

Title	Metabolic Engineering of the 5-Aminolevulinate Biosynthetic Pathway in E. coli Improves Efficiency of Hemoprotein-Based Biocatalysis
Author(s)	Kato, Shunsuke; Abe, Miteki; Okahashi, Nobuyuki et al.
Citation	Angewandte Chemie - International Edition. 2025, p. e202512156
Version Type	VoR
URL	https://hdl.handle.net/11094/102758
rights	This article is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License.
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

The University of Osaka



Communication

Angewandte

International Edition Chemie

www.angewandte.org

Biocatalysis

How to cite: Angew. Chem. Int. Ed. **2025**, e202512156 doi.org/10.1002/anie.202512156

Metabolic Engineering of the 5-Aminolevulinate Biosynthetic Pathway in *E. coli* Improves Efficiency of Hemoprotein-Based Biocatalysis

Shunsuke Kato,* Miteki Abe, Nobuyuki Okahashi, Shinya Ariyasu, Fumio Matsuda, Osami Shoji, and Takashi Hayashi*

Abstract: Biocatalysis using heme-dependent enzymes provides a powerful synthetic platform to facilitate a variety of chemical transformations required for organic synthesis. Despite recent advances in biocatalysis, recombinant expression systems for hemoproteins leave much room for improvement due to the strict regulation of heme biosynthesis in the host organism. To develop an efficient cofactor supplementation system for the expression of active holohemoproteins, we describe metabolic engineering of the heme biosynthetic pathway in *E. coli*. Through incorporation of a heterogeneous C4 pathway involving 5-aminolevulinic acid synthase of *Paracoccus denitrificans*, it was found that the concentrations of 5-aminolevulinic acid and heme in the engineered cells are increased during cultivation, and the expression level of the holohemoproteins is significantly improved. Notably, the heme content in the engineered cells is even higher than that produced by conventional cultivation methods, which add 5-aminolevulinic acid into the culture medium. Furthermore, we also demonstrate the application of this engineered *E. coli* cells in whole-cell and lysate-based biocatalysis using various types of heme-dependent enzymes. Considering the recent demand for biocatalysis, the system developed in this study will serve as a new practical and versatile platform for hemoprotein-based biocatalysis.

With recent significant advances in biotechnology, biocatalysis has emerged as a powerful synthetic platform which provides straightforward access to a wide variety of chemical feedstocks.^[1] In particular, the area of chemical transforma-

[*] Dr. S. Kato, M. Abe, Prof. Dr. T. Hayashi Department of Applied Chemistry, Graduate School of Engineering, The University of Osaka, 2-1 Yamadaoka, Suita, Osaka 565–0871, Japan

E-mail: s_kato@chem.eng.osaka-u.ac.jp thayashi@chem.eng.osaka-u.ac.jp

Dr. N. Okahashi, Prof. Dr. F. Matsuda Department of Bioinformatic Engineering, Graduate School of Information Science and Technology, The University of Osaka, 1–5 Yamadaoka, Suita, Osaka 565–0871, Japan

Dr. N. Okahashi, Prof. Dr. F. Matsuda Department of Biotechnology, The University of Osaka and Shimadzu Analytical Innovation Laboratories, Graduate School of Engineering, The University of Osaka, 2-1 Yamadaoka, Suita, Osaka 565–0871, Japan

Dr. N. Okahashi, Prof. Dr. F. Matsuda Industrial Biotechnology Initiative Division, Institute for Open and Transdisciplinary Research Initiatives, The University of Osaka, 2-1 Yamadaoka, Suita, Osaka 565–0871, Japan

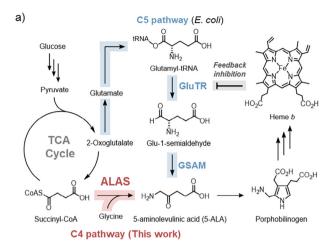
Dr. S. Ariyasu, Prof. Dr. O. Shoji Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464–8602, Japan

- Additional supporting information can be found online in the Supporting Information section
- © 2025 The Author(s). Angewandte Chemie International Edition published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

tion using hemoproteins is one of the most representative and well-studied models of biocatalysis. As exemplified by pioneering studies on the engineering of cytochrome P450s for carbene^[2-4] and nitrene^[5-8] transfer reactions, hemoproteins have demonstrated versatile catalytic activities not only for naturally occurring reactions found in nature but also for a variety of abiotic chemical transformations required for modern synthetic chemistry.[9-21] In spite of these significant advances, recombinant expression systems for hemoproteins still require improvements to encourage their use in practical applications. Because biosynthesis of the heme cofactor is tightly regulated in host organisms, [22] overexpression of recombinant hemoproteins often leads to the undesired accumulation of inactive apoenzymes. The exogenous addition of 5-aminolevulinic acid (5-ALA) into culture media is known to partially increase the production of holoenzymes. However, the cost of 5-ALA accounts for approximately 60% of the total cost of E. coli cell culture, [23] which would be a drawback for the practical applications of hemoprotein-based biocatalysis. To overcome this problem, we investigate metabolic engineering of the heme biosynthetic pathway in E. coli to enable recombinant expression of active holohemoproteins.

