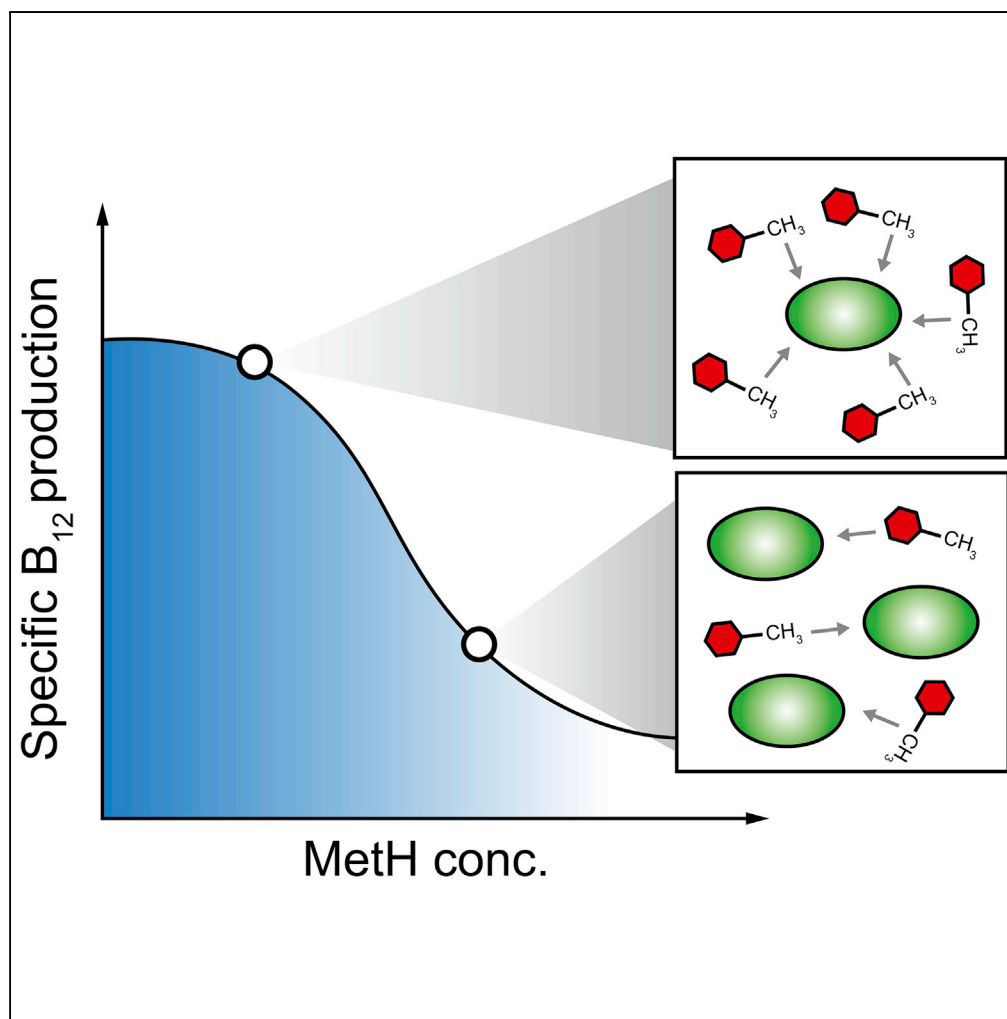


Article

Auxotrophic Selection Strategy for Improved Production of Coenzyme B₁₂ in *Escherichia coli*

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HIGHLIGHTS

The auxotrophic selection strategy was applied to coenzyme B₁₂ production

Coenzyme B₁₂-independent methionine synthase was deleted for auxotroph system

The auxotrophic strategy could significantly enhance the coenzyme B₁₂ production

Optimization of the auxotroph system further enhanced the coenzyme B₁₂ production

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Article

Auxotrophic Selection Strategy for Improved Production of Coenzyme B₁₂ in *Escherichia coli*

Myung Hyun Noh,^{1,4} Hyun Gyu Lim,^{1,4} Daeyeong Moon,¹ Sunghoon Park,³ and Gyoo Yeol Jung^{1,2,5,*}

SUMMARY

The production of coenzyme B₁₂ using well-characterized microorganisms, such as *Escherichia coli*, has recently attracted considerable attention to meet growing demands of coenzyme B₁₂ in various applications. In the present study, we designed an auxotrophic selection strategy and demonstrated the enhanced production of coenzyme B₁₂ using a previously engineered coenzyme B₁₂-producing *E. coli* strain. To select a high producer, the coenzyme B₁₂-independent methionine synthase (*metE*) gene was deleted in *E. coli*, thus limiting its methionine synthesis to only that via coenzyme B₁₂-dependent synthase (encoded by *metH*). Following the deletion of *metE*, significantly enhanced production of the specific coenzyme B₁₂ validated the coenzyme B₁₂-dependent auxotrophic growth. Further precise tuning of the auxotrophic system by varying the expression of *metH* substantially increased the cell biomass and coenzyme B₁₂ production, suggesting that our strategy could be effectively applied to *E. coli* and other coenzyme B₁₂-producing strains.

INTRODUCTION

Coenzyme B₁₂, also known as adenosylcobalamin, plays an important role in several metabolic reactions occurring in different organ systems of the body (Guo and Chen, 2018). For example, it is required for proper functioning of the nervous system and synthesis of red blood cells, fatty acids, and amino acids (Ko et al., 2014; Martens et al., 2002). The demand for large-scale production of coenzyme B₁₂ has steadily increased owing to its applications in food, feed additive, and pharmaceutical industries (Fang et al., 2018, 2017). However, chemical synthesis of coenzyme B₁₂ is highly complicated because of its complex structure. To overcome this shortcoming, industrial production of coenzyme B₁₂ through microbial fermentation has been regarded as an efficient method (Biedendieck et al., 2010).

Currently, microorganisms with the inherent ability to synthesize coenzyme B₁₂, including *Pseudomonas denitrificans* and *Propionibacterium freudenreichii* (the highest production was 214.3 and 206.0 mg/L, respectively), are widely employed for its industrial production (Fang et al., 2018; Lee et al., 2018; Martens et al., 2002). However, these strains are not well characterized; thus, only limited engineering tools, such as random mutagenesis and plasmid-based gene expression, have been utilized (Fang et al., 2018, 2017; Yin et al., 2019). In addition, these strains are known to have long fermentation cycles and expensive and complex medium requirements (Fang et al., 2017).

The use of genetically well-characterized bacteria can be a compelling strategy for the production of coenzyme B₁₂. In this regard, recent studies have reported the production of coenzyme B₁₂ by exploiting the representative microbial workhorse, *Escherichia coli* (Fang et al., 2018; Fowler et al., 2010; Ko et al., 2014). It has been demonstrated that *E. coli* could be used to synthesize coenzyme B₁₂ upon the addition of ado-cobinamide (AdoCbi) and dimethylbenzimidazole (DMBI) (Fowler et al., 2010; Jang et al., 2018) via the native coenzyme B₁₂ salvage pathway (Lawrence and Roth, 1996). Moreover, another group reconstructed the AdoCbi synthetic pathway from *P. denitrificans* and heterologously introduced it into *E. coli* BL21(DE3) (Ko et al., 2014). In their study, Ko et al. overexpressed 22 genes using three plasmids; the production of coenzyme B₁₂ (0.65 μg/g dry cell weight [DCW]) was confirmed even without the addition of AdoCbi. More recently, Fang et al. reported the production of unexpectedly high levels of coenzyme B₁₂ (307.00 μg/g DCW) through step-by-step optimization of the synthetic pathway (Fang et al., 2018). The entire synthetic pathway included heterologous expression of 28 genes, and the related genes from different microorganisms were screened for efficient production of coenzyme B₁₂.

