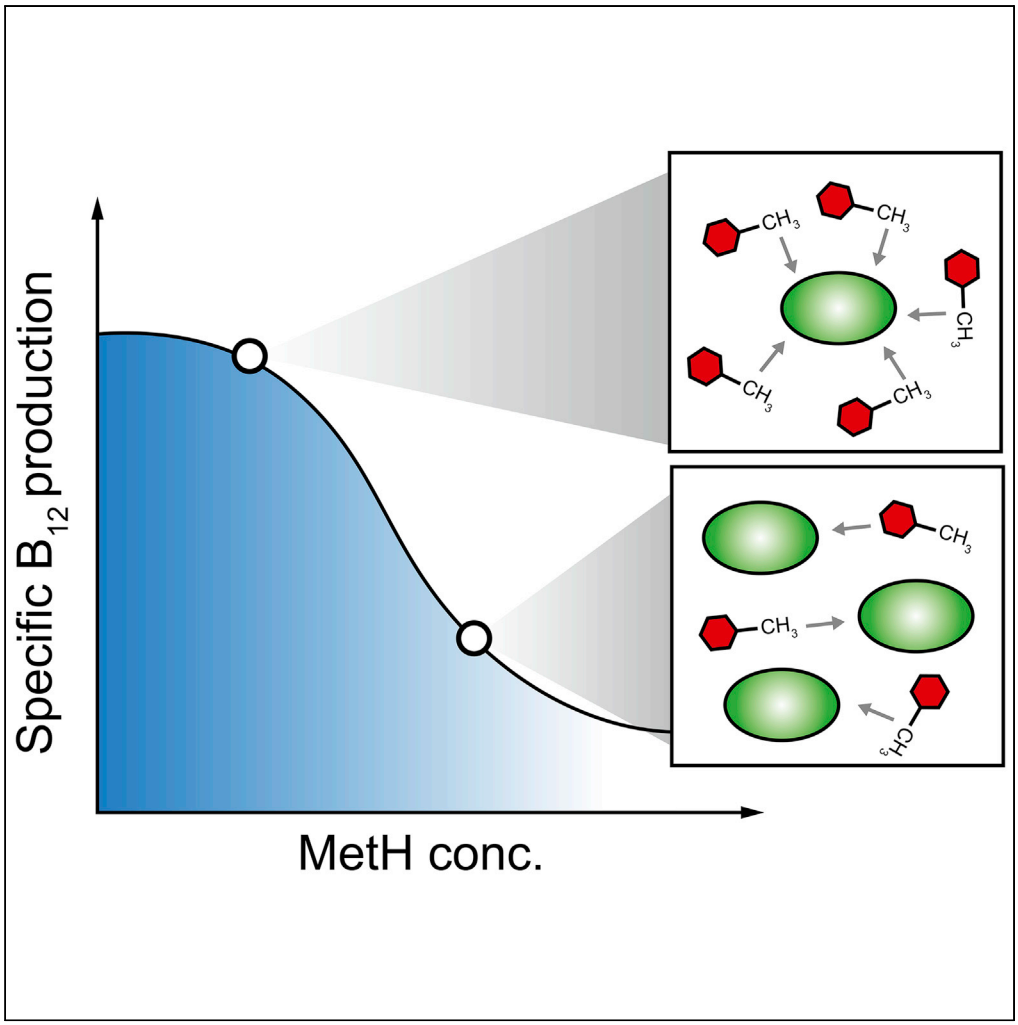
iScience

CellPress

Article

Auxotrophic Selection Strategy for Improved Production of Coenzyme B_{12} in *Escherichia coli*



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

gyjung@postech.ac.kr

HIGHLIGHTS

The auxotrophic selection strategy was applied to coenzyme B_{12} production

Coenzyme B_{12}-independent methionine synthase was deleted for auxotroph system

The auxotrophic strategy could significantly enhance the coenzyme B_{12} production

Optimization of the auxotroph system further enhanced the coenzyme B_{12} production

Noh et al., iScience *23*, 100890 March 27, 2020
https://doi.org/10.1016/j.isci.2020.100890

iScience

CellPress

Article

Auxotrophic Selection Strategy for Improved Production of Coenzyme B_{12} 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 B_{12} using well-characterized microorganisms, such as *Escherichia coli*, has recently attracted considerable attention to meet growing demands of coenzyme B_{12} in various applications. In the present study, we designed an auxotrophic selection strategy and demonstrated the enhanced production of coenzyme B_{12} using a previously engineered coenzyme B_{12}-producing *E. coli* strain. To select a high producer, the coenzyme B_{12}-independent methionine synthase (*metE*) gene was deleted in *E. coli*, thus limiting its methionine synthesis to only that via coenzyme B_{12}-dependent synthase (encoded by *metH*). Following the deletion of *metE*, significantly enhanced production of the specific coenzyme B_{12} validated the coenzyme B_{12}-dependent auxotrophic growth. Further precise tuning of the auxotrophic system by varying the expression of *metH* substantially increased the cell biomass and coenzyme B_{12} production, suggesting that our strategy could be effectively applied to *E. coli* and other coenzyme B_{12}-producing strains.