Heme biosynthesis begins with the production of 5-ALA. [24] In most bacteria including *E. coli*, 5-ALA is synthesized via the C5 pathway, which involves two key enzymes, glutamyl-tRNA reductase (GluTR) and glutamate-1-semialdehyde aminomutase (GSAM) (Figure 1a, pathway in blue). [25] GluTR promotes the reduction of glutamyl-tRNA to produce glutamate-1-semialdehyde, and GSA-AT converts glutamate-1-semialdehyde into 5-ALA via the pyridoxal 5'-phosphate (PLP)-mediated transamination reaction. Because the activity of GluTR is strictly regulated by feedback

15213773, 0. Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/anie.202512165 by The University Of Osaka, Wiley Online Library on [31/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons. Licensing



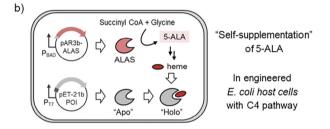


Figure 1. a) C5 and C4 pathways for the biosynthesis of 5-ALA. The activity of GluTR is strictly regulated by feedback inhibition of the end-product heme in E. coli. b) Self-supplementation strategy of 5-ALA via heterogeneous C4 pathway for the recombinant expression of holohemoproteins in E. coli host cells.

inhibition of the end-product heme, [22] the synthesis of 5-ALA generally serves as a bottleneck in the production of the heme cofactor. Previous studies have shown that engineering of the intrinsic C5 pathway can partially enhance the expression level of holohemoproteins in E. coli cells.[23,26,27] However, the engineered C5 pathway remains under the regulatory control of feedback inhibition, and overexpression of GluTR leads to growth inhibition, vide infra. To address this issue, we focused on a heterogeneous biosynthetic pathway of 5-ALA, which is known as the C4 pathway. This pathway is conserved in α -proteobacteria, fungi, and yeast (Figure 1a, pathway in red). The C4 pathway involves only one enzyme, 5-aminolevulinic acid synthase (ALAS), which produces 5-ALA by condensation of succinyl-CoA with glycine. [28-31] Since the activity of heterogeneous ALAS is not likely to be affected by the intrinsic regulation system of E. coli, the co-expression of ALAS is expected to increase the intracellular concentration of 5-ALA via the heterogeneous C4 pathway, thereby improving the efficiency of heme cofactor incorporation into the recombinant hemoproteins (Figure 1b). Based on this concept, we constructed an ALASbased synthetic C4 pathway for "self-supplementation" of 5-ALA in E. coli host cells and applied the engineered strain to whole-cell and lysate-based biocatalysis using various types of heme-dependent enzymes.

We first screened a panel of ALASs for the recombinant expression of holohemoproteins. The genes of ALASs from eight different microorganisms were selected from the

