L4[?]*sdhA* , under microaerobic conditions for enhanced 5-ALA biosynthesis.

Metabolic engineering to enhance 5-ALA biosynthesis under aerobic conditions

We also explore 5-ALA biosynthesis under aerobic conditions, which often facilitate carbon utilization and cell growth. While the DMH-L4 culture under AL-III showed effective glycerol dissimilation and cell growth, biosynthesis of 5-ALA and porphyrins was much lower than that of the DMH-L4 culture under AL-I (Fig. 6-I vs. Fig. 5-II), suggesting a potential limitation in succinyl-CoA precursor under aerobic conditions. Nevertheless, the significant enhancing effects of*hemB* -repression on 5-ALA biosynthesis were still observable under aerobic conditions by comparing the two cultures of DMH-L4 and DMH under AL-III (Fig. 6-I vs. Fig. 4-III). To overcome the limitation in succinyl-CoA under AL-III, we derived another mutant of DMH-L4[?]*iclR* with a deregulated glyoxylate shunt. Compared to the parental strain DMH-L4, DMH-L4[?]*iclR* had a much higher 5-ALA biosynthesis with effective glycerol dissimilation and cell growth under AL-III (Fig. 6-III vs. Fig 6-I), suggesting successful direction of the dissimilated carbon flux toward succinyl-CoA for 5-ALA biosynthesis via the glyoxylate shunt under aerobic conditions. For more effective carbon flux direction, we derived another double mutant of DMH-L4[?]*iclR* [?]*sdhA* with a disruptive oxidative TCA cycle such that the directed carbon flux at the succinate node via the glyoxylate shunt could be further directed toward succinyl-CoA via the reductive TCA branch. Compared to the parental strain DMH-L4[?]*iclR* , DMH-L4[?]*iclR* [?]*sdhA* had even higher 5-ALA biosynthesis under AL-III (Fig. 6-V vs. Fig. 6-III), achieving 6.93 g l^{-1} 5-ALA with 50.9% yield, though the single mutation of [?]*sdhA* appeared to be rather harmful to cell physiology and, therefore, culture performance (Fig. 6-II vs. Fig. 6-I). Note that the 5-ALA yield for DMH-L4[?]*sdhA* [?]*iclR* was 3.4-fold that for DMH-L4 and 1.3-fold that for DMH-L4[?]*iclR* . Also, note that the significant enhancing effects of *hemB* -repression on 5-ALA biosynthesis were further confirmed by comparing the two cultures of DMH-L4[?]*iclR* [?]*sdhA* and DMH[?]*iclR* [?]*sdhA* under AL-III (Fig. 6-V vs. Fig. 6-IV). On the other hand, the successful carbon flux direction toward the Shemin pathway via the glyoxylate shunt and reductive TCA branch for biosynthesis of both 5-ALA and porphyrin pigments can be also observed by comparing the two cultures of DMH[?]*iclR* [?]*sdhA* and DMH under AL-III (Fig. 6-IV vs. Fig. 4-III). These results successfully demonstrated the consolidated strategy based on carbon flux redirection in the TCA cycle toward the Shemin pathway with repressed *hemB* expression to enhance 5-ALA biosynthesis.

Discussion

While *E. coli* has the native C5 pathway for 5-ALA biosynthesis, we herein adopted the exogenous Shemin pathway since the intracellular level of the key precursor succinyl-CoA can be metabolically manipulated. Being a metabolic intermediate in the pathway for biosynthesis of essential porphyrins, 5-ALA hardly accumulates in*E. coli* . To prevent intracellular drainage of 5-ALA, we explored gene knockout of *hemB* , but failed to derive the corresponding mutant strain (data now shown), confirming that *hemB* is an essential gene. Hence, CRISPRi was applied to repress *hemB*expression, particularly in DMH-L1 and DMH-L4, such that 5-ALA could accumulate as a result of its reduced conversion. Our results further suggest that 5-ALA acts as a committed precursor for porphyrin biosynthesis. Despite the increased extracellular 5-ALA accumulation associated with the repressed *hemB* expression, suppression of carbon flux toward essential

porphyrin biosynthesis somewhat impacted cell growth upon cultivation.

