

Metabolic engineering of *Escherichia coli* for *de novo* biosynthesis of vitamin B₁₂

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ABSTRACT

The only known source of vitamin B₁₂ (adenosylcobalamin) is from bacteria and archaea, and the only unknown step in its biosynthesis is the production of the intermediate adenosylcobinamide phosphate. Here, using genetic and metabolic engineering, we generated an *Escherichia coli* strain that produces vitamin B₁₂ via an engineered *de novo* aerobic biosynthetic pathway. Excitingly, the BluE and CobC enzymes from *Rhodobacter capsulatus* transform L-threonine into (R)-1-Amino-2-propanol O-2-Phosphate, which is then condensed with adenosylcobyrinic acid to yield adenosylcobinamide phosphate by either CobD from the aerobic *R. capsulatus* or CbiB from the anaerobic *Salmonella typhimurium*. These findings suggest that the biosynthetic steps from co(II)byrinic acid a,c-diamide to adocobalamin are the same in both the aerobic and anaerobic pathways. Finally, we increased the vitamin B₁₂ yield of a recombinant *E. coli* strain by more than ~250-fold to 307.00 µg/g DCW via metabolic engineering and optimization of fermentation conditions. Beyond our scientific insights about the aerobic and anaerobic pathways and our demonstration of *E. coli* as a microbial biosynthetic platform for vitamin B₁₂ production, our study offers an encouraging example of how the several dozen proteins of a complex biosynthetic pathway can be transferred between organisms to facilitate industrial production.

KEY WORDS: vitamin B₁₂, *E. coli*, co(II)byrinic acid a,c-diamide, adenosylcobinamide phosphate, (R)-1-Amino-2-propanol O-2-Phosphate

INTRODUCTION

Strong demand from the food, feed additive, and pharmaceutical industries has recently sparked strong interest in the use of bacteria to produce vitamin B₁₂ (adenosylcobalamin)¹. To date, the industrial production of vitamin B₁₂ is mainly from fermentation of *Pseudomonas denitrifians* and *Propionibacterium shermanii*, but these strains grow slow and are difficult to engineer. Thus, the engineering of the *de novo* vitamin B₁₂ biosynthetic pathway in a fast-growing, genetically tractable species like *E. coli* is attractive as an alternative production platform. However, given that the *de novo* biosynthetic pathway contains around 30 genes that would need to be expressed heterologously, the successful production of vitamin B₁₂ represents a significant engineering challenge.

In nature, vitamin B₁₂ is synthesized *de novo* in some bacteria and archaea using one of two alternative pathways: the aerobic pathway (represented by *P. denitrifians*) and the anaerobic pathway (represented by *S. typhimurium*). The major differences between the two pathways relate to the time of cobalt insertion and the requirement for oxygen². In the aerobic pathway, cobalt insertion occurs on hydrogenobyric a,c-diamide (HBAD) and is catalyzed by the CobNST complex at a late pathway stage, and oxygen is required to promote ring-contraction³. In the anaerobic pathway, cobalt insertion occurs on factor II and is catalyzed by CbiK at an early stage⁴. Research using an ingenious enzyme-trap method to trap and identify pathway intermediates has been used to elucidate the biosynthetic mechanism of corrin ring formation for both the aerobic and anaerobic pathways^{5, 6}.

In the aerobic pathway, uroporphyrinogen III (Uro III) is converted to hydrogenobyric acid (HBA) via a series of C-methylations and decarboxylations as well as a ring contraction (Fig. 1). Subsequently, HBA is converted to HBAD through amidations of carboxyl groups at positions a and c by the CobB enzyme in *P. denitrifians*⁷. HBAD is converted to co(II)byric acid a,c-diamide (CBAD) after cobalt insertion, and CBAD then undergoes co(II)rrin reduction, adenylation, and amidations of carboxyl groups at positions b, d, e, and g to yield adenosylcobyrinic acid (AdoCby)⁸.