In addition to rational strain engineering strategies, selection-based engineering strategies have been valuable for improving the production capabilities of microorganisms. Indeed, recent studies have shown

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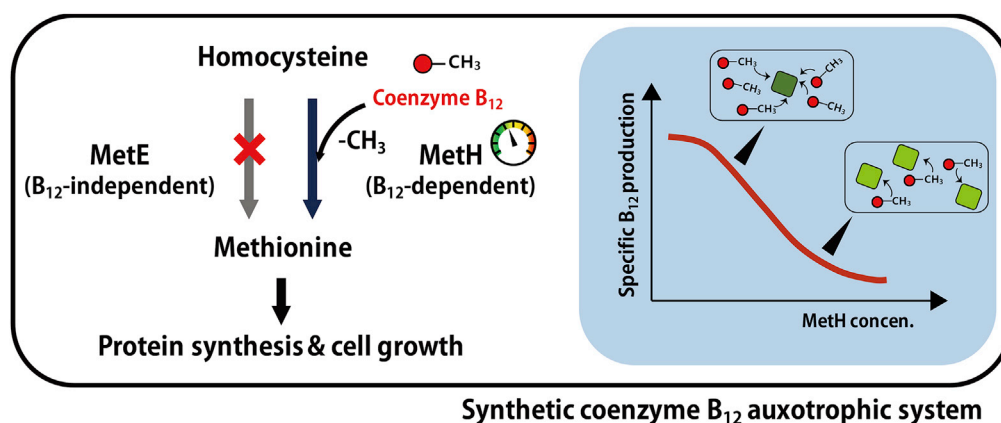


Figure 1. Schematic Diagram of the Overall Strategies Used in the Study

The synthetic coenzyme B₁₂ auxotroph could be constructed by the deletion of chromosomal *metE* (encoding cobalamin-independent homocysteine transmethylase). The expression of *metH* (encoding cobalamin-dependent homocysteine transmethylase) was varied using different synthetic promoters to obtain optimal production of coenzyme B₁₂. See also Figure S1.

tremendous improvement in the biochemical production by devising genetic circuits with biosensors to detect metabolite levels of interest. Moreover, coupling the production capability with cell survival has led to a remarkable improvement in the titers (Gao et al., 2019; Jang et al., 2019). For example, Xiao et al. have developed a circuit to control the expression of the genes responsible for antibiotic resistance or essential amino acid synthesis under the control of free fatty acid (FFA)-responsive promoter (P_{AR}), only allowing the strains with active production of FFA to grow (Xiao et al., 2016). Consequently, it was highly effective to control the population quality by minimizing the heterogeneity in biological systems, thereby significantly enhancing the production of FFA. Similarly, Rugbjerg et al. have introduced a genetic circuit to couple the gene expression of cell wall synthesis with that of mevalonate production (Rugbjerg et al., 2018); the introduction of the circuit enabled stable plasmid maintenance and consistent mevalonate production during long-term cultivation.

In the present study, an auxotrophic selection strategy was designed to increase the coenzyme B₁₂ production in *E. coli*. We leveraged the characteristics of methionine biosynthesis in *E. coli*; *E. coli* was engineered to synthesize methionine only if coenzyme B₁₂ existed with cobalamin-dependent homocysteine transmethylase (*metH*) by deleting the gene encoding for cobalamin-independent homocysteine transmethylase (*metE*). The growth rate of the *E. coli* strain lacking the gene *metE* was highly dependent on the concentration of exogenously added coenzyme B₁₂. When the strategy was applied to the EpACR^{coB} strain, a previously reported coenzyme B₁₂-producing strain (Ko et al., 2014), the specific coenzyme B₁₂ production was substantially improved by autonomous modulation of copy numbers of plasmids. The expression of *metH* was further varied to optimize the cell biomass and coenzyme B₁₂ production. The engineered strain exhibited significantly enhanced coenzyme B₁₂ production. We believe that this novel strategy would be useful not only for *E. coli*, but also for several other microorganisms for the production of coenzyme B₁₂.

RESULTS

Construction of Coenzyme B₁₂ Auxotroph System in *E. coli*

To develop the auxotrophic selection strategy for the production of coenzyme B₁₂, we initially sought an essential enzymatic reaction dependent on coenzyme B₁₂ in *E. coli*. The most well-known enzyme is cobalamin-dependent homocysteine transmethylase (encoded by *metH*, Figure 1), which catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to L-homocysteine to produce methionine and tetrahydrofolate (Lago and Demain, 1969; Neil and Marsh, 1999; Raux et al., 1996). Coenzyme B₁₂ is used as a direct mediator of the methyl group during the transfer process. In fact, *E. coli* possesses another cobalamin-independent enzyme, encoded by *metE*, to synthesize methionine (Davis and Mingioli, 1950; Mordukhova and Pan, 2013). Therefore, we decided to delete the *metE* gene in *E. coli* for auxotrophic selection. Additionally, we found an early stop codon at the 58th codon of *btuB* gene (KEGG number; ECD_03851,

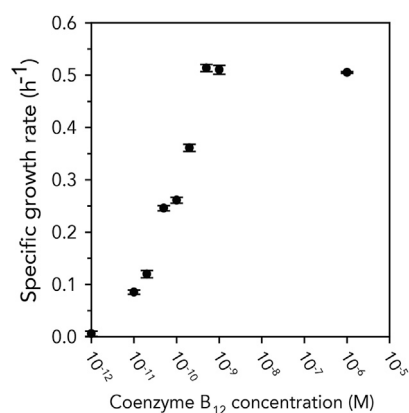


Figure 2. The Validation of Coenzyme B₁₂ Auxotrophic Cell Growth in the EDMB Strain

The specific cell growth rate (h^{-1}) was calculated and plotted on the y axis according to the coenzyme B₁₂ concentration (x axis). Error bars indicate the standard deviations from three independent cultures.

pseudogene encoding cobalamin outer membrane transporter) in the wild-type *E. coli* BL21(DE3) strain (Studier et al., 2009), a host used in the previous coenzyme B₁₂ production study (Ko et al., 2014). Because it plays an essential role in coenzyme B₁₂ import (Fowler et al., 2010), the early stop codon was replaced with “CAG” to incorporate glutamine, an identical codon used by other *E. coli* species (see the [Transparent Methods](#) section); thus, the disrupt *btuB* gene was functionally expressed and the B₁₂ auxotrophic growth was achieved following the addition of coenzyme B₁₂.

Upon deleting *metE*, methionine is produced only by coenzyme B₁₂-dependent MetH and the cells are able to grow in the presence of coenzyme B₁₂. To validate the coenzyme B₁₂-dependent cell growth, the resulting EDMB strain (*E. coli* BL21(DE3) with *metE* deletion and functional *btuB*, [Table S1](#)) was cultured in the B₁₂ auxotrophic medium containing all amino acids except methionine (see the [Transparent Methods](#) section). As expected, the strain exhibited negligible growth (growth rate $<0.01 \text{ h}^{-1}$) because of its inability to synthesize methionine. When the medium was supplemented with varying concentrations of coenzyme B₁₂ ranging from 0 M to 1 μM ($\sim 1.6 \text{ mg/L}$), the specific growth rate gradually increased as the concentration of coenzyme B₁₂ was increased to 500 pM ([Figure 2](#)), reaching that of the wild-type (0.51 h^{-1}) when a sufficient amount of coenzyme B₁₂ was present. Although the range of concentrations where the auxotrophic selection functions are at the picomolar level, given that the production of coenzyme B₁₂ is not high in *E. coli* (Ko et al., 2014), it is expected to be applicable to producing strains. Collectively, these results suggest that the coenzyme B₁₂ auxotrophic system was successfully constructed in *E. coli*.

Application of Auxotrophic System for Coenzyme B₁₂ Production

The auxotrophic system was applied to the previously reported coenzyme B₁₂-producing strain, EpACR^{cob} ([Tables S1](#) and [S2](#)). The three plasmids ([Table S1](#)) harboring AdoCbi synthetic pathway genes from the EpACR^{cob} strain (Ko et al., 2014) were introduced into the EDMB strain. The resulting EDMB^{cob} and EpACR^{cob} strains were initially cultured in the B₁₂ auxotrophic medium; however, a severely reduced cell growth was observed for the EDMB^{cob} strain (the growth rate was 0.03 h^{-1}). Therefore, we decided to use the RB₁₂ medium containing 10 g/L of tryptone instead of the individual amino acids to supplement the low amount of methionine and to enhance the protein synthesis at the early phase of culture. This supplementation was helpful to obtain higher biomass (0.60 g DCW/L , [Figure 3A](#)). However, the deletion of *metE* still resulted in the formation of significantly reduced biomass of the EDMB^{cob} strain ([Figure 3A](#), a 5.01-fold decrease). These results indicated that intracellular methionine synthesis was critical for cell growth and the EDMB^{cob} strain was still affected by the auxotrophic system.