While the effects of oxygen supply on 5-ALA biosynthesis were investigated in several organisms (Nishikawa et al. 1999; Yu et al. 2015), little effort on this front was made for *E. coli*. With the implemented the Shemin pathway, 5-ALA biosynthesis in *E. coli* can critically depend on the availability of the key precursor of succinyl-CoA, whose formation is rather oxygen-sensitive. In *E. coli*, succinate (and, therefore, succinyl-CoA) can be derived via three oxygen-dependent pathways in the TCA cycle, i.e., (i) reductive TCA branch, (ii) oxidative TCA cycle, and (iii) glyoxylate shunt (Fig. 1) (Cheng et al. 2013). Under anaerobic conditions, succinate accumulates as an end-product of mixed acid fermentation via the reductive TCA branch (Thakker et al. 2012). Although the reductive TCA branch can potentially yield high-level succinate, this pathway is generally unfavorable due to the limited availability of reducing equivalents (Skorokhodova et al. 2015). Under aerobic conditions, succinate is normally used up as a metabolic intermediate of the oxidative TCA cycle without accumulation, except for the conditions of oxidative stress and/or acetate/fatty-acid consumption under which succinate can be aerobically derived via the operational glyoxylate shunt (Thakker et al. 2012). Using the control strain DMH, the effects of oxygenic conditions on 5-ALA biosynthesis were systematically investigated for cell cultivation under microaerobic (AL-I), semiaerobic (AL-II), and aerobic (AL-III) conditions. Note that glycerol utilization and cell growth were severely retarded when DMH was cultivated under a strict anaerobic condition (data not shown). Our results show that biosynthesis of 5-ALA and porphyrins was favored by microaerobic conditions, though the low oxygenic level could trigger high-level acetogenesis and the associated physiological impacts. The results suggest that, in DMH, most of the dissimilated carbon flux was channeled into the Shemin pathway for biosynthesis of 5-ALA and porphyrins via the reductive TCA branch; and the oxidative TCA cycle and glyoxylate shunt contributed minimally toward such carbon flux channeling. Compared to DMH, blocking the oxidative TCA cycle in DMH[?]*sdhA*could potentially channel more dissimilated carbon flux toward succinyl-CoA via the reductive TCA branch under AL-I and, therefore, improve biosynthesis of 5-ALA and porphyrins with much reduced acetogenesis. With more dissimilated carbon flux channeling into the Shemin pathway, porphyrin biosynthesis was further reduced by repressing*hemB* expression to enhance 5-ALA accumulation in DMH-L4[?]*sdhA* under AL-I, achieving 5.95 g l^{-1}5-ALA and with 36.9% yield while minimizing porphyrin biosynthesis. Note that inactivating the oxidative TCA cycle and/or repressing*hemB* expression resulted in uncommon accumulation of formate with reduced acetogenesis, compared to the control strain DMH. Such observation suggests that, under these genetic backgrounds and microaerobic conditions, pyruvate formate lyase (PFL; via which formate is coproduced) could be more active than pyruvate dehydrogenase (PDH) for the conversion of pyruvate to acetyl-CoA. In *E. coli*, PDH and PFL are responsible for decarboxylation of pyruvate to form acetyl-CoA under aerobic and anaerobic conditions, respectively (Wang et al. 2010). It was also reported that acetate and formate could induce opposite proteome responses in*E. coli* as most proteins induced by one of these two acids are repressed by the other (Kirkpatrick et al. 2001). While the control strain DMH had a low-level biosynthesis of 5-ALA and porphyrins under aerobic conditions such as AL-III, implying a limited carbon flux contribution from the oxidative TCA cycle (and, therefore, limited succinyl-CoA precursor) into the Shemin pathway, repressing*hemB* expression could also increase 5-ALA accumulation significantly in DMH-L4. Inactivating the TCA oxidative cycle in DMH-L4[?]*sdhA* significantly retarded cell growth with limited glycerol dissimilation, cell growth, and metabolite production under AL-III, suggesting the critical metabolic roles of the TCA oxidative cycle for biomass formation and biosynthesis under aerobic conditions as per previous observations (Guest 1981; Steinsiek et al. 2011). To resolve the apparent succinyl-CoA limitation under AL-III, we explored channeling of the dissimilated carbon flux via a deregulated glyoxylate shunt by mutating *iclR* in DMH-L4[?]*iclR* and observed a significantly enhanced 5-ALA biosynthesis. Nevertheless, with an active TCA oxidative cycle in DMH-L4[?]*iclR*, the carbon flux arising from the deregulated glyoxylate shunt could divert at the succinate node to either the oxidative or re-