⁹. It has been assumed that adenosylcobinamide (AdoCbi) is synthesized via the attachment of (R)-1-Amino-2-propanol (AP) to AdoCby to yield AdoCbi in a single step reaction that is catalyzed by a two-component system (designated as α and β in *P. denitrificans* (Fig. 1); the β component is known to comprise the CobC and CobD enzymes in this organism, but the α protein component remains unidentified)⁹. AdoCbi then undergoes phosphorylation to form AdoCbi-P before the final addition of a GMP moiety by the bifunctional enzyme CobP to yield AdoCbi-GDP.

In the anaerobic pathway of *S. typhimurium*: AdoCbi-P is biosynthesized through the attachment of (R)-1-Amino-2-propanol O-2-Phosphate (APP) to AdoCby as catalyzed by an adenosylcobinamide-phosphate synthase CbiB (Fig. 1)¹⁰. APP is synthesized from L-threonine by PduX and CobD^{11, 12}. AdoCbi-P is transformed into AdoCbi-GDP through the addition of a GMP moiety by CobU¹³. Two additional known reactions transfer lower axial ligands onto AdoCbi-GDP, thus producing adenosylcobalamin (AdoCbl)^{14, 15}.

It has been reported that a recombinant *E. coli* expressing vitamin B₁₂ biosynthetic genes from *P. denitrificans* ATCC 13867 produced 0.65 ± 0.03 $\mu\text{g/g}$ DCW vitamin B₁₂¹⁶; however, this report did not include unambiguous chemical evidence of AdoCbl production, as the microbiological assay used is nonspecific for AdoCbl detection and is known to sometimes yield false positives¹⁷. Further, given that protein α has not been identified in the *de novo* aerobic vitamin B₁₂ biosynthetic pathway, AdoCbi-P (an intermediate of the pathway) was not produced in that study. Thus, to date there has been no demonstration of vitamin B₁₂ production in *E. coli*.

Here, seeking to engineer a *de novo* aerobic vitamin B₁₂ biosynthetic pathway in *E. coli*, we needed to determine how AdoCbi-P is synthesized. It also bears mention that there have been no reports CBAD production in a heterologous host, although *in vitro* assays have been reported previously. These two issues are the key problems that we tackle in this study. By comparing and contrasting *in vitro* and *in vivo* CBAD biosynthesis reactions, we determined that cobalt uptake transport proteins are necessary participants for cobalt chelation. We also confirmed that the *bluE*, *cobC*, and *cobD* genes from *R. capsulatus* are functional homologues of, respectively, *pduX*, *cobD*, and *cbiB* from *S.*

typhimurium.

We successfully engineered *E. coli* to produce vitamin B₁₂ by heterologously expressing a total of 28 genes from *R. capsulatus*, *B. melitensis*, *S. meliloti*, *S. typhimurium*, and *Rhodopseudomonas palustris* that were divided into 6 engineered modules. The enzymes of Module 1 together produce HBA from Uro III, and Module 2 produces CBAD from HBA. Module 3 includes the four cobalt transport proteins CbiM,N,Q,O enabling cobalt uptake from the environment. Module 4 synthesizes AdoCbi-P from CBAD and L-threonine. Module 5 includes the biosynthetic enzymes CobU, CobS, CobT, and CobC from the endogenous *E. coli* vitamin B₁₂ salvage pathway, and is used to convert AdoCbi-P to AdoCbl. Module 6 includes HemO, HemB, HemC, and HemD and was designed as a metabolic engineering booster to generate the aforementioned precursor Uro III to increase HBA biosynthesis. Each module was expressed on individual plasmids or was incorporated into the *E. coli* genome. The modules were assayed *in vitro* and/or *in vivo* to confirm their functions. After metabolic engineering of strains to ameliorate the cobalt chelation and Module 4 bottlenecks, as well as optimization of fermentation conditions, we finally improved the vitamin B₁₂ production to 307.00 µg/g DCW.

