Biosynthesis of organic photosensitizer Zn-porphyrin by diphtheria toxin repressor (DtxR)-mediated global upregulation of engineered heme biosynthesis pathway in Corynebacterium glutamicum

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Zn-porphyrin is a promising organic photosensitizer in various fields including solar cells, interface and biomedical research, but the biosynthesis study has been limited, probably due to the difficulty of understanding complex biosynthesis pathways. In this study, we developed a Corynebacterium glutamicum platform strain for the biosynthesis of Zn-coproporphyrin III (Zn-CP III), in which the heme biosynthesis pathway was efficiently upregulated. The pathway was activated and reinforced by strong promoter-induced expression of hemAM (encoding mutated glutamyl-tRNA reductase) and hemL (encoding glutamate-1-semialdehyde aminotransferase) genes. This engineered strain produced 33.54 ± 3.44 mg/l of Zn-CP III, while the control strain produced none. For efficient global regulation of the complex pathway, the dtxR gene encoding the transcriptional regulator diphtheria toxin repressor (DtxR) was first overexpressed in C. glutamicum with hemAM and hemL genes, and its combinatorial expression was improved by using effective genetic tools. This engineered strain biosynthesized 68.31 ± 2.15 mg/l of Zn-CP III. Finally, fed-batch fermentation allowed for the production of 132.09 mg/l of Zn-CP III. This titer represents the highest in bacterial production of Zn-CP III reported to date, to our knowledge. This study demonstrates that engineered C. glutamicum can be a robust biotechnological model for the production of photosensitizer Zn-porphyrin.

Porphyrims functioning as photo-electrochemical materials are of particular interest in a range of areas related to solar cell technology, battery chemistry, solid/liquid interfaces and biomedical research because of their functional and structural properties that incorporate a metal ion in the center and have great absorption bands in the visible spectrum1–7. In particular, Zn-porphyrins derivatives have recently attracted much attention for their great potential application as a promising raw material for sensitizing an organic solar cell in a photovoltaic system that harvests light to convert solar energy into electricity, or in catalyzing multistep reactions at interface5,6,8–13. In addition to electronic and physicochemical applications, Zn-porphyrins have been studied as photosensizers for their antitumor and antibacterial activity in photodynamic therapy-mediated biomedical applications14. Furthermore, iron-porphyrin has been used to synthesize bio-based magneto-electric nanocrystals by the heme polymerase enzyme complex and demonstrated as a monomer for advanced nanomaterial synthesis15. Most

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porphyrins used in applications are commonly synthesized from chemical reactions, which have also made great efforts for the high yieldable, cost-efficient and eco-friendly process24–28. Bio-based production of a useful compound is an attractive and alternative route to be able to yield high production levels through a microbial cell factory using a renewable, cost-efficient and eco-friendly source such as glucose. The biological synthesis for important biochemical compounds has recently attracted much attention in various scientific fields such as biotechnology, biomedical, chemical engineering and environmental science19–22. However, biosynthesis researches for most porphyrins were rarely reported until recently. In living organisms, porphyrins are commonly biosynthesized through the long and complex heme biosynthesis pathway regulated by eight to nine enzymes. This biosynthesis is initiated from the enzymatic condensation of 5-aminolevulinic acid (ALA) as a common precursor to yield porphobilinogen (PBG). After that, coproporphyrin III (CP III) or protoporphyrin (PP IX), acting as a chassis for various metallated porphyrin derivatives, is biosynthesized from PBG by five or six enzymatic reactions25. Metallated porphyrins have various biological and physiological functions. Heme functions as an enzymatic cofactor and electron transfer, chlorophyll is used in photosynthesis, and CP III is an element carrier via chelation of certain metals24,25.

Corynebacterium glutamicum, a gram-positive, nonpathogenic and facultative anaerobic organism, has been frequently used and developed for large-scale production of a variety of amino acids such as l-glutamate and l-lysine, proteins, and monomers for various chemical compounds from renewable biomass20–31. Recently, we reported that the engineered C. glutamicum produced ALA, a precursor in the heme biosynthesis pathway23,33. ALA biosynthesis in almost all bacteria containing Escherichia coli and C. glutamicum is the rate-limiting step in the heme biosynthesis pathway34–36. Hence, ALA biosynthesis has been overcome by two different approaches: 1) engineering of the C5 pathway initiated from l-glutamate or 2) engineering of the C4 pathway initiated from succinyl-CoA with glycine34–36. The C5 pathway is processed by three enzymes including glutamyl-tRNA synthetase (encoded by the gltX gene) producing t-glutamyl-tRNA from l-glutamate, glutamyl-tRNA reductase (encoded by the hemA gene) reducing t-glutamyl-tRNA to glutamate-1-semialdehyde, and glutamate-1-semialdehyde aminotransferase (encoded by the hemL gene) biosynthesizing ALA from glutamate-1-semialdehyde by transamination25. The C4 pathway is processed by the ALA synthase (encoded by the alas gene) biosynthesizing ALA from succinyl-CoA with glycine25. Unlike studies about ALA biosynthesis, as mentioned above, there are only a few studies on the biosynthesis of a porphyrin in bacteria27–29. Porphyrin overproduction containing heme, PP IX and/or CP III has been reported in metabolically engineered E. coli via overexpression of a number of genes related to the heme biosynthesis pathway through several vectors27,28. In the case of C. glutamicum, porphyrin production has only been reported as an iron additive for heme production by expressing ALA synthase29. In particular, study on the biosynthesis of Zn-porphyrin has been rarely reported, and none reported Zn-porphyrin production in a genetically engineered strain30,32.