ductive TCA direction. Importantly, the flux diversion could be prevented with the dissimilated carbon being effectively directed into the Shemin pathway by further mutating *sdhA* for inactivation of the oxidative TCA cycle in DMH-L4[?]*sdhA*[?]*iclR*, achieving 6.93 g l^{-1} 5-ALA with 50.9% yield upon its cultivation under AL-III while minimizing porphyrin biosynthesis. Such carbon flux rerouting effects under AL-III could also be observed by the enhanced biosynthesis of 5-ALA and porphyrins (reflected by significant pigmentation of the culture medium in Fig. 6-IV) in DMH[?]*sdhA*[?]*iclR* compared to DMH. Additionally, note that the retarded glycerol utilization and cell growth for DMH-L4Δ*sdhA* could be complemented by the *iclR*mutation, suggesting that both the oxidative TCA cycle and glyoxylate shunt contribute to active TCA operation for sustained cell growth and metabolic biosynthesis under aerobic conditions. While this study successfully demonstrated high-level biosynthesis of 5-ALA from structurally unrelated carbons under both microaerobic and aerobic conditions, the overall culture performance was limited by significant acetogenesis, particularly during extended fedbatch cultivation (data not shown).

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Competing financial interests

The authors declare no competing financial interests.

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Table captions

E. coli strains, plasmids, and oligonucleotides used in this study

Figure captions

1. **Schematic representation of the natural metabolism and the implemented Shemin pathway for 5-ALA and porphyrin biosynthesis in*E. coli* from glycerol.** Metabolic pathways outlined: glycolysis, glycine biosynthesis, pyruvate carboxylation, and oxidative TCA cycle (in black); glyoxylate shunt in the TCA cycle (in light brown); reductive branch of TCA cycle (in blue); Shemin pathway (in green); porphyrin biosynthesis (in red). Colored proteins: mutations (in red); overexpression (in purple). Metabolite abbreviations: 5,10-MTH, 5,10-methenyltetrahydrofolic acid; 5-ALA, 5-aminolevulinic acid; 3-PG, 3-phosphoglycerate; 3-PP, 3-phosphooxypyruvate; O-P-Serine, O-phospho-L-serine; PBG, porphobilinogen; PEP, phosphoenolpyruvate. The number of carbon atoms for each metabolite is specified in orange. Protein abbreviations: AceA, isocitrate lyase; AceB, malate synthase A; AceK, isocitrate dehydrogenase kinase/phosphatase; AckA, acetate kinase; AdhE, aldehyde-alcohol dehydrogenase; FHL, formate hydrogenlyase; HemA, 5-aminolevulinate synthase; HemB, 5-aminolevulinate dehydratase; IclR, AceBAK operon repressor; IDH, isocitrate dehydrogenase; IDH-P, isocitrate dehydrogenase-phosphate; LdhA, lactate dehydrogenase A; PC, pyruvate carboxylase; PckA, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase; PFL, pyruvate formate-lyase; PK, pyruvate kinase; PPC, phosphoenolpyruvate carboxylase; Pta, phosphotransacetylase; SdhA, succinate dehydrogenase complex (subunit A).
2. **Design strategy for CRISPRi-mediated *hemB* repression .**The three plasmids with their major genetic features, such as promoters, selection markers, key genes, are shown. The design of*hemB* -targeting sequences and their associated interacting spots in the *hemB* gene (i.e., L1, L2, L3, and L4) and the predicted repression efficiencies (numbers in parenthesis) are shown. The resulting *hemB* -repressed strains, i.e., DMH-CT (control), DMH-L1, DMH-L2, DMH-L3, and DMH-L4, were characterized for quantification of the relative *hemB* expression using qRT-PCR. All qRT-PCR values are reported as means ± SD (n = 2).
3. **Shake-flask cultivation of *hemB*-repressed strains for 5-ALA accumulation.** Strains compared include BW[?]*ldhA* , DMH, DMH-CT, DMH-L1, DMH-L2, DMH-L3, and DMH-L4. Results of the 48h shake-flask cultivation in (**a**) cell density (OD_{600}), (**b**) glycerol consumption, (**c**) 5-ALA titer and percentage yield, and (**d**) porphyrin biosynthesis (represented by the absorbance readings of the Soret peak (OD_{405}) and Q-band (OD_{495}) and the images of cell-free media) are shown. All values are reported as means +- SD (n = 3).
4. **Bioreactor cultivation of DMH for 5-ALA biosynthesis under different oxygenic conditions.** Time profiles of cell density (OD_{600}), glycerol consumption and metabolite production profiles,