INTRODUCTION

Coenzyme B_{12}, 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 B_{12} has steadily increased owing to its applications in food, feed additive, and pharmaceutical industries (Fang et al., 2018, 2017). However, chemical synthesis of coenzyme B_{12} is highly complicated because of its complex structure. To overcome this shortcoming, industrial production of coenzyme B_{12} through microbial fermentation has been regarded as an efficient method (Biedendieck et al., 2010).

Currently, microorganisms with the inherent ability to synthesize coenzyme B_{12}, 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 B_{12}. In this regard, recent studies have reported the production of coenzyme B_{12} 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 B_{12} upon the addition of ado-cobinamide (AdoCbi) and dimethylbenzimidazole (DMBI) (Fowler et al., 2010; Jang et al., 2018) via the native coenzyme B_{12} 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 B_{12} (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 B_{12} (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 B_{12}.

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

[1]Department of Chemical Engineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongsangbuk-do 37673, Korea

[2]School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongsangbuk-do 37673, Korea

[3]School of Energy and Chemical Engineering, Ulsan National Institute of Science and Technology, 50 UNIST-Ro, Ulju-gun, Ulsan, Korea

[4]These authors contributed equally

[5]Lead Contact

*Correspondence: gyjung@postech.ac.kr

https://doi.org/10.1016/j.isci.2020.100890

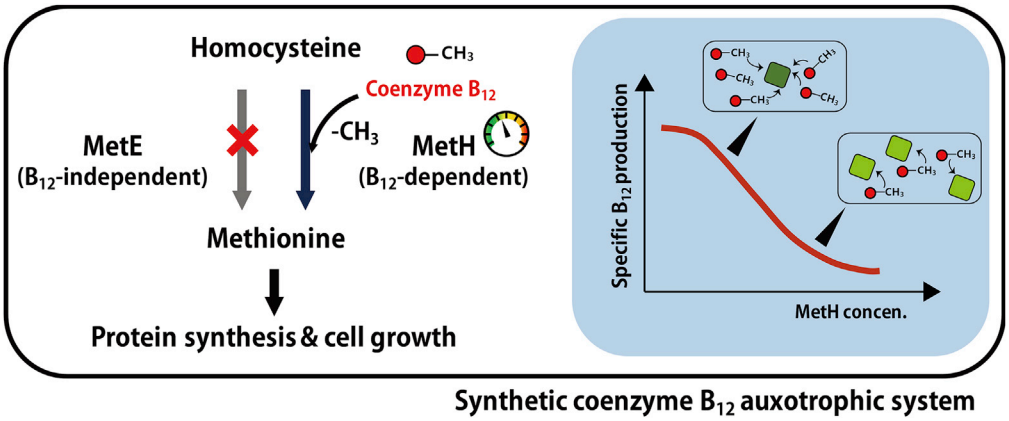


Figure 1. Schematic Diagram of the Overall Strategies Used in the Study

The synthetic coenzyme B_{12} 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 B_{12}. 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 B_{12} production in *E. coli*. We leveraged the characteristics of methionine biosynthesis in *E. coli*; *E. coli* was engineered to synthesize methionine only if coenzyme B_{12} 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 B_{12}. When the strategy was applied to the EpACR^{cob} strain, a previously reported coenzyme B_{12}-producing strain (Ko et al., 2014), the specific coenzyme B_{12} 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 B_{12} production. The engineered strain exhibited significantly enhanced coenzyme B_{12} 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 B_{12}.