The heme biosynthesis pathway is long, complicated, and tightly regulated by various factors such as feedback inhibition by the final product and repression of gene expression by the transcriptional regulator29,30,35,41–43. In recent years, transcriptional regulation related to iron and heme homeostasis have been studied in C. glutamicum44–47. It was revealed that the global transcriptional regulator called diphtheria toxin repressor (DtxR) controlled the expression of genes encoding a variety of membrane protein and transcriptional regulators involved in iron storage, uptake and utilization. In particular, it has been confirmed that heme and iron metabolism are closely involved with each other for homeostasis and are tightly regulated by DtxR protein under conditions dependent on iron concentration13,45–47. Additionally, it has revealed that the heme responsive regulator activator HrrA (encoded by the hrrA gene) repressed the transcription of partial genes included in heme operon43. Indeed, when the hrrA gene, repressed by the DtxR protein, was deleted from the genome of C. glutamicum, the mRNA expression level of partial heme biosynthesis-related genes was upregulated43. However, the cell growth of the hrrA gene deletion strain was significantly decreased. The direct impact of the DtxR protein for porphyrin production on the control of heme metabolism has not been studied43.

In this study, we describe the establishment of the C. glutamicum platform strain overproducing Zn-CP III and other porphyrins using metabolic engineering for the first time, to our knowledge. We selected C. glutamicum as a host strain for Zn-porphyrin production in this study, because this strain overproduces l-glutamate as a starting material in the heme biosynthesis pathway and is a well-developed ALA producer in our previous study32. Metabolic engineering for the increased production of Zn-CP III requires enhanced metabolic flux from various metabolites toward coproporphyrin III. Strategies for the enhancement of Zn-CP III production were designed to activate and upregulate the heme biosynthesis pathway and are pictured in Fig. 1a and Supplementary Fig. S1. The strategy for activating the heme biosynthesis pathway is the overexpression of hemA44 (mutated hemA) and hemL and is reinforced by expressing two genes under the control of a strong promoter. Following this strategy, to achieve the global upregulation of heme operons (Fig. 1b), we first overexpressed DtxR protein into C. glutamicum with Hema44 and HemL proteins. The combinatorial expression of target genes by effective genetic tools achieved the significant decrease of mRNA expression of the hrrA gene, the substantial increase of mRNA expression of heme biosynthesis-related genes, and the highest production of Zn-CP III over engineered strains. Finally, we successfully achieved the biosynthesis of Zn-CP III in fed-batch fermentation supplemented with a zinc source. This study demonstrates that engineered C. glutamicum has potential industrial applications as a platform for Zn-porphyrin production.

Results
Enhanced activation of the heme biosynthesis pathway by the expression of hemA44 and hemL genes under the strong tac promoter. ALA production is an important first step for enhanced production of porphyrin derivatives because almost all porphyrins are commonly biosynthesized in the heme biosynthesis pathway using eight molecules of ALA as the precursor32. We previously confirmed the potential of engineered C. glutamicum as an ALA-producing strain that coexpressed mutated hemA (derived from Salmonella
typhimurium) and hemL (derived from E. coli) genes by the expression vector pMT-trc containing the trc-derived promoter and pCG1 origin of replication, named the pHemAL vector (pMT-trc::hemAML through this article)32. In this study, to investigate its potential as a strain for the production of ALA and porphyrins, the engineered strain harboring the pMT-trc::hemAML vector was tested. Furthermore, for strong expression of target genes, hemA and hemL genes were cloned in the other expression vector pMT-tac replacing trc-derived promoter with the strong tac promoter (Supplementary Fig. S1). To analyze metabolites produced, the control strain harboring the empty vector and engineered C. glutamicum strains harboring the pMT-trc::hemAML and pMT-tac::hemAML vectors (named CGPB01 and CGPB02 throughout this article, respectively) were cultivated in modified CGXII medium, adding penicillin G at 12 h for l-glutamate overproduction. Compared with the CGPB01 strain, which produced 338.85 ± 5.44 mg/l of ALA, the CGPB02 strain showed a 1.77-fold increase in the