We further quantified the production of coenzyme B₁₂ in both strains to investigate the effect of the selection strategy. The EpACR^{cob} strain exhibited higher production of coenzyme B₁₂ ($2.43 \mu\text{g/g DCW}$) when compared with the previously reported value ($0.65 \mu\text{g/g DCW}$) (Ko et al., 2014). Given that most of the culture conditions are the same, this difference in productivity is probably owing to the use of RB₁₂ medium supplemented with glucose as a carbon source, unlike the previously used LB medium (see the [Transparent Methods](#) section). Surprisingly, the EDMB^{cob} strain with the *metE* deletion showed a 2.73-fold increase in specific coenzyme B₁₂ production compared with the EpACR^{cob} strain ([Figure 3B](#), $6.64 \mu\text{g/g DCW}$). The

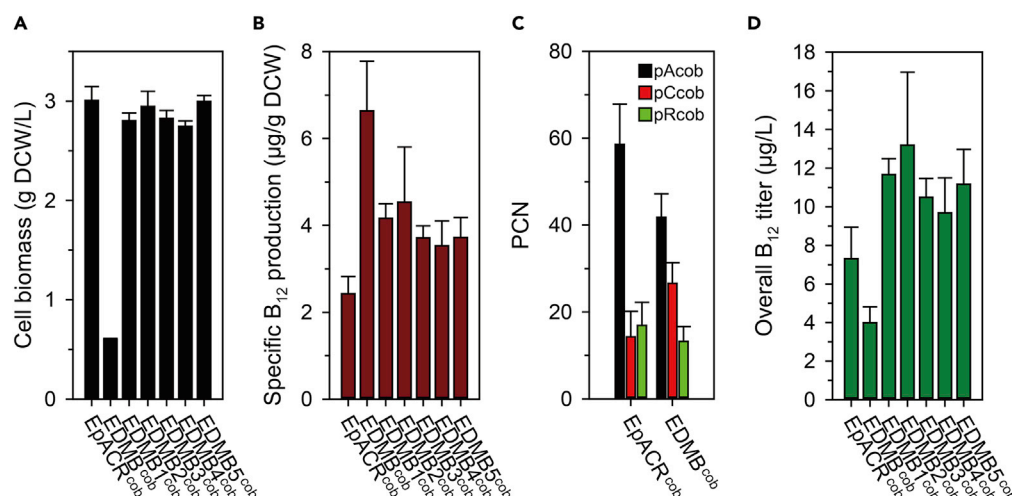


Figure 3. The Effect of Synthetic Auxotrophic System on Coenzyme B₁₂-Producing Strains

Comparison of cell biomass (A) (See also Figure S2), specific coenzyme B₁₂ production (B), and plasmid copy number (PCN) after 12-h cultivation (C) (see also Figure S3) and overall coenzyme B₁₂ titer (D) after 24-h cultivation. Error bars indicate the standard deviations from experiments conducted in triplicate.

result indicates that the auxotrophic selection system was effective in significantly improving the production of coenzyme B₁₂.

Because the only genomic differences are the deletion of *metE* and functional expression of *btuB*, it was hypothesized that the increase in the specific production could be attributed to the altered expression levels of AdoCbi synthetic genes. Plasmids typically exhibit a huge heterogeneity in their plasmid copy numbers (PCNs), which has often affected the production performance of microorganisms (Jahn et al., 2016; Kang et al., 2018). To test this hypothesis, the copy number of each plasmid in both strains was measured by quantitative PCR (qPCR). As mentioned, the copy numbers of the pCcob (CoIA origin) and pRcob (RSF1030 origin) plasmids in the EpACR^{cob} strain (Figure 3C) were observed to be relatively lower (14.2 and 16.9 copies per cell, respectively) than that known (20–40 copies and 100 copies, respectively), which could be due to different culture conditions, use of multiple plasmids, and metabolic burden from several heterologous gene expression (Jahn et al., 2016; Zhong et al., 2011). Meanwhile, the pAcob plasmid (p15A origin) exhibited a significantly higher PCN (58.5 copies/cell) than the known PCN (10–12 copies/cell). Moreover, the high PCN of pAcob is consistent with the previously measured high mRNA levels of the genes in the pAcob plasmid (Ko et al., 2014). The measurement revealed that the EDMB^{cob} strain showed dramatic changes in the copy number compared with the EpACR^{cob} strain. The copy number of the pCcob plasmid (26.6 copies/cell) was almost 2-fold higher than that in the EpACR^{cob} strain. The increased copy number of the pCcob plasmid led to enhanced expression of the genes responsible for converting hydrogenobyrinic acid a,c-diamide to AdoCbi (Figure S1). On the contrary, PCN of both pAcob and pRcob plasmids (41.8 and 13.2 copies/cell, respectively) was 1.40-fold and 1.28-fold lower than that of the EpACR^{cob} strain. The decreased copy number might be beneficial to minimize the wasteful usage of resources in gene expression. There might be other potential factors; nevertheless, the introduction of the auxotrophic selection system affected PCNs, improving the production of coenzyme B₁₂.

Optimization of *metH* Expression for Enhancing Coenzyme B₁₂ Production

Despite the enhanced specific production, the volumetric titer of the EDMB^{cob} strain was lower than that of the EpACR^{cob} strain by 1.83-fold (3.98 µg/L, Figure 3D) because of the decreased cell biomass of the EDMB^{cob} strain. This result suggested that an additional tuning of the auxotrophic system was required to restore the cell biomass and thereby increase the overall titer.

It has been known that MetH has a higher catalytic efficiency than MetE (Gonzalez et al., 1992). However, methionine is mostly synthesized by MetE and not MetH because the expression of *metH* is induced only when cobalamin is present in the media (Helliwell et al., 2011; Roth et al., 1996). Therefore, it was believed that reduced cell biomass of the EDMB^{cob} strain presumably resulted from insufficient methionine

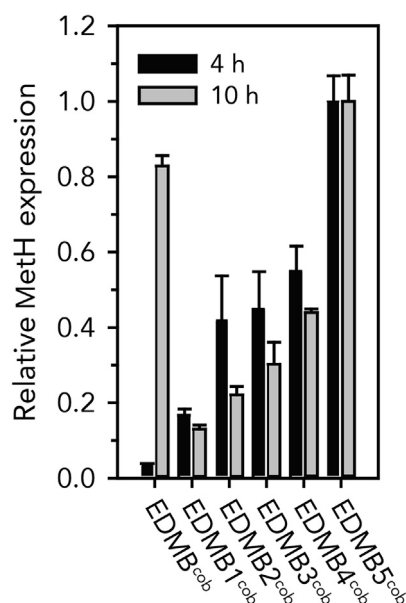


Figure 4. Comparison of Normalized Amounts of *metH* Transcript in Engineered Strains

Error bars indicate the standard deviations from experiments conducted in triplicate.

synthesis with low *metH* expression at an early stage of cultivation. To validate our assumption, the expression of *metH* was measured by qPCR (Figure 4). Indeed, the *metH* expression was very low at the beginning of the cultivation (4 h), reinforcing our assumption that this interfered with the early biomass accumulation. The expression of *metH* was subsequently induced after synthesis of coenzyme B₁₂ (10 h); however, the cell biomass could not be recovered (Figure S2). This is probably due to the metabolic imbalance that the proteins essential for cell growth could not be sufficiently synthesized along with the synthesis of numerous coenzyme B₁₂ (Darlington et al., 2018; Segall-Shapiro et al., 2014). Collectively, this result suggested that increasing the *metH* expression would improve the initial cell growth as well as the overall titer.

Therefore, we tried to deregulate the expression of *metH* using synthetic constitutive promoters (<http://parts.igem.org/Promoters/Catalog/Anderson>) (Kang et al., 2018; Noh et al., 2018, 2017). To restore the cell biomass, the *metH* expression needed to be increased than before as its insufficient expression produced less cell biomass. However, excessive expression could also lower the specific coenzyme B₁₂ production. Consequently, multiple strains with varied expression levels of *metH* were generated by introducing synthetic *metH* cassettes with different-strength constitutive promoters (Tables S1 and S2). The resultant strains (EDMB1-5^{cob}) were cultivated, and the expression of *metH* was measured in the same manner (Figure 4). Although there was a gap between predicted strength and measured *metH* expression levels (Iverson et al., 2016; Kelwick et al., 2015; Noh et al., 2017), varied *metH* expression levels (up to 7.84-fold) were successfully identified in these engineered strains as intended. Moreover, these strains showed higher *metH* expression up to 28.6-fold than the EDMB^{cob} strain at an early stage of cultivation and appeared to maintain a relatively constant level during cultivation.