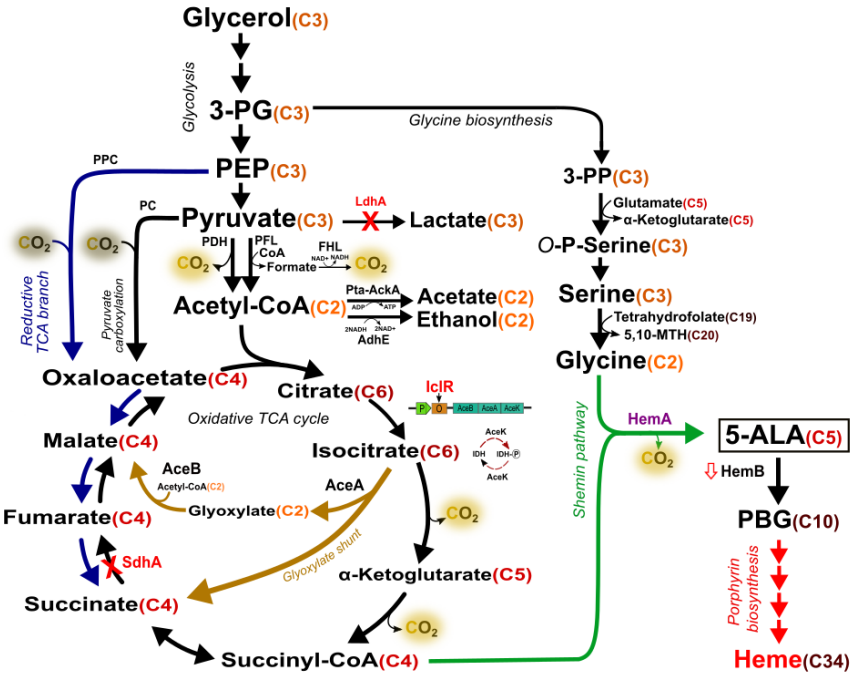
acetate and 5-APA percentage yields, and porphyrin biosynthesis (represented by the absorbance readings of the Soret peak in OD_{405} and Q-band in OD_{495} and the images of cell-free media) are shown. The percentage yields of acetate and 5-ALA are calculated based on the consumed glycerol at the end of cultivation. (**I**) AL-I: microaerobic, (**II**) AL-II: semi-aerobic, and (**III**) AL-III: aerobic. All values are reported as means +- SD (n = 2).