Figure 1. A schematic overview describing the target pathway and overall metabolic strategies for Zn-porphyrin production in C. glutamicum. (a) The heme biosynthesis pathway used in this study. Glucose starting from glucose is a chain of reactions to form pyruvate, which enters tricarboxylic acid (TCA) cycle with acetyl-CoA. TCA cycle is a series of biochemical reactions providing essential biochemical intermediates, such as 2-oxoglutarate, succinyl-CoA, etc., for cellular energy production and amino acid biosynthesis containing l-glutamate and l-glycine. Zinc-porphyrin biosynthesis is initiated from l-glutamate. The red letter represents the gene overexpression in C. glutamicum. The T-shape and red X-shape denote the gene repression by the transcriptional regulator and inactivation of the HrrA protein by gene repression by the DtxR protein, respectively. The solid-line arrow and dashed-line arrow indicate the general metabolic pathway and abbreviated metabolic pathway, respectively. The green arrow denotes enhanced metabolic flux by the combinatorial overexpression of hemA34, hemL and dtxR genes. The width of each green arrow is proportional to the result of the mRNA expression level of heme biosynthesis genes when the DtxR protein was overexpressed with HemA34 and HemL protein. Purple and black colored chemicals are the target product Zn-CP III and porphyrin chassis CP III, respectively. (b) The regulatory mechanism of the transcriptional regulator DtxR in C. glutamicum. The DtxR protein and HrrA protein bind in the promoter region in front of target genes, respectively, repress the gene expression of those43,45,47. Major symbols were described in the dashed black line box. The black line arrow shows processing of regulatory mechanisms of the HrrA protein. Abbreviations: GSA, l-glutamate 1-semialdehyde; ALA, 5-aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; UPG III, uroporphyrinogen III; CPG III, coproporphyrinogen III; CP III, coproporphyrin III; Zn-CP III, zinc-coproporphyrin III; UP III, uroporphyrin III; HemA, glutamyl-tRNA reductase; HemL, glutamate-1-semialdehyde 2,1-aminomutase; HemB, porphobilinogen synthase; HemC, hydroxymethylbilane synthase; HemD, uroporphyrinogen-III synthase; HemE, uroporphyrinogen decarboxylase; HemY, coproporphyrinogen III oxidase; DtxR, diphtheria toxin repressor; HrrA, heme responsive regulator activator.
Maximum cell growth was not affected by the expression of hemA M and Paracoccus denitrificans. It has been revealed that not only CP III acted as a chelate of zinc, but also Zn-CP III was biosynthesized from CP III produced in bacteria including wild type. Numbers per cell, and a pCG1 origin of replication contained in pMT-tac and pBL1 origin of replication. A pBL1 origin of replication contained in Shuttle vector pEKEx2 shows 8–30 copy numbers per cell, and a pCG1 origin of replication contained in pMT-tac shows 13–22 copy numbers per cell. Furthermore, while the control strain showed no production of any porphyrins except heme, the CGPB02 strain produced 33.32 ± 12.26 mg/l of uroporphyrin III (UP III), 23.68 ± 4.98 mg/l of CP III, and 16.56 ± 0.98 mg/l of heme, which was 7.79-fold, 3.04-fold, and 1.61-fold more than the CGPB01 strain, respectively (Fig. 2). Additionally, 22.38 ± 1.37 and 73.56 ± 17.89 mg/l of total porphyrins were produced in CGPB01 and CGPB02, respectively. Maximum cell growth was not affected by the expression of hemA M and hemL genes under the tac promoter (Supplementary Fig. S2). This result indicates that the expression of hemA M and hemL genes is important for activation of the heme biosynthesis pathway in C. glutamicum, and the expression under the tac promoter was strong enough to increase the metabolic flux of the heme biosynthesis pathway compared with the trc-derived promoter.

Enhanced metabolic flux toward a porphyrin chassis CP III by the combinatorial expression of the hemA M, hemL and dtxR genes. In this study, we selected CP III as a final chassis for Zn-porphyrin biosynthesis. It has been revealed that not only CP III acted as a chelate of zinc, but also Zn–CP III was biosynthesized from CP III produced in bacteria including wild type Paracoccus denitrificans and Streptomyces sp. in culture medium supplemented with zinc sulfate. As mentioned above, the transcription regulation mechanism between partial heme operon and the HrrA protein in C. glutamicum has been studied, although the cell growth of the hrrA gene deletion strain was significantly decreased. Hence, we hypothesized that the DtxR protein may serve as an effective enhancer of the heme biosynthesis pathway. To verify this hypothesis, the dtxR gene (derived from C. glutamicum) encoding the DtxR protein was introduced into the control strain and the CGPB02 strain, named the CGPB03 and CGPB04 strains, respectively. Contrary to expectations, while the production of CP III and UP III in the CGPB03 strain was not detected, the CGPB04 strain showed a 1.82-fold and 2.17-fold increase (60.90 ± 3.26 and 51.35 ± 1.69 mg/l) of UP III and CP III production over the CGPB02 strain, respectively (Fig. 3a). Although the overexpression of the hemA M and hemL genes somewhat delayed the exponential growth phase, whether or not the dtxR gene was present, the maximum cell growth and glucose consumption were not affected (Fig. 3b,c). These results show that the combinatorial expression of dtxR with hemA M and hemL not only overcomes the rate-limiting step but also improves CP III production by enhancing the metabolic flux of the heme biosynthesis pathway without the critical negative effect of cell growth.