The synthetic expression of *metH* caused noticeable changes in both cell biomass and specific coenzyme B₁₂ production. All engineered strains, EDMB1-5^{cob}, displayed notably increased cell biomass compared with the EDMB^{cob} strain (Figures 3A and S2). In particular, the cell biomass was generally enhanced at 12 and 18 h as the expression of *metH* increased (Figure S2), and almost 5-fold increased cell biomass was observed in all engineered strains at 24 h (Figure 3A). These values obtained at 24 h corresponded to the recovery of cell biomass to more than 90% of EpACR^{cob} strain without *metE* deletion. This indicated that sufficient methionine could be synthesized by the deregulated *metH* expression in engineered strains (Figure 4). On the contrary, specific coenzyme B₁₂ production was decreased as a result of the enhanced *metH* expression (Figure 3B). Especially, specific production generally decreased as the *metH* expression increased, up to 1.78-fold for the EDMB5^{cob} strain with the highest *metH* expression as intended. Nevertheless, the significantly enhanced cell biomass increased the overall titer (Figure 3D). Among the strains, the EDMB2^{cob} strain with moderate *metH* expression level showed the highest coenzyme B₁₂ production (13.2 μg/L), which was 3.31-fold and 1.80-fold higher than that of the EDMB^{cob} and EpACR^{cob} strains,

respectively. In addition, the PCNs of EDMB1-5^{cob} strains showed a similar tendency as that of EDMB^{cob} strain (Figure S3), indicating they were still affected by the synthetic auxotroph system. Collectively, these results show that the synthetic auxotroph system with precise controlled *metH* expression could be successfully applied to coenzyme B₁₂-producing strains.

DISCUSSION

Recent studies have successfully demonstrated the production of coenzyme B₁₂ in a well-known microbial workhorse *E. coli* (Fang et al., 2018; Ko et al., 2014). However, the heterologous expression of synthetic genes has been an obstacle to further improvement. In the present study, a novel strategy to enhance the coenzyme B₁₂ production was designed and applied to previously constructed coenzyme B₁₂-producing *E. coli* (Ko et al., 2014). Initially, the coenzyme B₁₂ synthetic auxotrophic system was constructed using the characteristics of methionine synthesis in *E. coli*. Next, this system was applied to the previously reported producer strain to greatly improve the coenzyme B₁₂ production. We found modulated copy numbers in the coenzyme B₁₂-producing plasmids after the application of the auxotrophic selection system, which could explain the observed improvement in the production.

Optimizing complex metabolic pathways such as coenzyme B₁₂ to enhance the production has been a labor-intensive work in metabolic engineering (Smanski et al., 2014). Our successful application of the auxotroph system, which could significantly enhance the coenzyme B₁₂ production without optimization of individual gene expressions, suggests enough potential that the auxotroph system can be effectively used for complex pathway optimization. In addition, it was shown that the selection efficiency could be optimized through precisely regulating the expression of a key enzyme, which implies that the system can be optimized for different production levels like other selection-based strategies (Rugbjerg et al., 2018; Xiao et al., 2016).

The strategy would be applied to other coenzyme B₁₂-producing strains such as *P. denitrificans* and *P. freudenreichii*. These strains also possess cobalamin-dependent homocysteine methyltransferase and are known to have a similar regulation system (Ainala et al., 2013; Falentin et al., 2010). Given that these strains produce high amounts of coenzyme B₁₂, the expression of cobalamin-dependent methyltransferase may need to be optimized at lower levels. Alternatively, the coenzyme B₁₂-binding residues could be intentionally disrupted to lower the affinity base on the elucidated structure of cobalamin-dependent methyltransferase (Drennan et al., 1994; Seo et al., 2018). In addition, other cobalamin-dependent enzymes, such as methylmalonyl-CoA mutase (essential for odd-chain fatty acid synthesis) and glycerol dehydratase (essential for glycerol utilization), could be utilized using a similar strategy (Banerjee and Ragsdale, 2003; Neil and Marsh, 1999). Moreover, these coenzyme B₁₂ synthetic auxotrophic systems could be applied to evolutionary engineering approaches (Lim et al., 2018; Seok et al., 2018). The short-term change in PCNs was validated to enhance the coenzyme B₁₂ production in the current study; however, it could be used to screen genetically effective mutants as a powerful screening method in long-term evolution. Taken together, we expect our strategy to be widely applied for the efficient production of coenzyme B₁₂.

Limitations of the Study

The system described in the present study may be limited by the binding affinity of MetH to coenzyme B₁₂. When this system is applied to a superior coenzyme B₁₂-producing strain (Fang et al., 2018), its operational range needs to be investigated or modified, if necessary. As discussed, the coenzyme B₁₂-binding residues or expression levels can be altered for effective tuning of the dynamic range. Furthermore, other enzymes involved in different reactions could be considered also. Nonetheless, the improved coenzyme B₁₂ production with our parental strain (EpACR^{cob}) shows great potential. The present study could be valuable for understanding the heterogeneity during biochemical production and its minimization by introducing a selection strategy.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.100890>.

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AUTHOR CONTRIBUTIONS

M.H.N., H.G.L., D.M., S.P., and G.Y.J. conceived the project. M.H.N. and H.G.L. designed and conducted the experiments. M.H.N., H.G.L., D.M., S.P., and G.Y.J. conducted data analysis and interpretation and wrote the manuscript. S.P. and G.Y.J. critically revised the manuscript. G.Y.J. supervised the project. All the authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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REFERENCES

- Ainala, S.K., Somasundar, A., and Park, S. (2013). Complete genome sequence of *Pseudomonas denitrificans* ATCC 13867. *Genome Announc.* *1*, e00257–13.
- Banerjee, R., and Ragsdale, S.W. (2003). The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes. *Annu. Rev. Biochem.* *72*, 209.
- Biedendieck, R., Malten, M., Barg, H., Bunk, B., Martens, J.H., Deery, E., Leech, H., Warren, M.J., and Jahn, D. (2010). Metabolic engineering of cobalamin (vitamin B12) production in *Bacillus megaterium*. *Microb. Biotechnol.* *3*, 24–37.
- Darlington, A.P.S., Kim, J., Jiménez, J.I., and Bates, D.G. (2018). Dynamic allocation of orthogonal ribosomes facilitates uncoupling of co-expressed genes. *Nat. Commun.* *9*, 695.
- Davis, B.D., and Mingioli, E.S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *J. Bacteriol.* *60*, 17–28.
- Drennan, C.L., Huang, S., Drummond, J.T., Matthews, R.G., and Ludwig, M.L. (1994). How a protein binds B12: a 3.0 Å x-ray structure of B12-binding domains of methionine synthase. *Science* *266*, 1669–1674.
- Falentin, H., Deutsch, S.M., Jan, G., Loux, V., Thierry, A., Parayre, S., Maillard, M.B., Dherbécourt, J., Cousin, F.J., Jardin, J., et al. (2010). The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1T, a hardy Actinobacterium with food and probiotic applications. *PLoS One* *5*, e11748.
- Fang, H., Kang, J., and Zhang, D. (2017). Microbial production of vitamin B12: a review and future perspectives. *Microb. Cell Fact.* *16*, 15.
- Fang, H., Li, D., Kang, J., Jiang, P., Sun, J., and Zhang, D. (2018). Metabolic engineering of *Escherichia coli* for de novo biosynthesis of vitamin B12. *Nat. Commun.* *9*, 4917.
- Fowler, C.C., Brown, E.D., and Li, Y. (2010). Using a riboswitch sensor to examine coenzyme B12 metabolism and transport in *E. coli*. *Chem. Biol.* *17*, 756–765.
- Gao, C., Xu, P., Ye, C., Chen, X., and Liu, L. (2019). Genetic circuit-assisted smart microbial engineering. *Trends Microbiol.* *27*, 1011–1024.
- Gonzalez, J.C., Banerjee, R.V., Huang, S., Sumner, J.S., and Matthews, R.G. (1992). Comparison of cobalamin-independent and cobalamin-dependent methionine synthases from *Escherichia coli*: two solutions to the same chemical problem. *Biochemistry* *31*, 6045–6056.
- Guo, M., and Chen, Y. (2018). Coenzyme cobalamin: biosynthesis, overproduction and its application in dehalogenation—a review. *Rev. Environ. Sci. Biotechnol.* *17*, 259–284.
- Helliwell, K.E., Wheeler, G.L., Leptos, K.C., Goldstein, R.E., and Smith, A.G. (2011). Insights into the evolution of vitamin B12 auxotrophy from sequenced algal genomes. *Mol. Biol. Evol.* *28*, 2921–2933.
- Iverson, S.V., Haddock, T.L., Beal, J., and Densmore, D.M. (2016). CIDAR MoClo: improved MoClo assembly standard and new *E. coli* part library enable rapid combinatorial design for synthetic and traditional biology. *ACS Synth. Biol.* *5*, 99–103.
- Jahn, M., Vorpahl, C., Hübschmann, T., Harms, H., and Müller, S. (2016). Copy number variability of expression plasmids determined by cell sorting and droplet digital PCR. *Microb. Cell Fact.* *15*, 211.
- Jang, S., Kim, M., Hwang, J., and Jung, G.Y. (2019). Tools and systems for evolutionary engineering of biomolecules and microorganisms. *J. Ind. Microbiol. Biotechnol.* *46*, 1313–1326.
- Jang, S., Jang, S., Noh, M.H., Lim, H.G., and Jung, G.Y. (2018). Novel hybrid input part using riboswitch and transcriptional repressor for signal inverting amplifier. *ACS Synth. Biol.* *7*, 2199–2204.
- Kang, C.W., Lim, H.G., Yang, J., Noh, M.H., Seo, S.W., and Jung, G.Y. (2018). Synthetic auxotrophs for stable and tunable maintenance of plasmid copy number. *Metab. Eng.* *48*, 121–128.
- Kelwick, R., Kopniczky, M., Bower, I., Chi, W., Chin, M.H.W., Fan, S., Pilcher, J., Strutt, J., Webb, A.J., and Jensen, K. (2015). A forward-design approach to increase the production of poly-3-hydroxybutyrate in genetically engineered *Escherichia coli*. *PLoS One* *10*, e0117202.
- Ko, Y., Ashok, S., Ainala, S.K., Sankaranarayanan, M., Chun, A.Y., Jung, G.Y., and Park, S. (2014). Coenzyme B12 can be produced by engineered *Escherichia coli* under both anaerobic and aerobic conditions. *Biotechnol. J.* *9*, 1526–1535.
- Lago, B.D., and Demain, A.L. (1969). Alternate requirement for vitamin B12 or methionine in mutants of *Pseudomonas denitrificans*, a vitamin B12-producing bacterium. *J. Bacteriol.* *99*, 347–349.
- Lawrence, J.G., and Roth, J.R. (1996). Evolution of coenzyme B12 synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. *Genetics* *142*, 11–24.
- Lee, J.H., Lama, S., Kim, J.R., and Park, S.H. (2018). Production of 1,3-propanediol from glucose by recombinant *Escherichia coli* BL21(DE3). *Biotechnol. Bioproc. Eng.* *23*, 250–258.
- Lim, H.G., Jang, S., Jang, S., Seo, S.W., and Jung, G.Y. (2018). Design and optimization of genetically encoded biosensors for