UniProt database (Table S1) and cloned into a pAR3b vector^[32] to construct a helper plasmid, pAR3b-ALAS, for self-supplementation of 5-ALA via the C4-pathway (Figure S1). pAR3b-ALAS is designed to encode the ALAS gene under the control of arabinose-inducible promoter P_{BAD}, thus allowing controllable and orthogonal ALAS expression independent of the pET expression system for hemoprotein. We also prepared helper plasmids, pAR3b-GluTR, which express 15 different types of GluTRs to enhance the flux of the intrinsic C5 pathway as a comparison (Figure S2 and Table S2).[23,26,27] These two types of helper plasmids were then transformed into the E. coli BL21-Gold(DE3) strain, and the resulting cells harboring pAR3b-ALAS or pAR3b-GluTR were used for recombinant expression of hemoproteins based on the pET system. In the initial screening, protoglobin from Aeropyrum pernix (ApPGb, UniProt ID: Q9YFF4) was selected as a model hemoprotein for the following three reasons: i) ApPGb has been widely utilized as a biocatalyst for abiotic chemical transformations.[33-38] ii) ApPGb can be expressed at high levels in E. coli, and iii) recombinant expression of ApPGb is known to require the addition of 5-ALA in the culture medium. Accordingly, the E. coli cells harboring pAR3b-ALAS/GluTR were transformed with the expression plasmid for ApPGb (pET21b-ApPGb) and cultivated in a normal LB medium using a 96-well microplate. During the log growth phase, the expression of ApPGb and ALAS/GluTR was induced by adding IPTG and arabinose, respectively, to investigate the effect of 5-ALA self-supplementation. The results showed that E. coli cell pellets expressing several ALASs and GluTRs exhibit the characteristic orange color corresponding to holo-ApPGb compared to negative control cells without the helper pAR3b plasmids (Figure 2a). In particular, ALAS from Bradyrhizobium diazoefficiens (BdALAS, UniProt ID: P08262), ALAS from Paracoccus denitrificans (PdALAS, UniProt ID: P43089), and ALAS from Caulobacter segnis (CsALAS, UniProt ID: D5VJ52), produce relatively high concentrations of holo-hemoproteins as estimated by UV-vis absorbance in the supernatant of E. coli cell lysates (Figure S3). Co-expression of these three ALASs did not interfere with the final concentration of E. coli cells nor the expression of ApPGb (Figures S4 and S5), while co-expression of GluTRs mostly results in lower cell densities and decreased expression levels. The excessive consumption of glutamyl-tRNA by GluTR may have disturbed protein synthesis and E. coli cell growth. These initial screening results led us to select BdALAS, PdALAS, and CsALAS for further investigations.

Next, we investigated the influence of ALAS coexpression in detail on a preparative scale using a 500 mL baffled flask. In addition to the pAR3b helper plasmid for BdALAS, PdALAS, and CsALAS, the helper plasmid for *E. coli* GluTR (EcGluTR) was also utilized as a negative control of the intrinsic C5 pathway similar to the previous studies. [23,26,27] The *E. coli* cells harboring pET21b-ApPGb and pAR3b-BdALAS/PdALAS/CsALAS/EcGluTR were cultivated in a normal LB medium in 100 mL volumes, and the expression of ApPGb and ALASs or EcGluTR was induced by adding IPTG and arabinose after OD₆₀₀ values reached approximately 0.7–0.9 (Figure S6). As observed in the

15213773, 0, I

wnloaded from https://onlinelibrary.wiley.com/doi/10.1002/anie.202512156 by The University Of Osaka, Wiley Online Library on [31/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons Licensu

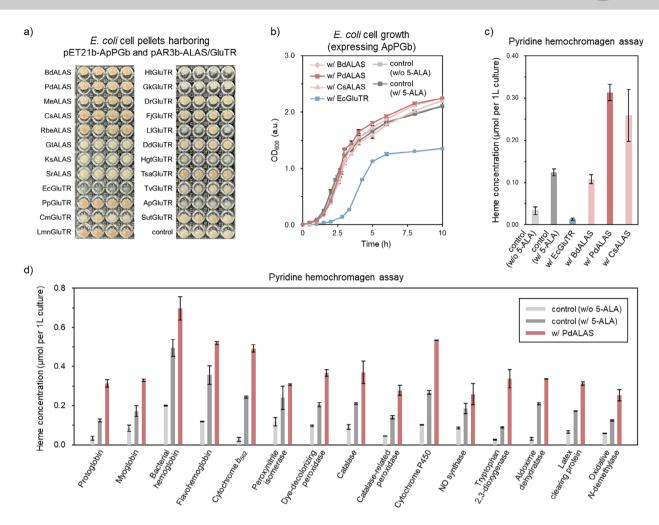


Figure 2. a) Photographs of the harvested *E. coli* cells harboring pET21b-ApPGb and pAR3b-ALAS/GluTR. The list of ALAS and GluTR enzymes is shown in Tables S1 and S2. control: *E. coli* cells harboring only pET21b-ApPGb were cultivated in the absence of 5-ALA. b) Growth curves of *E. coli* cells harboring pET21b-ApPGb and pAR3b-ALAS/GluTR indicated by OD₆₀₀ values. w/o 5-ALA: *E. coli* cells harboring only pET21b-ApPGb were cultivated in the absence of 5-ALA. w/ 5-ALA: *E. coli* cells harboring only pET21b-ApPGb were cultivated in the presence of 0.50 mM 5-ALA. After the OD₆₀₀ values reached approximately 0.7–0.9, IPTG (final conc. 0.20 mM) and arabinose (final conc. 2.0 μ g mL⁻¹) were added to induce the expression of ApPGb and ALAS/GluTR. Mean \pm SD (n=2, biological replicates). c) Heme concentrations in the supernatant of *E. coli* cell lysates expressing ApPGb. The concentrations of heme were calculated based on the pyridine hemochromagen assay. Mean \pm SD (n=2, biological replicates). d) The heme concentrations in the supernatant of *E. coli* cell lysates expressing various hemoproteins. Mean \pm SD (n=2, biological replicates).