To enhance the metabolic flux of the heme biosynthesis pathway toward CP III, the hemA M, hemL and dtxR genes were additionally cloned in an E. coli–C. glutamicum shuttle vector pEKEx2 containing the tac promoter and pBL1 origin of replication. A pBL1 origin of replication contained in Shuttle vector pEKEx2 shows 8–30 copy numbers per cell, and a pCG1 origin of replication contained in pMT-tac shows 13–22 copy numbers per cell. Expression of the hemA M, hemL and dtxR genes using the pEKEx2 vector was more effective at producing CP III than using the pMT-tac vector. Compared with the CGPB04 strain, the engineered strain harboring pEKEx2::hemA M::dtxR (named the CGPB07 strain herein) showed a 1.22-fold increase in CP III production over the CGPB04 strain (Supplementary Fig. S3). Although the overexpression of the hemA M and hemL genes somewhat delayed the exponential growth phase, whether or not the dtxR gene was present, the maximum cell growth and glucose consumption were not affected (Fig. 3b,c). These results show that the combinatorial expression of hemA M, hemL and dtxR not only overcomes the rate-limiting step but also improves CP III production by enhancing the metabolic flux of the heme biosynthesis pathway without the critical negative effect of cell growth.

The DtxR protein uses two ferrous iron as an essential cofactor. Hence, cultivation with various concentrations of iron was performed on the CGPB04 and CGPB07 strains for enhanced production of CP III. However, along with increasing the iron concentration, CP III production in both the CGPB04 and CGPB07 strains
decreased constantly while heme production was increased (Fig. 3d, Supplementary Fig. S4). These results mean that ferrous iron added in the culture medium may be used in processes to biosynthesize heme from CP III.

Global upregulation of the heme biosynthesis pathway by the transcriptional regulator DtxR. It has been reported that the mRNA expression level of hemA, hemC (encoding porphobilinogen deaminase), hemE (encoding uroporphyrinogen decarboxylase) and hemY was decreased when the hrrA gene was deleted in C. glutamicum 43. However, transcriptional analysis of hemB and hemD (encoding uroporphyrinogen-III synthase) genes in the hrrA gene deletion strain as well as the transcriptional regulation of the heme biosynthesis pathway by the overexpression of the DtxR protein has not been studied yet. In particular, the DtxR protein has never been used for porphyrins production, to our knowledge. To investigate the positive effect of the DtxR protein in enhancing the metabolic flux of the heme biosynthesis pathway, we analyzed the relative mRNA expression of heme biosynthesis-related genes when the DtxR protein was overexpressed. Compared to the CGPB02 strain, the mRNA expression levels of the hemA, hemL, hemC, hemD, hemE and hemY genes in the genome directly involved in CP III production after the precursor synthesis were all increased in the CGPB04 strain, which showed 10.83 ± 1.21-, 1.77 ± 0.46-, 1.48 ± 0.22-, 4.50 ± 0.55-, 2.15 ± 0.27- and 3.23 ± 1.19-fold increases respectively (Fig. 4). Furthermore, the mRNA expression level of the hrrA gene in the CGPB04 strain was significantly decreased 0.28 ± 0.02-fold over the CGPB02 strain. However, the hemB mRNA expression level did not change, and this result means that the hemB gene may not be regulated by the HrrA protein. Taken together, these data indicate that genes related to the heme biosynthesis pathway were mostly upregulated by hrrA gene repression via overexpression of the DtxR protein, which may result in increasing the total porphyrin production in the engineered strain. In addition, the mRNA expression levels of the hemA, hemL, hemC, hemD, hemE and hemY genes in the CGPB07 strain were more increased than those of the CGPB04 strain, and showed the 35.55 ± 3.37-, 1.89 ± 0.18-, 37.40 ± 8.58-, 5.79 ± 0.41-, 22.02 ± 2.20- and
7.92 ± 1.88-fold increase over the CGPB02 strain, respectively. The hrrA mRNA expression level in the CGPB07 strain was more decreased than that of the CGPB04 strain, showed the 0.14 ± 0.01-fold decrease over the CGPB02 strain. The result means that the mRNA expression of heme biosynthesis-related genes may be improved by the hrrA gene repression by the improved expression of the DtxR protein through the pEKEx2 vector.