- high-throughput screening of chemicals. *Curr. Opin. Biotechnol.* 54, 18–25.
- Martens, J.H., Barg, H., Warren, M., and Jahn, D. (2002). Microbial production of vitamin B12. *Appl. Microbiol. Biotechnol.* 58, 275–285.
- Mordukhova, E.A., and Pan, J.G. (2013). Evolved cobalamin-independent methionine synthase (MetE) improves the acetate and thermal tolerance of *Escherichia coli*. *Appl. Environ. Microbiol.* 79, 7905–7915.
- Neil, E., and Marsh, G. (1999). Coenzyme B12 (cobalamin)-dependent enzymes. *Essays Biochem.* 34, 139–154.
- Noh, M.H., Lim, H.G., Park, S., Seo, S.W., and Jung, G.Y. (2017). Precise flux redistribution to glyoxylate cycle for 5-aminolevulinic acid production in *Escherichia coli*. *Metab. Eng.* 43, 1–8.
- Noh, M.H., Lim, H.G., Woo, S.H., Song, J., and Jung, G.Y. (2018). Production of itaconic acid from acetate by engineering acid-tolerant *Escherichia coli* W. *Biotechnol. Bioeng.* 115, 729–738.
- Raux, E., Lanois, A., Levillayer, F., Warren, M.J., Brody, E., Rambach, A., and Thermes, C. (1996). *Salmonella typhimurium* cobalamin (vitamin B12) biosynthetic genes: functional studies in *S. typhimurium* and *Escherichia coli*. *J. Bacteriol.* 178, 753–767.
- Roth, J., Lawrence, J., and Bobik, T. (1996). Cobalamin (coenzyme B12): Synthesis and biological significance. *Annu. Rev. Microbiol.* 50, 137–181.
- Rugbjerg, P., Sarup-Lytzen, K., Nagy, M., and Sommer, M.O.A. (2018). Synthetic addiction extends the productive life time of engineered *Escherichia coli* populations. *Proc. Natl. Acad. Sci. U S A* 115, 2347–2352.
- Segall-Shapiro, T.H., Meyer, A.J., Ellington, A.D., Sontag, E.D., and Voigt, C.A. (2014). A 'resource allocator' for transcription based on a highly fragmented T7 RNA polymerase. *Mol. Syst. Biol.* 10, 742.
- Seo, J.H., Min, W.K., Lee, S.G., Yun, H., and Kim, B.G. (2018). To the final goal: can we predict and suggest mutations for protein to develop desired phenotype? *Biotechnol. Bioeng.* 23, 134–143.
- Seok, J.Y., Yang, J., Choi, S.J., Lim, H.G., Choi, U.J., Kim, K.J., Park, S., Yoo, T.H., and Jung, G.Y. (2018). Directed evolution of the 3-hydroxypropionic acid production pathway by engineering aldehyde dehydrogenase using a synthetic selection device. *Metab. Eng.* 47, 113–120.
- Smanski, M.J., Bhatia, S., Zhao, D., Park, Y.J., Woodruff, L.B.A., Giannoukos, G., Ciulla, D., Busby, M., Calderon, J., Nicol, R., et al. (2014). Functional optimization of gene clusters by combinatorial design and assembly. *Nat. Biotechnol.* 32, 1241–1249.
- Studier, F.W., Daegelen, P., Lenski, R.E., Maslov, S., and Kim, J.F. (2009). Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) and comparison of the *E. coli* B and K-12 genomes. *J. Mol. Biol.* 394, 653–680.
- Xiao, Y., Bowen, C.H., Liu, D., and Zhang, F. (2016). Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. *Nat. Chem. Biol.* 12, 339–344.
- Yin, J., Zheng, W., Gao, Y., Jiang, C., Shi, H., Diao, X., Li, S., Chen, H., Wang, H., Li, R., et al. (2019). Single-stranded DNA-binding protein and exogenous RecBCD inhibitors enhance phage-derived homologous recombination in *Pseudomonas*. *iScience* 14, 1–14.
- Zhong, C., Peng, D., Ye, W., Chai, L., Qi, J., Yu, Z., Ruan, L., and Sun, M. (2011). Determination of plasmid copy number reveals the total plasmid DNA amount is greater than the chromosomal DNA amount in *Bacillus thuringiensis* YBT-1520. *PLoS One* 6, e16024.