RESULTS

Construction of Coenzyme B_{12} Auxotroph System in *E. coli*

To develop the auxotrophic selection strategy for the production of coenzyme B_{12}, we initially sought an essential enzymatic reaction dependent on coenzyme B_{12} 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 B_{12} 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,

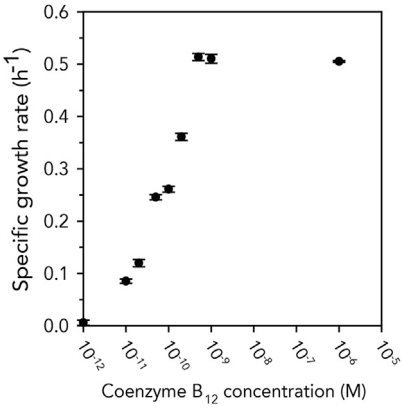


Figure 2. The Validation of Coenzyme B_{12} Auxotrophic Cell Growth in the EDMB Strain

The specific cell growth rate (h^{-1}) was calculated and plotted on the y axis according to the coenzyme B_{12} 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 B_{12} production study (Ko et al., 2014). Because it plays an essential role in coenzyme B_{12} 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 B_{12} auxotrophic growth was achieved following the addition of coenzyme B_{12}.

Upon deleting *metE*, methionine is produced only by coenzyme B_{12}-dependent MetH and the cells are able to grow in the presence of coenzyme B_{12}. To validate the coenzyme B_{12}-dependent cell growth, the resulting EDMB strain (*E. coli* BL21(DE3) with *metE* deletion and functional *btuB*, Table S1) was cultured in the B_{12} auxotrophic medium containing all amino acids except methionine (see the Transparent Methods section). As expected, the strain exhibited negligible growth (growth rate <0.01 h^{-1}) because of its inability to synthesize methionine. When the medium was supplemented with varying concentrations of coenzyme B_{12} ranging from 0 M to 1 μM (~1.6 mg/L), the specific growth rate gradually increased as the concentration of coenzyme B_{12} was increased to 500 pM (Figure 2), reaching that of the wild-type (0.51 h^{-1}) when a sufficient amount of coenzyme B_{12} was present. Although the range of concentrations where the auxotrophic selection functions are at the picomolar level, given that the production of coenzyme B_{12} 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 B_{12} auxotrophic system was successfully constructed in *E. coli*.

Application of Auxotrophic System for Coenzyme B_{12} Production

The auxotrophic system was applied to the previously reported coenzyme B_{12}-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 B_{12} 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 RB_{12} 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 B_{12} in both strains to investigate the effect of the selection strategy. The EpACR^{cob} strain exhibited higher production of coenzyme B_{12} (2.43 μg/g DCW) when compared with the previously reported value (0.65 μg/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 RB_{12} 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 B_{12} production compared with the EpACR^{cob} strain (Figure 3B, 6.64 μg/g DCW). The

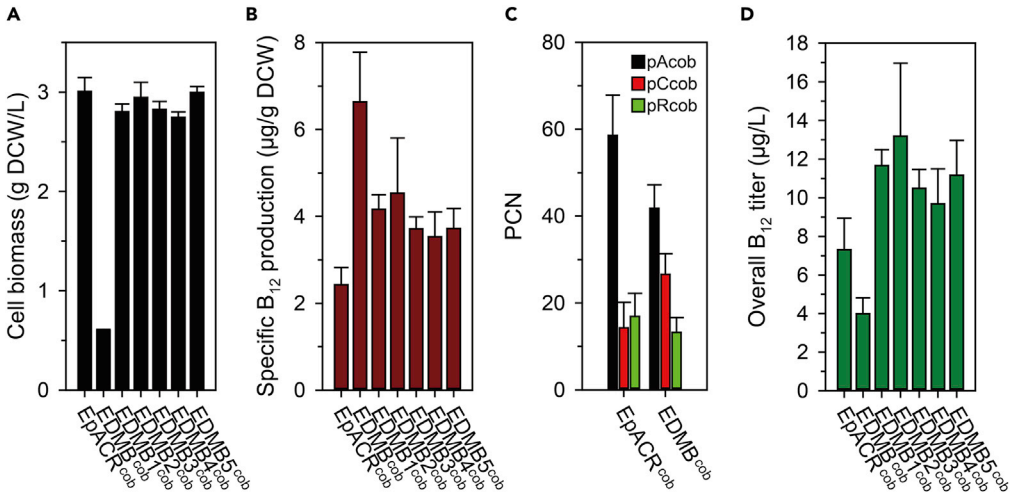


Figure 3. The Effect of Synthetic Auxotrophic System on Coenzyme B_{12}-Producing Strains

Comparison of cell biomass (A) (See also Figure S2), specific coenzyme B_{12} production (B), and plasmid copy number (PCN) after 12-h cultivation (C) (see also Figure S3) and overall coenzyme B_{12} 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 B_{12}.