Zn-CP III biosynthesis by engineered *C. glutamicum* strains by the combinatorial expression of the hemAM, hemL and dtxR genes. For Zn-CP III production, CP III-producing engineered strains were shake-cultivated in a glucose-minimal medium supplemented with zinc sulfate for 72 h, and most engineered strains except for the CGPB03 strain successfully produced Zn-CP III (Fig. 5a). Compared with the CGPB02 strain that produced 33.54 ± 3.44 mg/l of Zn-CP III, the CGPB04 and CGPB07 strains showed 1.65- and 2.04-fold increases in Zn-CP III production, corresponding to 55.44 ± 1.58 and 68.31 ± 2.15 mg/l, respectively. Similarly, as in the case of CP III, the CGPB07 strain showed the highest titer of Zn-CP III in bacteria reported to date, and the growth pattern and glucose consumption of all engineered strains were rarely affected (Fig. 5b,c).

Interestingly, relative to the total titer of CP III produced in the CGPB07 strain cultured without zinc sulfate, the total titer of Zn-CP III produced in the strain cultured with zinc sulfate was increased to 68.31 ± 2.15 mg/l from 62.54 ± 2.09 mg/l of CP III without certain genetic modifications, corresponding to a further 4.4% increase (Figs 3a, 5a). On the other hand, the total titer of heme produced in the strain cultured with zinc sulfate was decreased compared to that produced in the strain cultured without zinc sulfate, from 17.08 ± 0.10 mg/l to 2.64 ± 0.32 mg/l (Fig. 5d). This result means that CP III was partially used in Zn-CP III production rather than in heme production. The absorption spectrum of metabolites produced in the CGPB07 strain when zinc sulfate was supplemented showed not only the strongest feature at 406 nm but also minor features at 538 and 574 nm, representing optical characteristics of Zn-CP III (Supplementary Fig. S5) similar to that of previous studies. Typically, CP III has a maximum Soret band at 393 nm, consistent with the absorption maximum of metabolites produced in the strain cultured without zinc sulfate. These results suggest that engineered *C. glutamicum* strains successfully produced Zn-CP III, and efficiently used robust porphyrin metabolism to biosynthesize Zn-CP III.

Fed-batch fermentation for enhanced biosynthesis of Zn-CP III in engineered *C. glutamicum*. To test the potential of the CGPB07 strain for large-scale production of Zn-CP III, we carried out 1.8-l fed-batch fermentation in a 5 l fermenter (Fig. 6). Glucose feeding was initiated after 15 h of fermentation and was additionally injected whenever the glucose concentration was below 20 g/l. During fed-batch fermentation of the CGPB07 strain, the maximum titer reached 132.09 mg/l of Zn-CP III in 82 h of fermentation in which the total input of glucose was 350.5 g. The overall yield and productivity were 1.27 mg/g glucose and 1.61 mg/l/h, respectively. The specific growth rate in the exponential growth phase (between 8 and 20h) was 0.12/h. The maximum OD600 was 131.33, corresponding to the measured cell biomass of 37.04 g/l at the end of fermentation.