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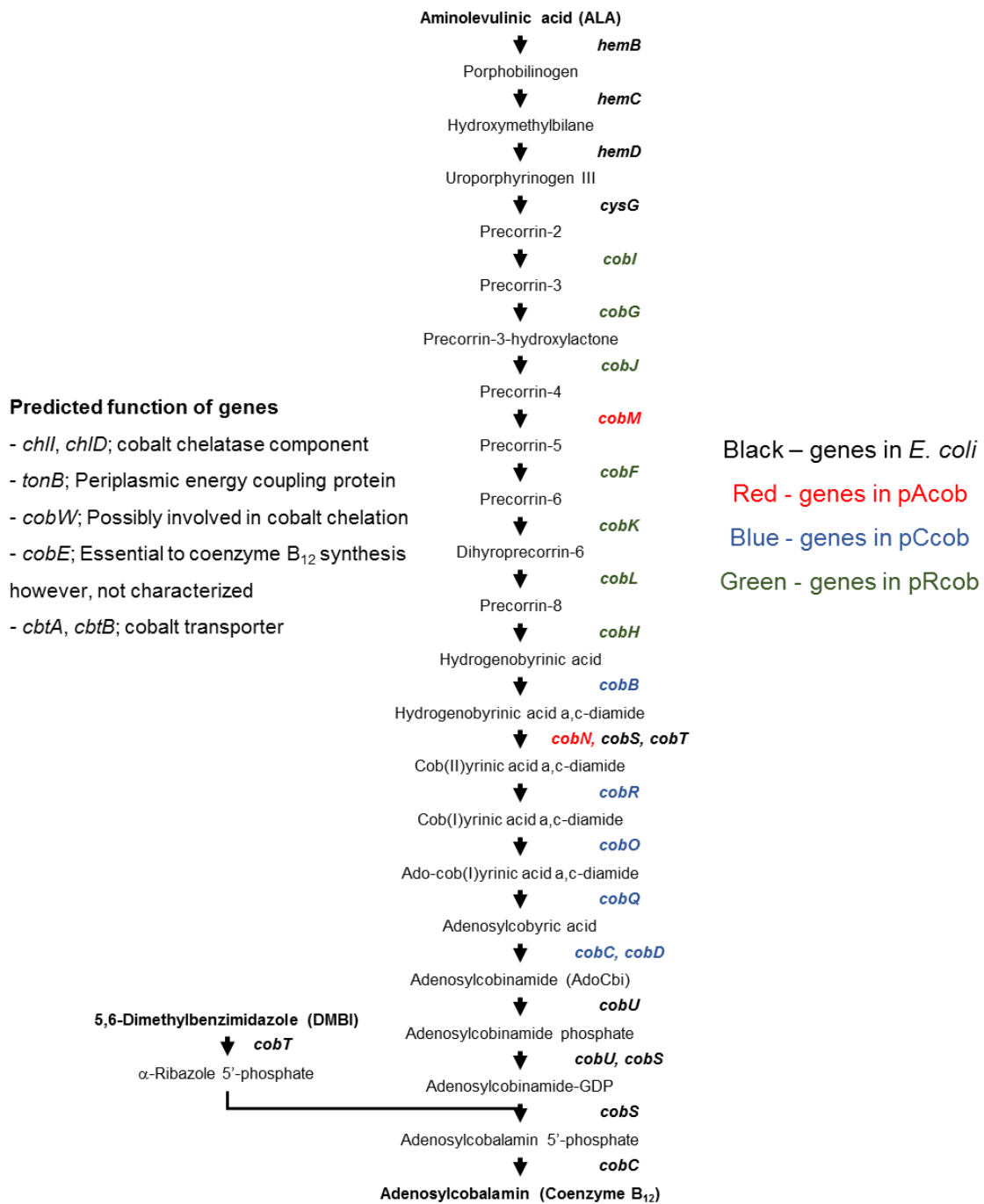
Supplemental Information

Auxotrophic Selection Strategy for Improved Production of Coenzyme B₁₂ in *Escherichia coli*

Myung Hyun Noh, Hyun Gyu Lim, Daeyeong Moon, Sunghoon Park, and Gyoo Yeol Jung

1 **Supplementary Figures**

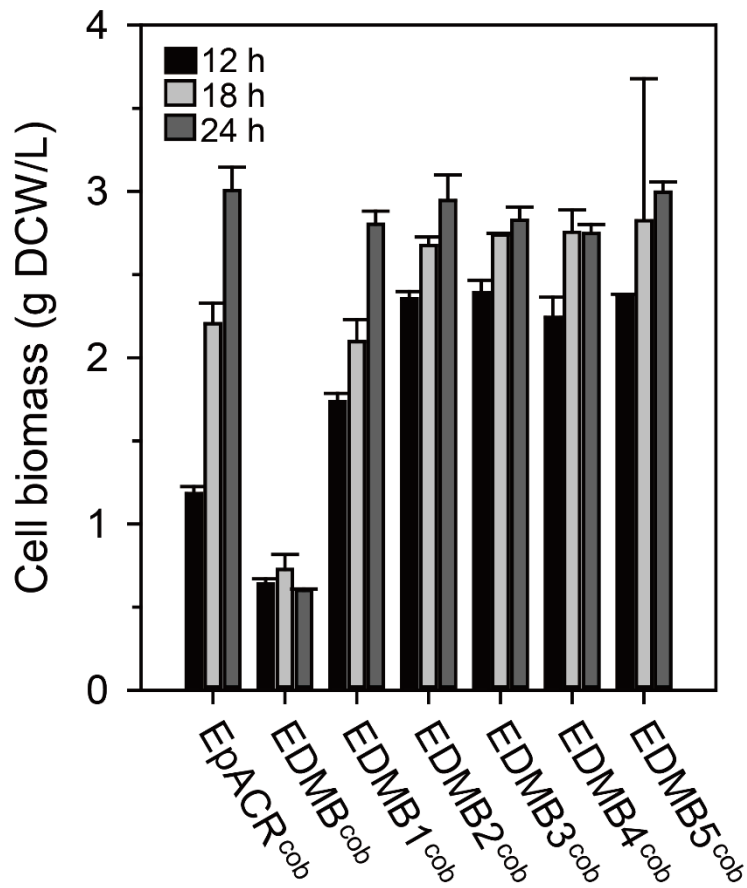
2 **Figure S1.** Coenzyme B₁₂ synthesis pathway and predicted function of genes (Ko et al., 2014),
 3 related to Figure 1.



4

1 **Figure S2.** The effect of synthetic auxotrophic system on obtained cell biomass of coenzyme
2 B₁₂-producing strains, related to Figure 3A. Error bars indicate the standard deviations from
3 three independent cultures.

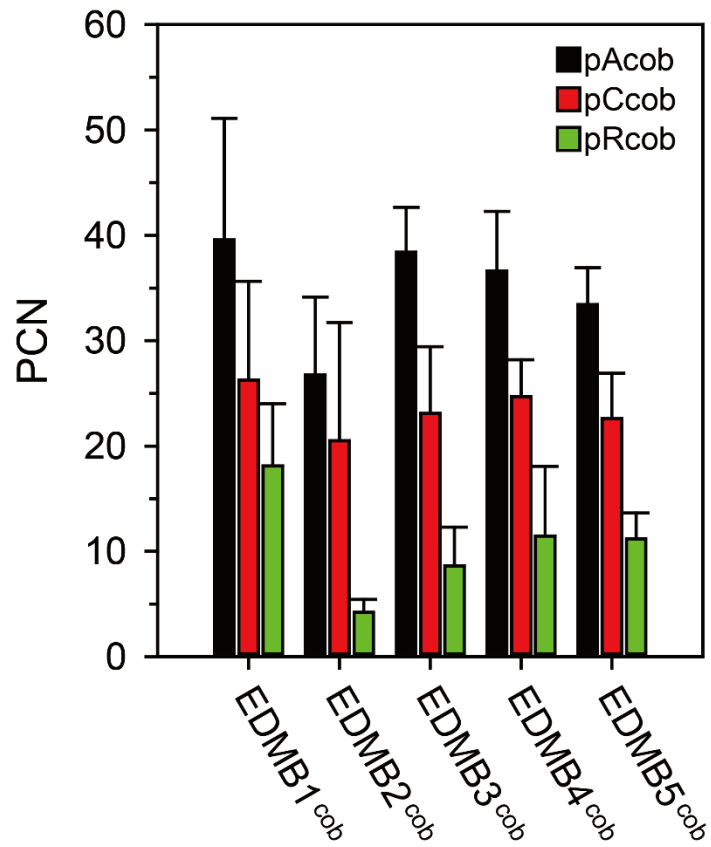
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6

1 **Figure S3.** The effect of synthetic auxotrophic system on plasmid copy number of coenzyme
2 B₁₂-producing strains, related to Figure 3C. Error bars indicate the standard deviations from
3 three independent cultures.

4



5

1 **Supplementary Tables**

2 **Table S1.** Bacterial strains and plasmids used in this study, related to Figures 1-4.