initial screening described above, the expression of EcGluTR largely inhibits the E. coli cell growth (Figure 2b). After 16 h of cultivation time, the mass of the cell pellets expressing EcGluTR was less than half of the mass of the positive control cells with only pET21b-ApPGb (Figure S7). On the other hand, the E. coli cells expressing ALASs exhibit an acceptable growth curve which is similar to that of the positive control cells (Figure 2b). The co-expression of these three ALASs did not affect the mass of obtained cell pellets (Figure S7) nor the expression level of target hemoprotein (Figure S8). These results highlight the feasibility and robustness of the ALASbased heterogeneous C4 pathway when compared with the results of GluTR coexpression system utilized in previous studies.^[23,26,27] Furthermore, the pyridine hemochromagen assay[39] revealed that the content of heme in ApPGb was significantly increased through the incorporation of the ALAS-based C4 pathway (Figures 2c and S9). In particular, the co-expression of PdALAS provides the highest concentration of heme cofactor. Although the exogenous addition of 0.5 mM 5-ALA into the LB medium partially increases the production of holohemoproteins, PdALAS still provides better performance than the conventional expression methods. This is also true for the ApPGb solution after the protein purification (Figure S10). After purification, a total of 2.33 mg of ApPGb was obtained from 130 mL of culture. The production of 5-ALA in the cell was confirmed by LC-MS analysis (Figure \$11). As expected, the incorporation of the PdALASbased C4 pathway significantly increases the intracellular concentration of 5-ALA, whereas exogenous addition of 5-ALA into the medium does not contribute significantly to the intracellular 5-ALA content. This might be due to the rapid consumption of 5-ALA during the cell cultivation and/or the poor activity of the 5-ALA transport system in E. coli. [40] The concentration of 5-ALA in the PdALAS-expressing E. coli culture was calculated to be 26 ± 1 mg L⁻¹. This value is much lower than the concentration (exceeding 1.0 g L^{-1})

wnloaded from https://onlinelibrary.wiley.com/doi/10.1002/anie.202512156 by The University Of Osaka, Wiley Online Library on [31/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons Licensu

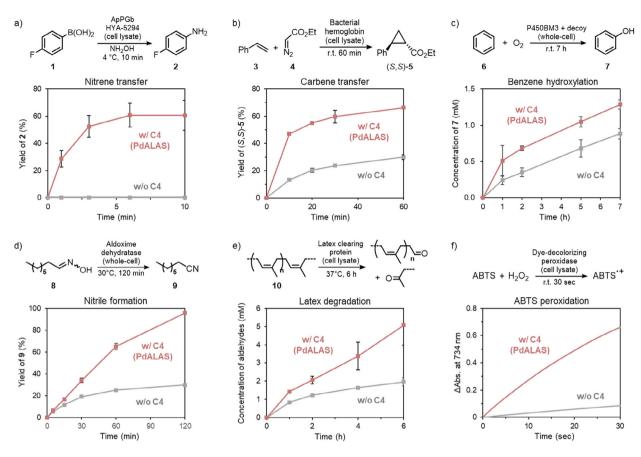


Figure 3. Time course plots of hemoprotein-catalyzed chemical transformations. E. coli cells with and without the C4 pathway were used for the expression of the corresponding hemoproteins. Mean \pm SD (n=2, biological replicates). a) Amination of aryl boronic acid 1 catalyzed by engineered ApPGb HYA-5294. b) Cyclopropanation of 3 with 4 catalyzed by bacterial hemoglobin A8IPS1. c) Hydroxylation of benzene catalyzed by cytochrome P450BM3 with a decoy system. d) Dehydration of aldoxime 8 catalyzed by aldoxime dehydratase. e) Degradation of natural rubber latex 10 catalyzed by latex clearing protein. f) Peroxidation of ABTS catalyzed by dye-decolorizing peroxidase. For more details of the reaction conditions, see the Supplementary Information.

reported in previously engineered *E. coli* strains designed for 5-ALA overproduction. [28-31] Excessive 5-ALA production may deplete cellular energy resources, such as nitrogen sources, required for hemoprotein biosynthesis. Therefore, continuous and appropriately balanced intracellular supply of 5-ALA is considered crucial for enhancing hemoprotein expression. LC-MS analysis also revealed increased levels of heme cofactor production within the cells, consistent with the results of the pyridine hemochromagen assay (Figure S12a). On the other hand, the concentration of protoporphyrin IX, an intermediate in heme biosynthesis, was not affected by the co-expression of PdALAS (Figure S12b). Since the biosynthetic intermediates of the heme cofactor exhibit cytotoxicity, this feature could provide benefits for cell growth.