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 (ColA 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 B_{12}.

Optimization of *metH* Expression for Enhancing Coenzyme B_{12} 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

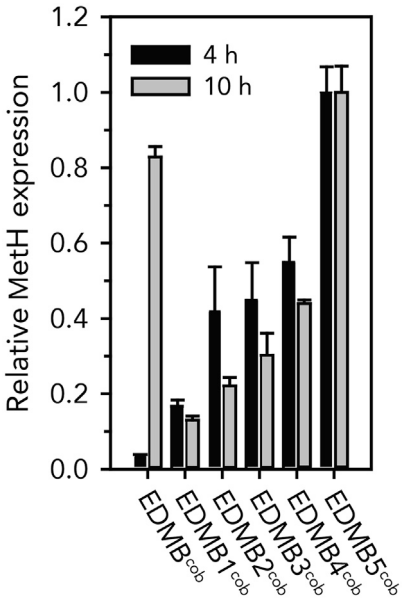


Figure 4. Comparison of Normalized Amounts of *metH* Transcript in Engineered Strains

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 B_{12} (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 B_{12} (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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12}-producing strains.

DISCUSSION

Recent studies have successfully demonstrated the production of coenzyme B_{12} 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 B_{12} production was designed and applied to previously constructed coenzyme B_{12}-producing *E. coli* (Ko et al., 2014). Initially, the coenzyme B_{12} 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 B_{12} production. We found modulated copy numbers in the coenzyme B_{12}-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 B_{12} 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 B_{12} 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 B_{12}-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 B_{12}, the expression of cobalamin-dependent methyltransferase may need to be optimized at lower levels. Alternatively, the coenzyme B_{12}-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 B_{12} 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 B_{12} 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 B_{12}.

Limitations of the Study

The system described in the present study may be limited by the binding affinity of MetH to coenzyme B_{12}. When this system is applied to a superior coenzyme B_{12}-producing strain (Fang et al., 2018), its operational range needs to be investigated or modified, if necessary. As discussed, the coenzyme B_{12}-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 B_{12} 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.

ACKNOWLEDGMENTS

This research was supported by the C1 Gas Refinery Program (NRF-2018M3D3A1A01055754) and the National Research Foundation of Korea grant (NRF-2019R1A2C2084631) funded by the Ministry of Science and ICT. This research was also supported by the Korea Institute of Energy Technology Evaluation and Planning (KETEP) and the Ministry of Trade, Industry and Energy (MOTIE) of the Republic of Korea (no. 20194030202330).

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.

Received: October 29, 2019
Revised: January 17, 2020
Accepted: February 3, 2020
Published: March 27, 2020

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. Biopro. Eng. *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.

iScience, Volume *23*

Supplemental Information

Auxotrophic Selection Strategy for Improved Production of Coenzyme B_{12} in *Escherichia coli*

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

Supplementary Figures

Figure S1. Coenzyme B_{12} synthesis pathway and predicted function of genes (Ko et al., 2014), related to Figure 1.