Name	Description^a	Source
Strains		
<i>E. coli</i> Mach1-T1 ^R	Cloning host	Invitrogen
<i>E. coli</i> BL21(DE3)	Expression host	Invitrogen
EpACR ^{cob}	<i>E. coli</i> BL21(DE3) / pAcob / pCob / pRcob	(Ko et al., 2014)
EDM	<i>E. coli</i> BL21(DE3) Δ <i>metE</i>	This study
EDMB	<i>E. coli</i> BL21(DE3) Δ <i>metE</i> <i>btuB</i> (*58Q)	This study
EDMB0	<i>E. coli</i> BL21(DE3) Δ <i>metE</i> <i>btuB</i> (*58Q) Δ <i>metH</i>	This study
EDMB1	EDMB0 <i>att</i> _{HK022} ::J23118_nativeUTR_metH_terT7	This study
EDMB2	EDMB0 <i>att</i> _{HK022} ::J23108_nativeUTR_metH_terT7	This study
EDMB3	EDMB0 <i>att</i> _{HK022} ::J23110_nativeUTR_metH_terT7	This study
EDMB4	EDMB0 <i>att</i> _{HK022} ::J23115_nativeUTR_metH_terT7	This study
EDMB5	EDMB0 <i>att</i> _{HK022} ::J23100_nativeUTR_metH_terT7	This study
EDMB ^{cob}	EDMB / pAcob / pCcob / pRcob	This study
EDMB1 ^{cob}	EDMB1 / pAcob / pCcob / pRcob	This study
EDMB2 ^{cob}	EDMB2 / pAcob / pCcob / pRcob	This study
EDMB3 ^{cob}	EDMB3 / pAcob / pCcob / pRcob	This study
EDMB4 ^{cob}	EDMB4 / pAcob / pCcob / pRcob	This study
EDMB5 ^{cob}	EDMB5 / pAcob / pCcob / pRcob	This study
Plasmids		
pAcob	p15A ori, Cm ^R , P _{T7} - <i>cobWN</i> - tet _{T7} , P _{T7} - <i>cbtAB_cobEM</i> -tet _{T7}	(Ko et al., 2014)
pCcob	ColA ori, Sm ^R , P _{T7} - <i>chIID</i> - tet _{T7} , P _{T7} - <i>tonB_cobOBRDCQ</i> - tet _{T7}	(Ko et al., 2014)
pRcob	RSF1030 ori, Km ^R , P _{T7} - <i>cobGHJJ</i> - tet _{T7} , P _{T7} - <i>cobLFK</i> - tet _{T7}	(Ko et al., 2014)
pKD46	Red recombinase expression vector, Amp ^R	(Datsenko and Wanner, 2000)
pCP20	FLP expression vector, Amp ^R , Cm ^R	(Datsenko and Wanner, 2000)
pM_FKF	PCR template for FRT-Kan ^R -FRT, pMB1 ori, Amp ^R , Km ^R	(Noh et al., 2018)
pAH69	CloDF13 ori, Sm ^R , <i>E. coli</i> expression vector	(Haldimann and Wanner, 2001)
pBAC-L6-P _{T3} T4Ei	PCR template for <i>att</i> _{HK022} -FRT-Cm ^R -FRT, R6K γ ori, Cm ^R	(Lee et al., 2016)
pCDFduet-1	ColA ori, Sm ^R , <i>E. coli</i> expression vector	Novagen

pCDFHM1-5

ColA ori, Sm^R, *att*_{HK022}-FRT-Cm^R-FRT, P_{BBa_J231XX}-
nativeUTR-*metH*-tet₇

This study

- 1 ^aAbbreviations: Amp, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Km, kanamycin;
2 R, resistance.
3

1 **Table S2.** Oligonucleotides sequences used in this study, related to Figures 1-4.

Name	Sequence (5'-3') ^a
metE_del_F	atgacaatattgaatcacaccctcggttccctcgcgttggcctgcgtcggcatgaccggcgcgatgc
metE_del_R	gctcagcggatctcatgcctcatccccgacgcaaattctgcgccctgcaccatgttcgccagtgcg
btuB_s.d.m	ttgaccaaccaccggtgtgaccgctcaggatcgcaccgctggcagtcgacctcggtaaatgatgtgctg cgccgtcttcgggctc
metH_del_F	atgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagcatgaccggcgcgatgc
metH_del_F	tcagtccgcgtcataccccagattcgggtccagccagcgcctcaacttcgggctcagcggatctcatgcgc
pCDF_F	ggtatgacgcggactgactcagaatcagtttaacctaggctgctgccacc
pCDF_R	ggggcttcccggatcaacaggacaccagttaatgtaagttagctcactcattaggcac
HK022_F	ctggtgtccctgttgataccgggaagcccc
HK022_R	actgagctagccgtcaagaattcgtactgctgcagaacagtagtacttgacagac
metH_F1 118	cagtagcgaattc <u>ctgacggctagctcagtcctaggtattgtgctagct</u> ggtgaattttattaaatctgggtg agcg
metH_F2 108	cagtagcgaattc <u>ctgacagctagctcagtcctaggtataatgctagct</u> ggtgaattttattaaatctgggtg agcg
metH_F3 110	cagtagcgaattc <u>ttacggctagctcagtcctaggtacaatgctagct</u> ggtgaattttattaaatctgggtg agcg
metH_F4 115	cagtagcgaattc <u>ttatagctagctcagcccttggtaacaatgctagct</u> ggtgaattttattaaatctgggtg gcg
metH_F5 100	cagtagcgaattc <u>ctgacggctagctcagtcctaggtacagtgctagct</u> ggtgaattttattaaatctgggtg agcg
metH_R	tcagtccgcgtcataccccagatt
metH_int_F	ctggtgtccctgttgatacc
metH_int_R	cgatgggatccatgctagtattgctcagcgggtg
metH_RT_F	catacggaaaaagccaccatctg
metH_RT_R	ggtaacgctcatacctttacgg
cysG_RT_F	cagcaaaagctgattgaacacg
cysG_RT_R	aacaaccgaccaataataattagc
RSF_RT_F	cttgagaaaaccaccgttgta
RSF_RT_R	aacggaatagctgttcgttgac
CDF_RT_F	gtggtttttcgtttacaggca
CDF_RT_R	aatctagagcgggtcagtagaaaa
ACY_RT_F	tttagcttccttagctcctgaaaatc
ACY_RT_R	gtaagaggtccaacttcaccata
polA_RT_F	gcgagcgateccagaagatct
polA_RT_R	gattatgggtaaaggatgccaca

2 ^aUnderlined nucleotides indicate synthetic promoter (P_{BBa_J231XX}, <http://parts.igem.org>)

3

1 **Transparent Methods**

2 **Reagents, oligonucleotides, and media**

3 The extraction of plasmid and genomic DNA was conducted using the GeneAll^R Plasmid
4 SV kit and the GeneAll^R ExgeneTM Cell SV kit (GeneAll; Seoul, Korea), respectively. The
5 GeneAll^R ExpinTM Gel SV and GeneAll^R ExpinTM CleanUp SV kits were used for the
6 purification of DNA. The Q5^R High-Fidelity DNA polymerase, T4 DNA ligase, restriction
7 enzymes, NEBuilder^R HiFi DNA assembly reagents were purchased from New England
8 Biolabs (Ipswich, MA, USA). Synthetic oligonucleotides (Table S2) were purchased from
9 Cosmo Genetech (Seoul, Korea). Other reagents for cell culture were purchased from Sigma-
10 Aldrich (St. Louis, MO, USA).

11 For a rich nutrient environment, as required for the production of coenzyme B₁₂, the RB₁₂
12 medium containing 0.5 g/L MgSO₄·7H₂O, 2.0 g/L NH₄Cl, 2.0 g/L NaCl, 10 g/L tryptone, 0.1
13 mM 5,6-dimethylbenzimidazole (DMBI), 10 mg/L 5-aminolevulinic acid, 10 mg/L
14 CoCl₂·6H₂O, 100 mM potassium phosphate buffer (pH 7.0), and 20 g/L glucose as the carbon
15 source was used. The B₁₂ auxotrophic medium is a modified version of the RB₁₂ medium and
16 is obtained by replacing 10 g/L tryptone with defined concentrations of all amino acids
17 except methionine (0.28 g/L alanine, 0.04 g/L cysteine, 0.22 g/L histidine, 0.65 g/L lysine,
18 0.86 g/L proline, 0.10 g/L tryptophan, 0.33 g/L arginine, 1.87 g/L glutamate, 0.44 g/L
19 isoleucine, 0.50 g/L serine, 0.18 g/L tyrosine, 0.65 g/L aspartic acid, 0.17 g/L glycine, 0.76
20 g/L leucine, 0.39 g/L threonine, and 0.55 g/L valine). LB medium was used for routine
21 genetic manipulations.

22 **Construction of strains and plasmids**

1 All bacterial strains and plasmids used in the study are listed in Table S1. The EpACR^{cob}
2 strain and three compatible plasmids (Ko et al., 2014) were a kind gift from Prof. Sunghoon
3 Park (Ulsan National Institute of Science and Technology). Sequences of the synthetic
4 promoters (J231 promoter series) were obtained from the Registry of Standard Biological
5 Parts (<http://parts.igem.org>). Plasmid cloning was performed using *E. coli* Mach1-T1^R
6 (Thermo Scientific; Waltham, MA, USA) as the host.

7 For the inactivation of chromosomal *metE* of *E. coli* BL21(DE3), the lambda-red
8 recombination method was used with pKD46 and pCP20 plasmids (Datsenko and Wanner,
9 2000). An FRT-Kan^R-FRT fragment was prepared by amplifying the pM_FKF plasmid (Noh
10 et al., 2018) with a *metE_del_F/R* primer pair. For replacing the early stop codon on *btuB*, the
11 *btuB_s.d.m* oligonucleotide was directly introduced into the EDM strain via electroporation.