Motivated by these promising results of the PdALAS-based C4 pathway, we next examined the generality of the engineered *E. coli* cell for recombinant expression of diverse hemoproteins. In addition to ApPGb, the *E. coli* cells with pAR3b-PdALAS were found to significantly increase the content of the heme cofactor in various classes of hemoproteins (Figure 2d, Table S3). In particular, it was found that the engineered cell could be widely applied for expression of myoglobin (UniProt

ID: P02185), bacterial hemoglobin (UniProt ID: A8IPS1), flavohemoglobin (UniProt ID: P49852), cytochrome b_{562} (UniProt ID: P0ABE7), peroxynitrite peroxidase (UniProt ID: O64527), dye-decolorizing peroxidase (UniProt ID: P76536), catalase (UniProt ID: P14412), catalase-related peroxidase (UniProt ID: B7X4G2), cytochrome P450BM3 (UniProt ID: P14779), NO synthase (UniProt ID: O34453), tryptophan 2,3-dioxygenase (UniProt ID: A0A1M6U789), aldoxime dehydratase (UniProt ID: UPI000400A70A), latex clearing protein (UniProt ID: D1ADK3), and oxidative Ndemethylase (UniProt ID: A4XXY9) as confirmed by the pyridine hemochromagen assay and SDS-PAGE (Figures S13-S19). In all cases, the E. coli cells harboring pAR3b-PdALAS produce higher heme content relative to the control cells cultivated by conventional methods (Figures 2d). When PdALAS was co-expressed with high-molecular-weight proteins, such as catalase (84.5 kDa) and cytochrome P450BM3 (117 kDa), the expression levels of these hemoproteins decreased due to competition for intracellular energy resources. To mitigate this expression interference, we adopted sequential induction of the two genes. Specially, the pET expression system was first induced with IPTG, and then PdALAS expression was induced with arabinose after 3 h of

15213773, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/anie.202512156 by The University Of Osaka, Wiley Online Library on [31/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Centive Commons Licensus



incubation (Figure S20). The orthogonal expression system enabled by pAR3b-PdALAS provides the practical advantages in balancing the trade-off between 5-ALA synthesis and hemoprotein expression.

Finally, as a demonstration, biocatalysis using a series of heme-dependent enzymes was performed to take advantage of the engineered E. coli cells with the PdALAS-based C4 pathway (Figure 3). We first tested biocatalytic nitrene transfer reactions using an ApPGb variant designated HYA-5294, which was reported by F. H. Arnold and E. Alfonzo et al. (Figure 3a).[37] E. coli cells with and without pAR3b-PdALAS were cultivated for the expression of ApPGb HYA-5294, and the lysates obtained from the same amount of cell culture were subjected to the heme-nitrene-mediated amination reaction of 4-fluorophenylboronic acid (1). Consequently, the cells with the C4 pathway were found to efficiently catalyze the target amination reaction and afforded 4-fluoroaniline (2) in 61% yield, while the cells without the C4 pathway show little activity (Figures 3a and S21). The introduction of the PdALAS-based C4 pathway significantly promotes the "holo" expression of ApPGb HYA-5294 (Figure S22), thereby improving the performance of the lysate-based biocatalyst. It was found that the engineered cell could be applied to abiotic carbene transfer reactions using bacterial hemoglobins from Azorhizobium caulinodans (Uniprot ID: A8IPS1) (Figure 3b).[41] The initial rate of the cyclopropanation reaction of styrene (3) with ethyl diazoacetate (4) is significantly improved by the cell lysate with the PdALASbased C4 pathway (Figure 3b). The desired product, ethyl 2-phenylcyclopropanecarboxylate (S,S)-5, was obtained in 66% yield with very high diastereo- and enantio-selectivities (>99% de, >99% ee). In contrast, the yield of (S,S)-5 is decreased to less than half the level provided in the absence of the C4 pathway (Figures 3b, S23, and S24). Furthermore, the E. coli cells with pAR3b-PdALAS were found to accelerate a wide range of biocatalytic transformations, including hydroxylation of benzene (6) using cytochrome P450BM3 with a decoy system^[42-45] (Figures 3c and \$25), dehydration reaction of octanal oxime (8) using aldoxime dehydratase^[46,47] (Figures 3d and \$26), degradation of natural rubber latex 10 using latex clearing protein^[48] (Figures 3e and \$27), peroxidation of ABTS using dye-decolorizing peroxidase^[49] (Figure 3f), and degradation of H₂O₂ using catalase^[50] (Figure S28). In all cases, incorporation of the C4 pathway significantly improves the catalytic performance of the whole-cell and lysate-based biocatalysts compared to the cells without the C4 pathway. Furthermore, the biocatalytic system using pAR3b-PdALAS is applicable to preparative scale synthesis. E. coli cells expressing aldoxime dehydratase with the C4 pathway efficiently converted 573 mg of octanal oxime (8) into octanenitrile (9) in 97% isolated yield (Figure S29), while E. coli cells lacking the C4 pathway provided 9 as a mixture with unreacted starting material 8 in 49% yield (Figure \$30). These results clearly demonstrate the versatility of our PdALAS-based C4 pathway for the preparation of hemoprotein-based biocatalysts.