Discussion

Much research has recently advanced Zn-porphyrin and porphyrin derivatives as promising raw material for solar cell devices, solid/liquid interface and photodynamic therapy over the past several years. Until now, the development of porphyrin production in bacteria has lagged behind precursor ALA production presumably because of the complex synthetic processes and regulatory mechanisms of the heme biosynthetic pathway. Toriya et al.
found that wild type Streptomyces sp. strain AC8007 biosynthesizes Zn-CP III (10 mg/l), but there was no metabolic design for overproduction of Zn-CP III in this wild type strain. Kwon et al. reported successful production of CP III, PP IX and heme (89 ± 9 μmol/l of total porphyrins, corresponding to approximately 50 mg/l of total porphyrins) in E. coli by overexpressing eight genes related to heme biosynthesis. However, the introduction of many genes and vectors may require excessive use of antibiotic resistance markers and reduce cell growth by metabolic burden. Indeed, engineered E. coli strains, named as the ECPB01, ECPB02 and ECPB03 strains, less produced Zn-CP III than engineered C. glutamicum strains, corresponding to 1.93 ± 0.05 mg/l, 1.87 ± 0.07 mg/l and 1.84 ± 0.05 mg/l, respectively (Supplementary Fig. S6). The porphyrin production in the engineered E. coli strain overexpressing the HemA and HemL proteins (C5 pathway) in this study was similar to that in the engineered strain overexpressing the ALA synthase (C4 pathway) used in the study of Kwon et al. Furthermore, the DtxR protein only worked in C. glutamicum, but not in E. coli (Fig. 3a, Supplementary Figs S3, S6). This result means that C. glutamicum is suitable for Zn-CP III and have the possibility of performing the further metabolic engineering. In the field of microbial engineering, pertinent gene expression and regulation of the target biosynthesis pathway are important factors for high-yield production of a target product. In this study, we confirmed the potential of C. glutamicum as a platform strain for Zn-CP III and porphyrin production. The activation and upregulation of the long and complex heme biosynthesis pathway by the transcriptional regulator DtxR alongside HemA and HemL proteins in C. glutamicum can allow for efficient biosynthesis of Zn-CP III. Furthermore, target gene expression by the efficient genetic tool such as replacement of the strong promoter and origin of replication achieved the high production of CP III, Zn-CP III and Heme. Furthermore, the mRNA expression of most heme biosynthesis genes except for the hemB gene was significantly improved in the CGPB07 strain (Fig. 4). Interestingly, while the hemD gene, which is located at 500-bp upstream of the hemB gene, was upregulated by
the hrrA protein repressed, the hemB gene expression was not affected in both the CGPB04 and CGPB07 strains. Even though the hemB gene is close to the hemD gene in genome, their expression may be regulated by different expression factors containing a promoter and repressor because it is predicted that they do not exist as an operon (an operon was predicted at http://www.coryneregnet.de/)43. Based on these results, it can be speculated that hemB and hemD genes are individually expressed by different factors and that there exists no binding site for the HrrA protein in the promoter region of the hemB gene. Frunzke et al. has identified that the HrrA protein bound in the promoter region of hemA and hemE genes although the promoter region of hemD and hemB genes remained unclear41. Nonetheless, the enhanced mRNA expression level of most heme biosynthesis genes by the combinatorial expression of hemAM, hemL and dtxR genes induced substantial porphyrin production (Figs 3a, 4, Supplementary Fig. S3). Interestingly, in the CGPB03 strain overexpressing the dtxR gene alone, most of the porphyrins except for heme were not produced (Supplementary Fig. S3). We suggest the reason may be because the native HemA protein overexpressed by the DtxR protein is feedback-inhibited by the final product heme41–43.

In terms of the insertion of a zinc ion into metal-free porphyrin, CP III may be more suitable for Zn-porphyrin synthesis than PP IX because CP III has the characteristic of spontaneous chelation of a zinc ion without any energy requirement while PP IX requires an enzyme reaction for Zn-PP IX biosynthesis41,42,44. Indeed, as described in our results regarding CP III, Zn-CP III and heme concentration, CP III generated for subsequent biosynthesis pathways was used for Zn-CP III production instead of heme production (Figs 3, 5). Dailey et al. recently revealed that the Actinobacteria and Firmicutes uses CP III instead of PP IX in heme biosynthesis44. C. glutamicum, which belongs to phylum Actinobacteria, is able to biosynthesize heme from a coproporphyrin-dependent pathway. As reported in Fig. 3, CP III accumulation was shown in metabolites produced in the CGPB02, CGPB04, and CGPB07 strains. However, none of the engineered strains constructed in this study produced PP IX despite increased mRNA expression levels of the hemN gene (encoding coproporphyrinogen III oxidase) (Supplementary Fig. S7). Furthermore, we confirmed that PP IX was produced when coproporphyrinogen III oxidase derived from E. coli with hemA41, hemL and dtxR genes was heterogeneously expressed in C. glutamicum (Supplementary Fig. S8). This observation suggests that C. glutamicum may not have a protoporphyrin-dependent pathway for heme biosynthesis, and therefore is suitable in terms of being a coproporphyrin-producing platform strain.

Even though the mRNA expression levels of the hemA, hemL, hemB, hemC, hemD, hemE and hemY genes for CP III production, as well as that of the hemH gene, were increased, heme production in the CGPB04 strain was slightly decreased, compared with the CGPB02 strain (Supplementary Figs S3, S7). Regarding this result, we speculate that the DtxR protein and heme competed for ferrous ion as a cofactor and required metal ions, respectively. Although the heme production from CP III was increased in the culture supplemented with iron sulfate of the CGPB07 strain, the bioconversion yield from CP III to heme was still low at 31.55 ± 3.39% (Fig. 3d). It is tempting to speculate that the final biosynthetic section from CP III to heme may be a major bottleneck for heme biosynthesis. Kwon et al. suggested that the limited transport of a metal ion into the cell and the low activity of the HemH protein in vivo may limit heme biosynthesis in the recombinant E. coli37. It has been recently reported that heme biosynthesis in the coproporphyrin-dependent pathway is performed by the HemQ protein which decarboxylates coproheme resulting in heme45–47. Hence, a mechanistic and enzymatic perspective of the coproporphyrin-dependent pathway in C. glutamicum is required for enhanced heme production. Nevertheless, our result regarding heme concentration in the CGPB07 strain showed the high-level production, corresponding to 29.57 ± 2.46 mg/l (Fig. 3d).