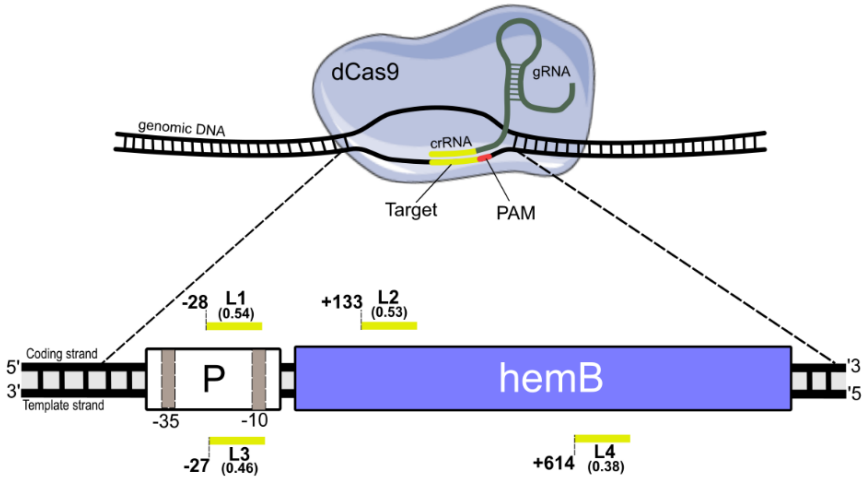
5. **Bioreactor cultivation of engineered *E. coli* for 5-ALA biosynthesis under microaerobic (AL-I) conditions.** Strains compared include DMH[?]*sdhA* , DMH-L4, and DMH-L4[?]*sdhA* . Time profiles of cell density (OD_{600}), glycerol consumption and metabolite production profiles, acetate and 5-APA percentage yields, and porphyrin biosynthesis (represented by the absorbance readings of the Soret peak in OD_{405} and Q-band in OD_{495} and the images of cell-free media) are shown. The percentage yields of acetate and 5-ALA are calculated based on the consumed glycerol at the end of cultivation. (**I**) DMH[?]*sdhA* , (**II**) DMH-L4, and (**III**) DMH-L4[?]*sdhA* . All values are reported as means +- SD (n = 2).
6. **Bioreactor cultivation of engineered *E. coli* for 5-ALA biosynthesis under aerobic (AL-III) conditions.** Strains compared include DMH-L4, DMH-L4[?]*sdhA* , DMH-L4[?]*iclR* , DMH[?]*sdhA* [?]*iclR* , and DMH-L4[?]*sdhA* [?]*iclR* . Time profiles of cell density (OD_{600}), glycerol consumption and metabolite production profiles, acetate and 5-APA percentage yields, and porphyrin biosynthesis (represented by the absorbance readings of the Soret peak in OD_{405} and Q-band in OD_{495} and the images of cell-free media) are shown. The percentage yields of acetate and 5-ALA are calculated based on the consumed glycerol at the end of cultivation. (**I**) DMH-L4 (**II**) DMH-L4[?]*sdhA* , (**III**) DMH-L4[?]*iclR* , (**IV**) DMH[?]*sdhA* [?]*iclR* , and (**V**) DMH-L4[?]*sdhA* [?]*iclR* . All values are reported as means +- SD (n = 2).