In summary, we have engineered the heme biosynthetic pathway in *E. coli* to develop an efficient cofactor supplementation system for the recombinant expression of

hemoproteins. Through a series of enzyme screenings, ALAS from Paracoccus denitrificans (PdALAS), which efficiently increases the intracellular concentration of 5-ALA, was identified for the metabolic engineering. The incorporation of the PdALAS-based heterogeneous C4 pathway significantly increases the expression level of holohemoproteins. Notably, the heme content of the engineered cells is even higher than the heme content provided by the conventional cultivation method in which 5-ALA is externally added into culture media. Thus, the engineered E. coli cells were shown to be versatile for the expression of various hemoproteins (including 15 examples). Compared to previous studies utilizing the C5 pathway, [23,26,27] the use of the heterogeneous C4 pathway and the orthogonal expression plasmid pAR3b-ALAS offers clear advantages in terms of both hemoprotein expression and E. coli growth. Furthermore, we applied the engineered E. coli cells for whole-cell and lysate-based biocatalysis using a series of heme-dependent enzymes, including protoglobin, cytochrome P450, aldoxime dehydratase, and several others. In all cases, the engineered E. coli cells with the heterogeneous C4 pathway exhibit improved catalytic activity relative to E. coli cells without the C4 pathway. Given the increasing demand for biocatalysis in recent years, we are convinced that the system developed in this study will provide a powerful and practical platform for the implementation of the hemoprotein-based biocatalysis.

Supporting Information

Supplementary methods for sample preparation, experimental details, analytical data, amino acid and DNA sequence, and characterization data. The authors have cited additional references within the Supporting Information.^[51,52]

Acknowledgements

Deproteinized natural rubber was kindly gifted by Prof. Seiichi Kawahara and Associate Prof. Daisuke Kasai (Nagaoka University of Technology). The authors thank Ayumi Kawakami (The University of Osaka), and Dr. Qiuyi Wang and Dr. Junko Iida (Shimadzu Corporation) for their skillful support with LC-QTOF-MS analysis. This work was supported by JSPS KAKENHI Grant Number JP25H01579 (ForecastBiosyn), JP24H01136 (Bottom-up Biotech), JP23H04554 (ForecastBiosyn), JP22H05421 (Bottomup Biotech), JP25H00887, JP24K01630, JP22K21348, JP22K14783, JP21K20535, and JST ACT-X Grant Number JPMJAX22B6 (Environments and Biotechnology). S.K. acknowledges supports from Kaneko-Narita research fund (Protein Research Foundation), and Daiichi-Sankvo "Habataku" Support Program for the Next Generation of Researchers (Naedoko Grant).

Conflict of Interests

The authors declare no conflict of interest.

Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/anie.202512156 by The University Of Osaka, Wiley Online Library on [31/08/2025]. See the Terms

and Conditions (https://onlinelibrary.wiley.com/term

and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons



Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: 5-Aminolevulinic acid • Biocatalysis • Heme • Hemoprotein • Metabolic engineering