12 To control the expression level of *metH*, the native *metH* was replaced and synthetic
13 expression cassettes were introduced. Initially, the native gene was removed similarly to the
14 *metE* deletion except that a *metH_del_F/R* primer pair was used. The synthetic expression
15 cassettes for *metH* expression were integrated using the phage-integration method
16 (Haldimann and Wanner, 2001). To construct the pCDFHMI1-5 plasmids, required for the
17 integration method, a vector fragment was amplified using pCDF_F/R and pCDFduet-1 as a
18 template. The vector fragments were assembled with fragments amplified using HK022_F/R
19 with pBAC-L₆-P_{T3}T₄Ei as a template and *metH_F1-5/metH_R* with genomic DNA of
20 BL21(DE3) strain, respectively. Thereafter, the fragments for integration were amplified with
21 *metH_int_F/R* and pCDFHM1-5 plasmids as a template to remove replication origin. The
22 fragments were subsequently digested using *Bam*HI and re-circularized for genomic
23 integration (Lee et al., 2016).

24

1 **Cell culture medium and conditions**

2 To validate coenzyme B₁₂ auxotrophic growth, a seed culture was performed in the RB₁₂
3 medium. After 12 h cultivation, cell pellets were washed twice with the B₁₂ auxotrophic
4 medium. Next, the cultures were inoculated in a 15-mL test tube containing 3 mL of the B₁₂
5 auxotrophic medium at an optical density at 600 nm (OD₆₀₀) of 0.05. OD₆₀₀ values were
6 monitored with cultivating the cells at 30°C with agitation at 200 rpm.

7 The culture conditions for B₁₂ production were determined by referring to the previously
8 optimized conditions (Ko et al., 2014). It was conducted in 300-mL Erlenmeyer flasks
9 containing 50 mL of the RB₁₂ medium. For seed culture, a single colony was inoculated in a
10 15-mL test tube containing 3 mL of the RB₁₂ medium. After 12 h, the seed culture was
11 inoculated in the fresh RB₁₂ medium at OD₆₀₀ of 0.05 and cultured. When the OD₆₀₀ reached
12 0.4, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM for
13 induction. OD₆₀₀ values were monitored with cultivating the cells at 30°C with agitation at
14 200 rpm. The pH was periodically measured using an Orion™ 8103BN ROSS™ pH meter
15 (Thermo Scientific) and adjusted to 7.0 with a 10 M NaOH solution.

16 All analytical cell cultures were performed in biological triplicate. To maintain the
17 plasmids, appropriate concentrations of antibiotics were added to the media (50 µg/mL
18 streptomycin, 50 µg/mL kanamycin, and 34 µg/mL chloramphenicol).

19

20 **Quantification of cell biomass and coenzyme B₁₂**

21 Cell biomass was measured using a UV-1700 spectrophotometer (Shimadzu; Kyoto,
22 Japan) at a wavelength of 600 nm, and one OD₆₀₀ unit was converted to 0.31 g/L of dry cell
23 weight (DCW) (Jo et al., 2019). For validation of coenzyme B₁₂ auxotrophic growth, OD₆₀₀
24 was measured using the VICTOR 1420 Multilabel Counter (PerkinElmer; Waltham, WA,

1 USA). Similarly, OD₆₀₀ was measured at 12, 18, and 24 h for quantification of cell biomass
2 during cell culture for coenzyme B₁₂ production.

3 To quantify the amount of coenzyme B₁₂, cell pellets after 24 h cultivation were
4 harvested, washed twice, and resuspended with 50 mM sodium acetate buffer (pH 4.0). The
5 cells were completely lysed using the French^R Press FA-078A (Thermo Electron Co.,
6 Waltham, MA, USA). The cell lysate was centrifuged and the supernatant was filtered using a
7 0.22- μ m syringe filter to remove the cell debris. Coenzyme B₁₂ was purified from the filtered
8 supernatant using EASI-EXTRACT^R VITAMIN B₁₂ immunoaffinity column (R-Biopharm
9 AG; Darmstadt, Germany) (Ko et al., 2014). The resultant samples were analyzed with ICP-
10 MS (Element XR; Thermo Scientific) at the RIST Analysis & Assessment Center (Pohang,
11 Gyeongbuk, Korea). The amount of cobalt ion, which corresponds to coenzyme B₁₂ (Karmi et
12 al., 2011; Ko et al., 2014) was measured. High-purity argon gas was used as the reaction gas
13 and the operation condition was as follows: auxiliary gas flow, 0.80 L/min; sample gas flow,
14 1.1 L/min; and ICP-RF power, 1250 W. The measurement was performed for biological
15 triplicate.

16

17 **Quantification of plasmid copy number and gene transcripts**

18 The PCN was determined as previously reported (Kang et al., 2018; Škulj et al., 2008).
19 Briefly, cultured samples were harvested and denatured by heating at 95°C for 10 min.
20 Especially, to remove a potential interference, RNAs were removed by the addition of RNase
21 (Sigma-Aldrich). RSF_RT_F/R, CDF_RT_F/R, and ACY_RT_F/R primer pairs were utilized
22 to quantify the PCN of pRcob, pCcob, and pAcob, respectively. A polA_RT_F/R primer pair
23 was also used to quantify the copy number of the chromosome as a reference (Kang et al.,
24 2018). For qPCR, the StepOnePlus Real-time PCR system (Applied Biosystems; Foster City,

1 CA, USA) and TOPrealTM qPCR 2X PreMIX (Enzynomics; Daejeon, Korea) were utilized.

2 To measure the amount of *metH* transcripts, total RNAs were extracted using RibospinTM
3 and RiboclearTM plus (GeneAll) from the cell culture. Reverse transcription was conducted to
4 obtain complementary DNA using the SuperScript III Reverse Transcriptase (Invitrogen;
5 Carlsbad, CA, USA). The *metH*_RT_F/R and *cysG*_RT_F/R primer pairs were used for
6 quantifying *metH* and *cysG* (as internal standards), respectively. The amount of transcripts
7 was calculated using the comparative C_T method (Schmittgen and Livak, 2008). All
8 experiments were performed in technical triplicate of a single strain.

9

1 **References**

- 2 Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in
3 *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6640–
4 6645.
- 5 Haldimann, A., Wanner, B.L., 2001. Conditional-replication, integration, excision, and
6 retrieval plasmid-host systems for gene structure-function studies of bacteria. *J.*
7 *Bacteriol.* 183, 21.
- 8 Jo, M., Noh, M.H., Lim, H.G., Kang, C.W., Im, D.K., Oh, M.K., Jung, G.Y., 2019. Precise
9 tuning of the glyoxylate cycle in *Escherichia coli* for efficient tyrosine production from
10 acetate. *Microb. Cell Fact.* 18, 57.
- 11 Kang, C.W., Lim, H.G., Yang, J., Noh, M.H., Seo, S.W., Jung, G.Y., 2018. Synthetic
12 auxotrophs for stable and tunable maintenance of plasmid copy number. *Metab. Eng.* 48,
13 121–128.
- 14 Karmi, O., Zayed, A., Baragethi, S., Qadi, M., Ghanem, R., 2011. Measurement of vitamin
15 B12 concentration: A review on available methods. *IIOAB J.* 2, 23–32.
- 16 Ko, Y., Ashok, S., Ainala, S.K., Sankaranarayanan, M., Chun, A.Y., Jung, G.Y., Park, S.,
17 2014. Coenzyme B12 can be produced by engineered *Escherichia coli* under both
18 anaerobic and aerobic conditions. *Biotechnol. J.* 9, 1526–1535.
- 19 Lee, J.W., Gyorgy, A., Cameron, D.E., Pyenson, N., Choi, K.R., Way, J.C., Silver, P.A., Del
20 Vecchio, D., Collins, J.J., 2016. Creating single-copy genetic circuits. *Mol. Cell* 63,
21 329–336.
- 22 Noh, M.H., Lim, H.G., Woo, S.H., Song, J., Jung, G.Y., 2018. Production of itaconic acid
23 from acetate by engineering acid-tolerant *Escherichia coli* W. *Biotechnol. Bioeng.* 115,
24 729–738.

- 1 Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative
2 CTmethod. *Nat. Protoc.* 3, 1101–1108.
- 3 Škulj, M., Okršlar, V., Jalen, Š., Jevševar, S., Slanc, P., Štrukelj, B., Menart, V., 2008.
4 Improved determination of plasmid copy number using quantitative real-time PCR for
5 monitoring fermentation processes. *Microb. Cell Fact.* 7, 6.
6