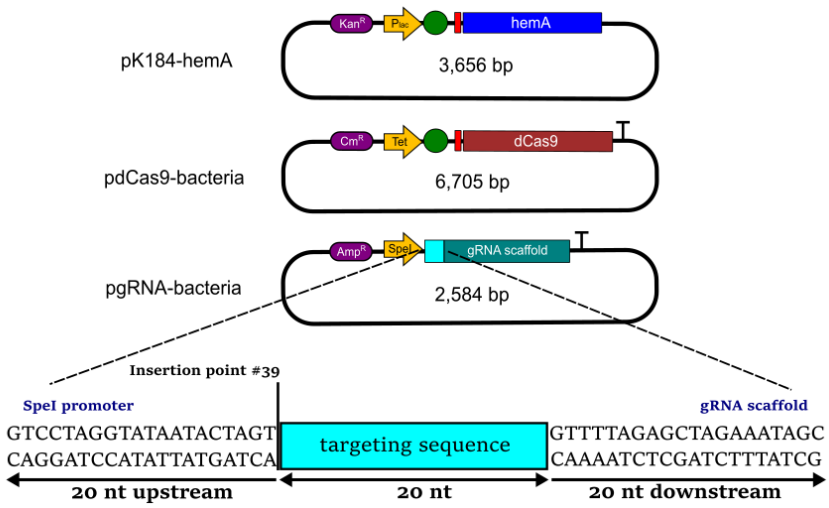
Name	**Description, relevant genotype or primer/oligo sequence (5' 3')**	**Source**
E. coli **host strains**		
DH5α	F-, *ενδA1, γλν˝44, τηι-1, ρεςA1, ρελA1, γψρA96, δεοP, νυπΓ φ80δ λaςZ[;]aςZδ λaδλaςZΨA – aργΦ) ϒ169, ησδP17(ρK-μK +),* λ-	Lab stock
BW25113	F-, [?]*(araD-araB)567, [?]lacZ4787(::rrnB-3),* -, *rph-1, [?](rhaD-rhaB)568, hsdR514*	(Datsenko and Wanner 2000)
BW[?]*ldhA*	BW25113 *ldhA* null mutant	(Srirangan et al. 2014)
DMH	BW[?]*ldhA*/pK-hemA	This study
DMH[?]*sdhA*	*sdhA* null mutant of DMH *sdhA*	This study This study
DMH[?]*sdhA*[?]*iclR*	and *iclR* mutants of DMH	
DMH-CT	DMH/pK-hemA/pgRNA-bacteria/pdcas9-bacteria	This study
DMH-L1	DMH/pK-hemA/pgRNA-L1/pdcas9-bacteria	This study
DMH-L2	DMH/pK-hemA/pgRNA-L2/pdcas9-bacteria	This study
DMH-L3	DMH/pK-hemA/pgRNA-L3/pdcas9-bacteria	This study
DMH-L4	DMH/pK-hemA/pgRNA-L4/pdcas9-bacteria	This study
DMH-L4[?]*sdhA*	*sdhA* null mutant of DMH-L4	This study
DMH-L4[?]*iclR*	*iclR* null mutant of DMH-L4	This study

Name	Description, relevant genotype or primer/oligo sequence (5' 3')	Source
DMH-L4[?]*sdhA*[?]*iclR*	*sdhA* and *iclR* null mutants of DMH-L4	This study
Plasmids		
pCP20 pK184	Flp+, λ cI857+, λ pR Rep(pSC101 ori)ts, ApR, CmR	(Cherepanov and Wackernagel 1995) (Jobling and Holmes
	p15A ori, KmR, P*lac::lacZ'*	1990)
pdcas9-bacteria	p15A ori, P_{Tet}-dCas9	(Qi et al. 2013)
pgRNA-bacteria	ColE1 origin, P_{J23119}-gRNA	(Qi et al. 2013)
pgRNA-L1	Derived from pgRNA-bacteria, P_{speI}::*hemB*-gRNA-L1	This study
pgRNA-L2	Derived from pgRNA-bacteria, P_{speI}::*hemB*-gRNA-L2	This study
pgRNA-L3	Derived from pgRNA-bacteria, P_{speI}::*hemB*-gRNA-L3	This study
pgRNA-L4	Derived from pgRNA-bacteria, P_{speI}::*hemB*-gRNA-L4	This study
pK-hemA	Derived from pTrc99a, P_{trc}::*phaAB*	This study
Primers/oligo sequences		
v-ldhA	GATAACGGAGATCGGGAATGATTAA; GGTTTAAAAGCGTCGATGTCCAGTA	(Alkawi et al. 2015)
v-sdhA	CTCTGCGTTCACCAAAGTGT; ACACACCTTCACGGCAGGAG	This study
v-iclR	GGTGGAATGAGATCTTGCGA; CCGACACGCTCAACCCAGAT	This study
c-frt	AGATTGCAGCATTACACGTCTTGCTG; CCAGCTGCATTAATGAATCGGGCCATGGTCCATATGAATATCCTCC	(Srirangan et al. 2014)