In summary, we demonstrated the possibility of C. glutamicum as a platform strain for the microbial production of Zn-porphyrin Zn-CP III as well as heme, and the titer of the porphyrins in the final engineered strain CGPB07 are the highest, reported to date in bacteria (Figs 3d, 6, Supplementary Fig. S3). These bio-based
compounds can be used as a valuable material in various research related to solar cell photosensitizers, biological sensors as well as biomedical application such as antimicrobial, antitumor and anticancer photodynamic therapy\(^1\)\(^{-15}\),\(^\text{58,59}\). In further study, large scale production of Zn-porphyrin in bacteria by microbial and metabolic engineering can be directly applied to solar cell technology and biomedical research. Indeed, Toriya et al. has directly applied Zn-CP III biosynthesized from wild type Streptomyces sp. used in their previous study to photodynamic therapy for antitumor effect in mice\(^60\),\(^\text{59}\). To enhance porphyrin biosynthesis and increase the diversity of types of porphyrins, further microbial engineering in C. glutamicum should be performed, including rational pathway engineering of the endogenous metabolism toward increased availability of an additional rate-limiting step, fine-tuning of enzyme engineering for improving target enzymes representing low activity, and the development of transport systems for efficient substrate supply.

**Materials and Methods**

**Reagents and chemicals.** Cell culture media used in this study was purchased from Daejung chemical Co. (Korea) and BD Bioscience (USA). Genomic DNA and plasmid DNA were prepared using Wizard\textsuperscript{\textregistered} Genomic DNA Purification Kit (Promega, USA) and GeneAll\textsuperscript{\textregistered} Exprep Plasmid Kit (GeneAll, Korea), respectively. Oligo synthesis and DNA sequencing were performed by Macrogen (Korea) and Cosmogenetech (Korea), respectively. Restriction enzymes, Taq DNA polymerase and T4 DNA ligase were purchased from New England Biolabs (USA), GeneAll (Korea) and Promega (USA), respectively. DNA fragments treated by either polymerase, ligase or restriction enzymes were purified by GeneAll\textsuperscript{\textregistered} Expin\textsuperscript{TM} Gel SV Kit (GeneAll, Korea). HPLC-grade chemicals such as acetonitrile and methanol were purchased from Duksan (Korea). 5-Aminolevulinic acid hydrochloride (Sigma-Aldrich, USA), Uroporphyrin III dihydrochloride (Echelon Biosciences, USA), coproporphyrin III dihydrochloride (Santa Cruz biotechnology, USA), protoporphyrin IX (Sigma-Aldrich) and heme (Sigma-Aldrich, USA) were used as standard chemicals for the quantitative analysis. Zn-CP III was prepared by the modified method described by Anttila et al.\(^24\). For quantitative real time-PCR (qRT-PCR) analysis, Hybrid-R prep kit, M-MLV reverse transcriptase and HiPi Real-Time PCR 2x Master Mix (SYBR green) were purchased from GeneAll (Korea), Pioneer (Korea) and Elpisbio (Korea), respectively.

**Strains and plasmids.** All plasmids and bacterial strains constructed and used in this study are listed in Table 1. E. coli DH5\textalpha was chosen as the cloning host strain, and C. glutamicum ATCC 13826 was used in the construction of recombinant strains and experiments for porphyrin production in this study. S. typhimurium, E. coli and C. glutamicum strains were used to obtain hemA, hemL and dtxR genes from genomic DNA, respectively.