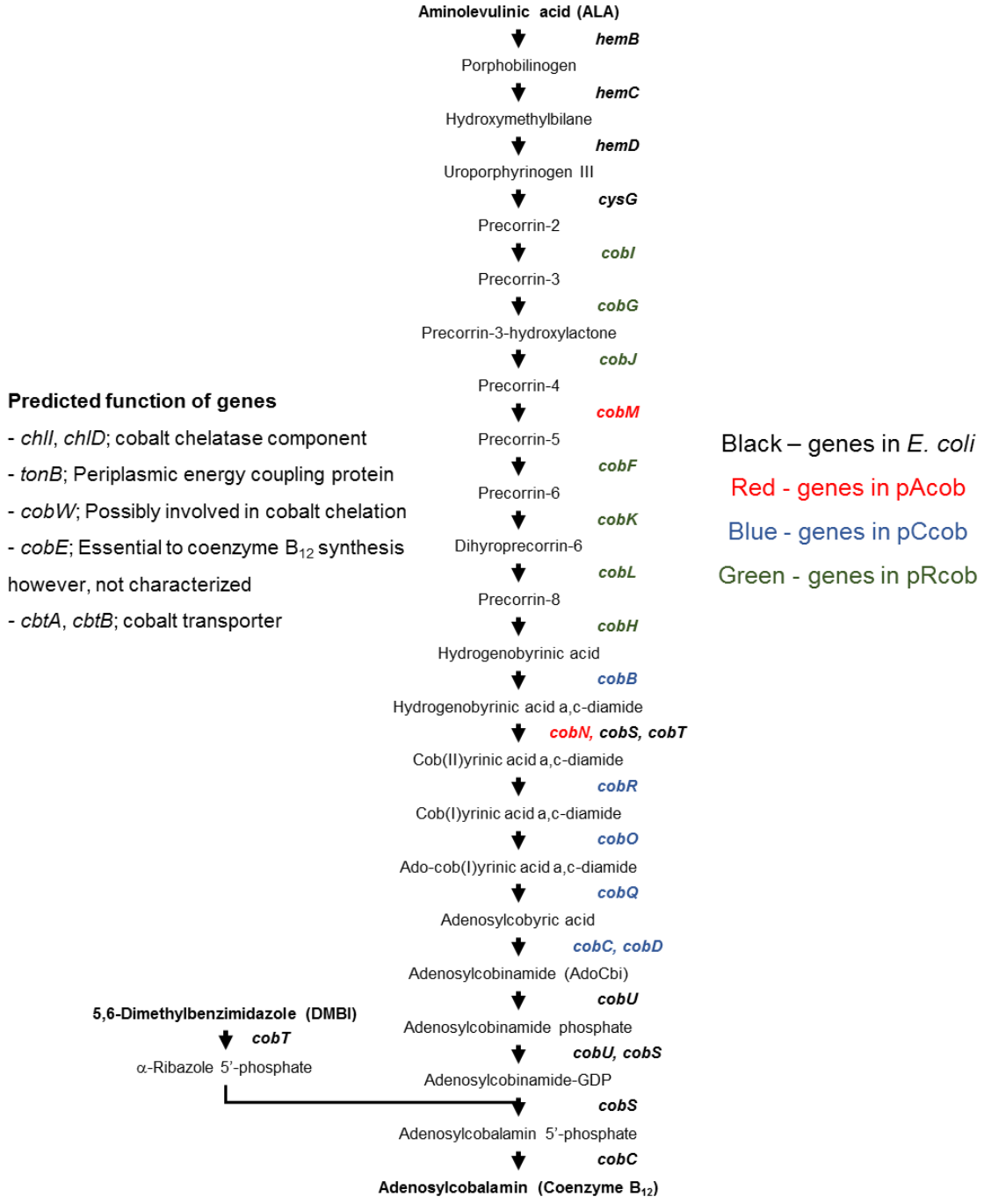


Figure S2. The effect of synthetic auxotrophic system on obtained cell biomass of coenzyme B_{12}-producing strains, related to Figure 3A. Error bars indicate the standard deviations from three independent cultures.