- [1] E. O. Romero, A. T. Saucedo, J. R. Hernández-Meléndez, D. Yang, S. Chakrabarty, A. R. H. Narayan, JACS Au 2023, 3, 2073-2085.
- [2] P. S. Coelho, E. M. Brustad, A. Kannan, F. H. Arnold, Science **2013**, *339*, 307–310.
- [3] P. S. Coelho, Z. J. Wang, M. E. Ener, S. A. Baril, A. Kannan, F. H. Arnold, E. M. Brustad, Nat. Chem. Biol. 2013, 9, 485-487.
- [4] R. K. Zhang, K. Chen, X. Huang, L. Wohlschlager, H. Renata, F. H. Arnold, Nature 2019, 565, 67-72.
- [5] E. W. Svastits, J. H. Dawson, R. Breslow, S. H. Gellman, J. Am. Chem. Soc. 1985, 107, 6427-6428.
- [6] J. A. McIntosh, P. S. Coelho, C. C. Farwell, Z. J. Wang, J. C. Lewis, T. R. Brown, F. H. Arnold, Angew. Chem. Int. Ed. 2013, 52, 9309-9312.
- [7] T. K. Hyster, C. C. Farwell, A. R. Buller, J. A. McIntosh, F. H. Arnold, J. Am. Chem. Soc. 2014, 136, 15505-15508.
- [8] C. K. Prier, R. K. Zhang, A. R. Buller, S. Brinkmann-Chen, F. H. Arnold, Nat. Chem. 2017, 9, 629-634.
- [9] A. Dennig, N. Lulsdorf, H. Liu, U. Schwaneberg, Angew. Chem. Int. Ed. 2013, 52, 8459-8462.
- [10] M. Bordeaux, V. Tyagi, R. Fasan, Angew. Chem. Int. Ed. 2015, 54, 1744-1748.
- [11] S. B. Kan, R. D. Lewis, K. Chen, F. H. Arnold, *Science* **2016**, *354*, 1048-1051.
- [12] S. B. J. Kan, X. Huang, Y. Gumulya, K. Chen, F. H. Arnold, Nature 2017, 552, 132-136.
- [13] T. Betke, M. Maier, H. Gruber-Wölfler, H. Gröger, Nat. Commun. 2018, 9, 5112.
- [14] Q. Zhou, M. Chin, Y. Fu, P. Liu, Y. Yang, Science 2021, 374, 1612-1616.
- [15] M. Pott, M. Tinzl, T. Hayashi, Y. Ota, D. Dunkelmann, P. R. E. Mittl, D. Hilvert, Angew. Chem. Int. Ed. 2021, 60, 15063–15068.
- [16] L. E. Zetzsche, J. A. Yazarians, S. Chakrabarty, M. E. Hinze, L. A. M. Murray, A. L. Lukowski, L. A. Joyce, A. R. H. Narayan, Nature 2022, 603, 79-85.
- [17] I. Kalvet, M. Ortmayer, J. Zhao, R. Crawshaw, N. M. Ennist, C. Levy, A. Roy, A. P. Green, D. Baker, J. Am. Chem. Soc. 2023, 145, 14307-14315.
- [18] S. Gergel, J. Soler, A. Klein, K. H. Schülke, B. Hauer, M. Garcia-Borràs, S. C. Hammer, Nat. Catal. 2023, 6, 606-617.
- [19] K. Oohora, H. Meichin, L. Zhao, M. W. Wolf, A. Nakayama, J. Y. Hasegawa, N. Lehnert, T. Hayashi, J. Am. Chem. Soc. 2017, 139, 17265-17268.
- [20] Y. Kagawa, K. Oohora, T. Himiyama, A. Suzuki, T. Hayashi, Angew. Chem. Int. Ed. 2024, 63, e202403485.
- [21] S. Kato, M. Abe, H. Gröger, T. Hayashi, ACS Catal. 2024, 14, 13081-13087.
- [22] M. Schobert, D. Jahn, J. Mol. Microbiol. Biotechnol. 2002, 4, 287-294.
- [23] I. N. Harnastai, A. A. Gilep, S. A. Usanov, Protein Expr. Purif. **2006**, 46, 47–55.
- [24] G. Layer, Biochim. Biophys. Acta, Mol. Cell Res. 2021, 1868, 118861.