All oligonucleotides for the construction of recombinant vectors were listed in Supplementary Table S1. pMT-\textit{tac} and pEKEx2 vectors were expressed as E. coli and C. glutamicum shuttle vectors and for the expression of target genes in C. glutamicum. To express \textit{hemA}\textsuperscript{M} and \textit{hemL} genes under the control of the \textit{tac} promoter, the \textit{hemA}\textsuperscript{M} and \textit{hemL} gene operon was amplified with \textit{HemA}\textit{M} BamHI F and \textit{HemL} NotI R primers using pMT-\textit{tac}:\textit{hemA}\textsuperscript{M}L vector as a template. The \textit{hemA}\textsuperscript{M}:\textit{hemL} gene fragment was ligated into \textit{BamHI}-\textit{NotI} restriction sites of the pMT-\textit{tac} vector by T4 DNA ligation, resulting in the pMT-\textit{tac}:\textit{hemA}\textsuperscript{M}L vector. For the construction of pMT-\textit{tac}:\textit{hemA}\textsuperscript{M}SL and pMT-\textit{tac}:\textit{hemA}\textsuperscript{M}L vectors, the \textit{dtxR} gene was amplified with DtxR NotI F and DtxR NotI R primers using pMT-\textit{tac}:\textit{hemA}\textsuperscript{M}L and pMT-\textit{tac} vectors as a template, respectively. To prevent self-ligation by one-cut restriction digestion, the digested sample was treated with Cloned Alkaline Phosphatase (Takara, Japan). The \textit{dtxR} gene fragment was ligated into the NotI restriction sites of pMT-\textit{tac}:\textit{hemA}\textsuperscript{M}L and pMT-\textit{tac} vectors, resulting in pMT-\textit{tac}:\textit{hemA}\textsuperscript{M}LdtxR and pMT-\textit{tac}:dtxR vectors. In the same manner, the pEKEx2:2:hemA\textsuperscript{M}L vector was constructed by means of restriction digestion and ligation into \textit{BstI}-\textit{Sall} sites of the pEKEx2 vector. The \textit{dtxR} gene was also introduced into \textit{BamHI} sites of pEKEx2 and pEKEx2:hemA\textsuperscript{M}L vectors, resulting in pEKEx2:dtxR and pEKEx2:hemA\textsuperscript{M}LdtxR. Except for an adjacent gene from a promoter, a ribosomal binding site (AAGGAG) was inserted between downstream of the stop codon and upstream of the start codon in individual genes. Transformation of all recombinant vectors in E. coli and C. glutamicum was performed by the methods previously described\(^27\).

**Culture medium and conditions.** For the construction of engineered strains, E. coli and C. glutamicum were grown overnight in shaking cultivation at 37 °C and 30 °C, respectively, and 200 rpm in Luria–Bertani medium. C. glutamicum wild type and engineered strains were grown overnight in shaking cultivation at 30 °C and 150 rpm in brain–heart infusion (BHI) –. The seed culture for porphyrin production was washed once with modified CGXII medium and transferred to fermenter containing 1.8 l modified CGXII medium, 40 g/l glucose, 1 mM IPTG and 25 mg/l kanamycin. The fermentation was performed at 30 °C and 300 to 600 rpm. The pH was automatically maintained at 6.8 by 4 M NaOH and 5 M H\textsubscript{2}SO\textsubscript{4}. Air aeration rate and dissolved oxygen were maintained at 1...
Analytical methods.  Cell growth of all strains used in this study was measured at OD$_{600}$ using a UV–vis spectrophotometer (Mecasys Co., Ltd., Korea). The dry cell weight was experimentally determined on the basis of the correlation model OD$_{600}$ = 0.282 g dry cell weight (DCW)/l and used for the calculation of biomass concentration of fed-batch fermentation. Glucose concentration was estimated using a Glucose (GO) Assay Kit (Sigma-Aldrich). When glucose was lower than 20 g/l, 50% glucose stock solution was manually fed.

The absorbance was determined at 400 nm. The data were estimated using Waters Empower-3 software. ALA concentration was measured using the colorimetric assay called Ehrlich's reagent$^{61}$. One volume of the supernatant (Sigma-Aldrich, USA). For measurement of intracellular metabolites, 1 ml of cell pellet was harvested by centrifugation (13,000 rpm at 4 °C for 3 minutes), and the supernatant excluding the cell pellet was used for the measurement of extracellular metabolites. For the extraction of the metabolite from cells, including inter alia, heme (Sigma-Aldrich). When glucose was lower than 20 g/l, 50% glucose stock solution was manually fed.

Table 1.  Bacterial strains and plasmids used in this study.  1Invitrogen Corporation, Carlsbad, California, USA.  bAmerican Type and Culture Collection, Manassas, USA.
RNA Preparation and qRT-PCR. To prepare total RNA, the CBGP02 and CBGP04 strains were cultivated in modified CGXII medium and harvested at 12 h. Total RNA in the cell pellet was extracted using a Hybrid-R prep kit (GeneAll, Korea) according to the manufacturer’s instructions. Total RNA with random primer was denatured at 65 °C for 10 minutes, and reverse transcriptionally transcribed to cDNA templates by M-MLV reverse transcriptase. All oligonucleotides for qRT-PCR were designed based on target gene sequence (Supplementary Table S2). qRT-PCR analysis was determined using a StepOnePlus thermocycler (Thermo Fisher Scientific, USA) with HiPi Real-Time PCR 2x Master Mix (SYBR green) according to the manufacturer’s instructions. The mRNA level of each gene of the CBGP04 strain was calculated relative to those of the CBGP02 strain using the comparative ΔΔCT method and was always normalized using the 16S rRNA level as an internal standard.

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Author Contributions

Y.J.K. and S.O.H. designed research. Y.J.K. performed research and wrote the manuscript. Y.C.J., J.E.H., S.W.K., C.P. and S.O.H. revised and edited the manuscript. Y.J.K., E.L., M.E.L. and J.S. performed fed-batch fermentation. All authors reviewed and commented on the manuscript.

Additional Information

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