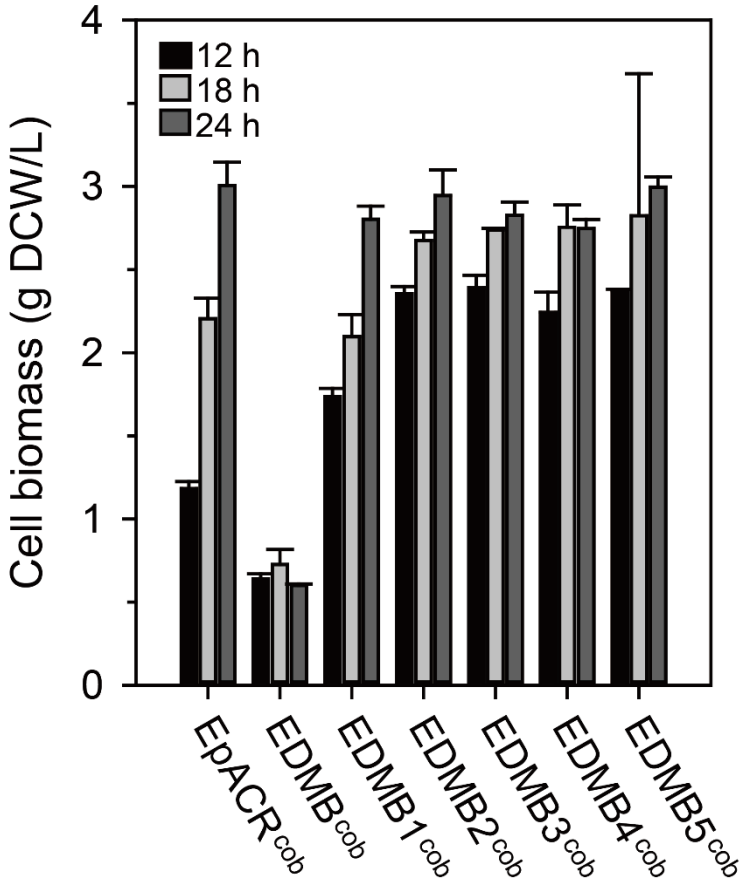
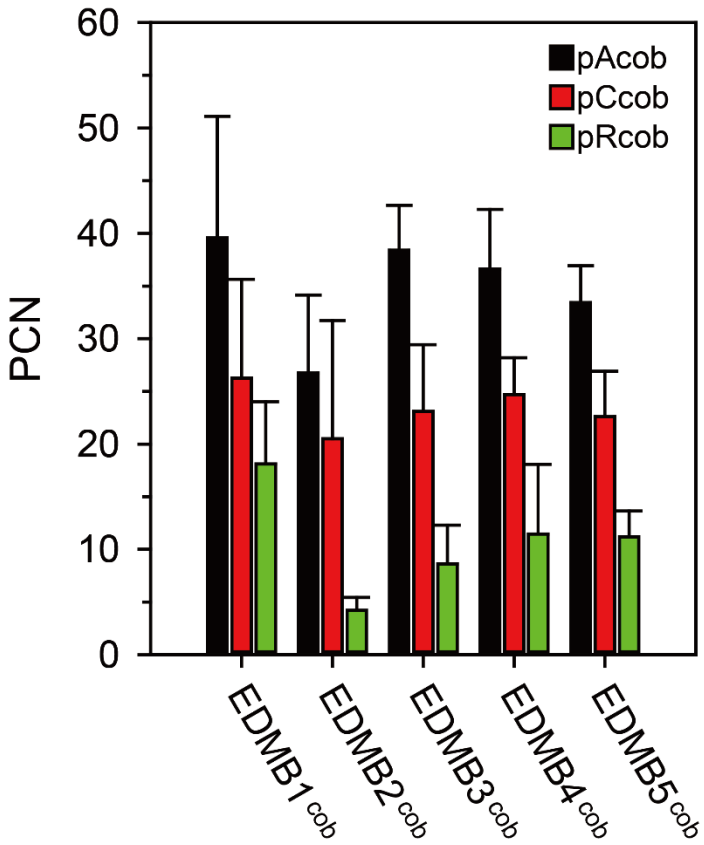


Figure S3. The effect of synthetic auxotrophic system on plasmid copy number of coenzyme B_{12}-producing strains, related to Figure 3C. Error bars indicate the standard deviations from three independent cultures.



Supplementary Tables

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

Name	Description[a]	Source
Strains		
E. coli Mach1-T1^R	Cloning host	Invitrogen
E. coli BL21(DE3)	Expression host	Invitrogen
EpACR^{cob}	*E. coli* BL21(DE3) / pAcob / pCob / pRcob	(Ko et al., 2014)
EDM	*E. coli* BL21(DE3)Δ*metE*	This study
EDMB	*E. coli* BL21(DE3)Δ*metE btuB*(*58Q)	This study
EDMB0	*E. coli* BL21(DE3)Δ*metE btuB*(*58Q) Δ*metH*	This study
EDMB1	EDMB0 att_{HK022}::J23118_nativeUTR_metH_ter_{T7}	This study
EDMB2	EDMB0 att_{HK022}::J23108_nativeUTR_metH_ter_{T7}	This study
EDMB3	EDMB0 att_{HK022}::J23110_nativeUTR_metH_ter_{T7}	This study
EDMB4	EDMB0 att_{HK022}::J23115_nativeUTR_metH_ter_{T7}	This study
EDMB5	EDMB0 att_{HK022}::J23100_nativeUTR_metH_ter_{T7}	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}-*cobWN*- tet_{T7}, P_{T7}-*cbtAB_cobEM*-tet_{T7}	(Ko et al., 2014)
pCcob	ColA ori, Sm^R, P_{T7}-*chlID*- tet_{T7}, P_{T7}-*tonB_cobOBRDCQ*- tet_{T7}	(Ko et al., 2014)
pRcob	RSF1030 ori, Km^R, P_{T7}-*cobGHIJ*- tet_{T7}, P_{T7}-*cobLFK*- 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, *E. coli* expression vector	(Haldimann and Wanner, 2001)
pBAC-L_6-$P_{T3}T_4$Ei	PCR template for att_{HK022}-FRT-Cm^R-FRT, R6Kγori, Cm^R	(Lee et al., 2016)
pCDFduet-1	ColA ori, Sm^R, *E. coli* expression vector	Novagen

pCDFHM1-5	ColA ori, Sm^R, att_{HK022}-FRT-Cm^R-FRT, P_{BBa_J231XX}-nativeUTR-*metH*-tet_{T7}	This study

[a]Abbreviations: Amp, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Km, kanamycin; R, resistance.