- [25] M. Jiang, K. Hong, Y. Mao, H. Ma, T. Chen, Z. Wang, Front. Bioeng. Biotechnol. 2022, 10, 841443.
- [26] C. Feng, M. Pan, L. Tang, Biotechnol. Lett. 2022, 44, 271–277.
- [27] J. Ge, X. Wang, Y. Bai, Y. Wang, Y. Wang, T. Tu, X. Qin, X. Su, H. Luo, B. Yao, H. Huang, J. Zhang, Cell Fact 2023, 22, 59.
- [28] W. Wang, Y. Xiang, G. Yin, S. Hu, J. Cheng, J. Chen, G. Du, Z. Kang, Y. Wang, J. Agric. Food Chem. 2024, 72, 8006-8017.
- [29] T. Li, Y.-Y. Guo, G.-Q. Qiao, G.-Q. Chen, ACS Synth. Biol. 2016, 5, 1264-1274,
- [30] W. Pu, J. Chen, Y. Zhou, H. Qiu, T. Shi, W. Zhou, X. Guo, N. Cai, Z. Tan, J. Liu, J. Feng, Y. Wang, P. Zheng, J. Sun, Biofpr. 2023,
- [31] H. Zhou, C. Zhang, Z. Li, M. Xia, Z. Li, Z. Wang, G.-Y. Tan, Y. Luo, L. Zhang, W. Wang, Trends Biotechnol. 2024, 42, 1479-
- [32] J. Pérez-Pérez, J. Gutiérrez, Gene 1995, 158, 141-142.
- [33] N. J. Porter, E. Danelius, T. Gonen, F. H. Arnold, J. Am. Chem. Soc. 2022, 144, 8892-8896.
- [34] E. Danelius, N. J. Porter, J. Unge, F. H. Arnold, T. Gonen, J. Am. Chem. Soc. 2023, 145, 7159-7165.
- [35] L. Schaus, A. Das, A. M. Knight, G. Jimenez-Osés, K. N. Houk, M. Garcia-Borràs, F. H. Arnold, X. Huang, Angew. Chem. Int. Ed. 2023, 62, e202208936.
- [36] E. Alfonzo, D. Hanley, Z.-Q. Li, K. M. Sicinski, S. Gao, F. H. Arnold, J. Am. Chem. Soc. 2024, 146, 27267-27273.
- [37] D. Hanley, Z.-Q. Li, S. Gao, S. C. Virgil, F. H. Arnold, E. Alfonzo, J. Am. Chem. Soc. 2024, 146, 19160-19167.
- [38] T. Rogge, Q. Zhou, N. J. Porter, F. H. Arnold, K. N. Houk, J. Am. Chem. Soc. 2024, 146, 2959-2966.
- [39] I. Barr, F. Guo, Bio-protocol 2015, 5, e1594.
- [40] E. Verkamp, V. M. Backman, J. M. Björnsson, D. Söll, G. Eggertsson, J. Bacteriol. 1993, 175, 1452-1456.
- [41] S. Kato, K. Takeuchi, M. Iwaki, K. Miyazaki, K. Honda, T. Hayashi, Angew. Chem. Int. Ed. 2023, 62, e202303764.
- [42] N. Kawakami, O. Shoji, Y. Watanabe, Angew. Chem. Int. Ed. **2011**, *50*, 5315–5318.
- [43] O. Shoji, T. Kunimatsu, N. Kawakami, Y. Watanabe, Angew. Chem. Int. Ed. 2013, 52, 6606-6610.
- [44] O. Shoji, S. Yanagisawa, J. K. Stanfield, K. Suzuki, Z. Cong, H. Sugimoto, Y. Shiro, Y. Watanabe, Angew. Chem. Int. Ed. 2017, 56, 10324-10329,
- [45] M. Karasawa, J. K. Stanfield, S. Yanagisawa, O. Shoji, Y. Watanabe, Angew. Chem. Int. Ed. 2018, 57, 12264-12269.
- [46] K. Chen, Z. Wang, K. Ding, Y. Chen, Y. Asano, Green Synth. Catal. 2021, 2, 179–186.
- [47] A. Hinzmann, T. Betke, Y. Asano, H. Gröger, Chem. Eur. J. **2021**, 27, 5313-5321.
- [48] L. Ilcu, W. Röther, J. Birke, A. Brausemann, O. Einsle, D. Jendrossek, Sci. Rep. 2017, 7, 6179.
- [49] X. Liu, Z. Yuan, J. Wang, Y. Cui, S. Liu, Y. Ma, L. Gu, S. Xu, Biochem. Biophys. Res. Commun. 2017, 484, 40-44.
- [50] R. Singh, B. Wiseman, T. Deemagarn, V. Jha, J. Switala, P. C. Loewen, Arch. Biochem. Biophys. 2008, 471, 207-214.
- [51] K. Nishimoto, N. Okahashi, M. Maruyama, Y. Izumi, K. Nakatani, Y. Ito, J. Iida, T. Bamba, F. Matsuda, Sci. Rep. 2023, 13, 18549.
- [52] S. Komori, N. Okahashi, J. Iida, F. Matsuda, J. Biosci. Bioeng. **2023**, 135, 102-108.

Manuscript received: June 04, 2025 Revised manuscript received: July 29, 2025 Accepted manuscript online: July 29, 2025 Version of record online: ■■, ■