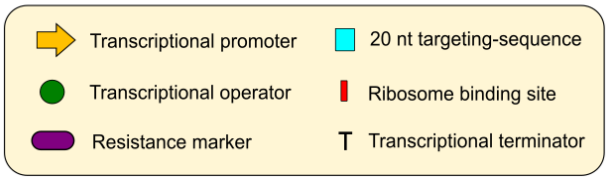
Name	Description, relevant genotype or primer/oligo sequence (5' 3')	Source
c-ptrc cf-gRNA q-hemB q-rrsA	CCGATTCATTAATGCAGCTGG;	(Srirangan et al. 2014) This
g-hemA g-pK-hemA g-pgRNA	GGTCTGTTTCCTGTGT-	study This study This study
hemA-gRNA-L1	GAAATTGTTA	This study This study This
hemA-gRNA-L2	ATCTTTGACAGC-	study This study This study
hemA-gRNA-L3	TAGCTCAGTCC;	This study This study
hemA-gRNA-L4	CAAGCTTCAAAAAAAG-	
	CACCGA	
	GCGCGCTATGTTTGAA-	
	GAGA;	
	TCGGTATGGTGAGAGAT-	
	GCC	
	TCCAGGTGTAGCGGT-	
	GAAAT;	
	TGAGTTTTAACCTTGCG-	
	GCC	
	CACAGGAAACAGCTAT-	
	GACCATGGACTA-	
	CAATCTGGCACTCGA;	
	GAGCTCGAATTCGTAAT-	
	CATTCAGGCAACGAC-	
	CTCGGC	
	GCGCCGAGGTCGTTGCCT-	
	GAATGATTACGAATTC-	
	GAGCTCGGTAC;	
	AGTGCCAGATTGTAGTC-	
	CATGGTCATAGCTGTTTC-	
	CTGTGTG	
	GTTTTAGAGCTA-	
	GAAATAGCAAGTT;	
	ACTAGTATTATACCTAG-	
	GACTGAGC	
	GTCCTAGGTATAATAC-	
	TAGT*CCCTCGATTCCACAAACATC*GTTTTAGAGCTAGAAATAGC;	
	GCTATTTCTAGCTC-	
	TAAAAC*GATGTTTGTGGAATCGAGGG*ACTAGTATTATACCTAGGAC	
	GTCCTAGGTATAATAC-	
	TAGT*AAAGCCGTTGAAGCCATGCC*GTTTTAGAGCTAGAAATAGC;	
	GCTATTTCTAGCTC-	
	TAAAAC*GGCATGGCTTCAACGGCTTT*ACTAGTATTATACCTAGGAC	
	GTCCTAGGTATAATAC-	
	TAGT*GCCTGATGTTTGTGGAATCG*GTTTTAGAGCTAGAAATAGC;	
	GCTATTTCTAGCTC-	
	TAAAAC*CGATTCCACAAACATCAGGC*ACTAGTATTATACCTAGGAC	
	GTCCTAGGTATAATAC-	
	TAGT*ATGCGCTTCCGGCAGCTTCA*GTTTTAGAGCTAGAAATAGC	
	GCTATTTCTAGCTC-	
	TAAAAC*TGAAGCTGCCGGAAGCGCAT*ACTAGTATTATACCTAGGAC	







hemB-gRNA Gibson oligo design



gRNA	20 nt targeting-sequence
hemB-gRNA-L1	CCCTCGATTCCACAAACATC
hemB-gRNA-L2	AAAGCCGTTGAAGCCATGCC
hemB-gRNA-L3	GCCTGATGTTTGTGGAATCG
hemB-gRNA-L4	ATGCGCTTCCGGCAGCTTCA

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`image11.emf` available at https://authorea.com/users/302765/articles/432841-strain-engineering-for-high-level-5-aminolevulinic-acid-production-in-escherichia-coli

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`image14.emf` available at https://authorea.com/users/302765/articles/432841-strain-engineering-for-high-level-5-aminolevulinic-acid-production-in-escherichia-coli