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

Name	Sequence (5′−3′)[a]
metE_del_F	atgacaatattgaatcacaccctcggtttccctcgcgttggcctgcgtcggcatgaccggcgcgatgc
metE_del_R	gctcagcggatctcatgcgctcatccccgacgcaaattctgcgccgcctgcaccatgttcgccagtgccg
btuB_s.d.m	ttgcaccaaccaccgttgtgacccgtcaggatatcgaccgctggcagtcgacctcggtcaatgatgtgctg cgccgtcttccgggcgtc
metH_del_F	atgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagcatgaccggcgcgatgc
metH_del_F	tcagtccgcgtcataccccagattcggtgccagccagcgctcaacttcgggctcagcggatctcatgcgc
pCDF_F	ggtatgacgcggactgactcgagaatcagtttaacctaggctgctgccacc
pCDF_R	ggggcttcccggtatcaacagggacaccagttaatgtaagttagctcactcattaggcac
HK022_F	ctggtgtccctgttgataccgggaagcccc
HK022_R	actgagctagccgtcaagaattcgctactgctgcagaacagtagtacttgcacagac
metH_F1 118	cagtagcgaattcttgacggctagctcagtcctaggtattgtgctagctgttgaatttttattaaatctgggttg agcg
metH_F2 108	cagtagcgaattcctgacagctagctcagtcctaggtataatgctagctgttgaatttttattaaatctgggttg agcg
metH_F3 110	cagtagcgaattctttacggctagctcagtcctaggtacaatgctagctgttgaatttttattaaatctgggttg agcg
metH_F4 115	cagtagcgaattctttatagctagctcagcccttggtacaatgctagctgttgaatttttattaaatctgggttga gcg
metH_F5 100	cagtagcgaattcttgacggctagctcagtcctaggtacagtgctagctgttgaatttttattaaatctgggttg agcg
metH_R	tcagtccgcgtcataccccagatt
metH_int_F	ctggtgtccctgttgatacc
metH_int_R	cgtatgggatccatgctagttattgctcagcggtgg
metH_RT_F	catacggaaaaagccaccatctg
metH_RT_R	ggtaacgctcatacctttacgg
cysG_RT_F	cagcaaaagctgattgaacacg
cysG_RT_R	aacaacccgaccaataataattagc
RSF_RT_F	cttgagaaaaccaccgttggta
RSF_RT_R	aacggaatagctgttcgttgac
CDF_RT_F	gtggtttttcgtttacagggca
CDF_RT_R	aatctagagcggttcagtagaaaa
ACY_RT_F	tttagcttccttagctcctgaaaatc
ACY_RT_R	gtaagaggttccaactttcaccata
polA_RT_F	gcgagcgatccagaagatct
polA_RT_R	gattatgggtaaaggatgccaca

[a]Underlined nucleotides indicate synthetic promoter (P_{BBa_J231XX} , http://parts.igem.org)

Transparent Methods

Reagents, oligonucleotides, and media

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

For a rich nutrient environment, as required for the production of coenzyme B_{12}, the RB_{12} medium containing 0.5 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L NH_4Cl, 2.0 g/L NaCl, 10 g/L tryptone, 0.1 mM 5,6-dimethylbenzimidazole (DMBI), 10 mg/L 5-aminolevulinic acid, 10 mg/L $CoCl_2 \cdot 6H_2O$, 100 mM potassium phosphate buffer (pH 7.0), and 20 g/L glucose as the carbon source was used. The B_{12} auxotrophic medium is a modified version of the RB_{12} medium and is obtained by replacing 10 g/L tryptone with defined concentrations of all amino acids except methionine (0.28 g/L alanine, 0.04 g/L cysteine, 0.22 g/L histidine, 0.65 g/L lysine, 0.86 g/L proline, 0.10 g/L tryptophan, 0.33 g/L arginine, 1.87 g/L glutamate, 0.44 g/L isoleucine, 0.50 g/L serine, 0.18 g/L tyrosine, 0.65 g/L aspartic acid, 0.17 g/L glycine, 0.76 g/L leucine, 0.39 g/L threonine, and 0.55 g/L valine). LB medium was used for routine genetic manipulations.

Construction of strains and plasmids

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

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

To control the expression level of *metH*, the native *metH* was replaced and synthetic expression cassettes were introduced. Initially, the native gene was removed similarly to the *metE* deletion except that a metH_del_F/R primer pair was used. The synthetic expression cassettes for *metH* expression were integrated using the phage-integration method (Haldimann and Wanner, 2001). To construct the pCDFHMI1-5 plasmids, required for the integration method, a vector fragment was amplified using pCDF_F/R and pCDFduet-1 as a template. The vector fragments were assembled with fragments amplified using HK022_F/R with pBAC-L_6-$P_{T3}T_4$Ei as a template and metH_F1-5/metH_R with genomic DNA of BL21(DE3) strain, respectively. Thereafter, the fragments for integration were amplified with metH_int_F/R and pCDFHM1-5 plasmids as a template to remove replication origin. The fragments were subsequently digested using *BamHI* and re-circularized for genomic integration (Lee et al., 2016).

Cell culture medium and conditions

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

The culture conditions for B_{12} production were determined by refering to the previously optimized conditions (Ko et al., 2014). It was conducted in 300-mL Erlenmeyer flasks containing 50 mL of the RB_{12} medium. For seed culture, a single colony was inoculated in a 15-mL test tube containing 3 mL of the RB_{12} medium. After 12 h, the seed culture was inoculated in the fresh RB_{12} medium at OD_{600} of 0.05 and cultured. When the OD_{600} reached 0.4, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM for induction. OD_{600} values were monitored with cultivating the cells at 30°C with agitation at 200 rpm. The pH was periodically measured using an Orion™ 8103BN ROSS™ pH meter (Thermo Scientific) and adjusted to 7.0 with a 10 M NaOH solution.

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

Quantification of cell biomass and coenzyme B_{12}

Cell biomass was measured using a UV-1700 spectrophotometer (Shimadzu; Kyoto, Japan) at a wavelength of 600 nm, and one OD_{600} unit was converted to 0.31 g/L of dry cell weight (DCW) (Jo et al., 2019). For validation of coenzyme B_{12} auxotrophic growth, OD_{600} was measured using the VICTOR 1420 Multilabel Counter (PerkinElmer; Waltham, WA,

USA). Similarly, OD_{600} was measured at 12, 18, and 24 h for quantification of cell biomass during cell culture for coenzyme B_{12} production.

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

Quantification of plasmid copy number and gene transcripts

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

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

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

References

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. 97, 6640–6645.

Haldimann, A., Wanner, B.L., 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J. Bacteriol. 183, 21.

Jo, M., Noh, M.H., Lim, H.G., Kang, C.W., Im, D.K., Oh, M.K., Jung, G.Y., 2019. Precise tuning of the glyoxylate cycle in Escherichia coli for efficient tyrosine production from acetate. Microb. Cell Fact. 18, 57.

Kang, C.W., Lim, H.G., Yang, J., Noh, M.H., Seo, S.W., Jung, G.Y., 2018. Synthetic auxotrophs for stable and tunable maintenance of plasmid copy number. Metab. Eng. 48, 121–128.

Karmi, O., Zayed, A., Baraghethi, S., Qadi, M., Ghanem, R., 2011. Measurement of vitamin B12 concentration: A review on available methods. IIOAB J. 2, 23–32.

Ko, Y., Ashok, S., Ainala, S.K., Sankaranarayanan, M., Chun, A.Y., Jung, G.Y., Park, S., 2014. Coenzyme B12 can be produced by engineered Escherichia coli under both anaerobic and aerobic conditions. Biotechnol. J. 9, 1526–1535.

Lee, J.W., Gyorgy, A., Cameron, D.E., Pyenson, N., Choi, K.R., Way, J.C., Silver, P.A., Del Vecchio, D., Collins, J.J., 2016. Creating single-copy genetic circuits. Mol. Cell 63, 329–336.

Noh, M.H., Lim, H.G., Woo, S.H., Song, J., Jung, G.Y., 2018. Production of itaconic acid from acetate by engineering acid-tolerant Escherichia coli W. Biotechnol. Bioeng. 115, 729–738.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CTmethod. Nat. Protoc. 3, 1101–1108.

Škulj, M., Okršlar, V., Jalen, Š., Jevševar, S., Slanc, P., Štrukelj, B., Menart, V., 2008. Improved determination of plasmid copy number using quantitative real-time PCR for monitoring fermentation processes. Microb. Cell Fact. 7, 6.