RESULTS

Biosynthesis of CBAD in *E. coli*

We started the construction of the *de novo* vitamin B₁₂ biosynthetic pathway using *E. coli* strain MG1655 (DE3) into which we transformed the pET28-HBA plasmid (Supplementary Data 1) to enable it to synthesize HBA, resulting in strain FH001. FH001 was cultured in 2×YT medium, and produced 0.73 mg/g DCW HBA (HPLC analysis). We then expressed the pCDFDuet-1 plasmid harboring *cobB* from *R. capsulatus* in FH001 and thereby generated the HBAD-producing strain FH131 (Supplementary Fig. 1); LC-MS analysis showed that FH131 could produce 0.17 mg/g DCW HBAD (Fig. 2b).

We conducted a series of *in vitro* assays to support our identification of functional CobN, CobS, and CobT enzymes for the production of CBAD. To increase the likelihood of obtaining functional versions of these three enzymes, three variants from *Sinorhizobium meliloti* 320¹⁸, *Brucella melitensis*, and *R. capsulatus* were tested (total of 9 enzymes tested). Note that the substrate HBAD is not commercially available, so we purified it from an HBAD-producing strain via an enzyme-trap method. Previous work has established that purified CobN protein can bind to its substrate HBAD³, so we expressed the *cobN* genes from *B. melitensis*, *S. meliloti*, and *R. capsulatus* with N terminal fusion hexa-histidine tags using the pCDF-cobB plasmid and transformed these into the FH001 strain, respectively generating strains FH159, FH160, and FH161. Cultures of these strains were used to purify CobN and HBAD complexes via the enzyme-trap method detailed in the Methods section, and after boiling, LC-MS analysis showed that HBAD could be detected in all samples (Supplementary Fig. 2), thereby demonstrating that CobN can bind its substrate HBAD without the aid of CobS or CobT.

We next attempted to purify CobS and CobT from *S. meliloti* 320, *B. melitensis*, and *R. capsulatus*. Unfortunately, the CobT proteins were prone to precipitate during purification. We therefore temporarily shifted our focus to examine CobT denaturation: given that CobS and CobT have a common evolutionary origin with, respectively, the magnesium chelatase subunits BchI and BchD¹⁹, and considering that BchI is known to be a chaperone essential for the function of the BchD magnesium chelatase subunit²⁰, we hypothesized that CobT needs CobS to assist with its protein folding. To test this hypothesis, CobS and CobT from *S. meliloti* 320, *B. melitensis*, or *R. capsulatus* (each with a C-terminal hexa-histidine tag) were purified via affinity chromatography (SDS-PAGE analysis shown in Supplementary Fig. 3). We found that when CobS was present we did not observe CobT precipitation. Having overcome this issue with CobT, we next conducted *in vitro* assays with HBAD, CobN, CobS, and CobT and used LC-MS to confirm their activity: CBAD was formed when the substrate HBAD, the three enzymes, and cobalt chloride were all present, but was not detected in the control group lacking cobalt chloride (Supplementary Fig. 4, 5 and Supplementary Note 1).

Having confirmed the ability of CobN, CobS, and CobT to produce CBAD *in vitro*,

we next expressed these proteins *in vivo* to synthesize CBAD. Initially we used the *cobB* gene from *R. capsulatus* and *cobN* (with a fused N-terminal hexa-his tag to increase translation efficiency; SDS-PAGE analysis shown in Supplementary Fig. 7), *cobS*, and *cobT* genes from *S. meliloti* 320, *B. melitensis* or *R. capsulatus* (expressed as pseudo-operons) on a single plasmid (Module 2). LC-MS analysis of these strains did not detect CBAD, and these discrepancies in the results from the *in vivo* vs. *in vitro* assays prompted us to consider how cobalt metabolism may have affected biosynthesis in our *E. coli* cells. It has been previously reported that the heterologous expression in *E. coli* of the CbiM,N,Q,O proteins from *S. typhimurium* or from *R. capsulatus* enables cobalt uptake²¹. To this end, a plasmid carrying genes for these four proteins (Module 3) was co-transformed into the FH001 strain alongside plasmids harboring Module 2, generating strains FH163, FH164, and FH165 (a separate strain for each of the Module 2 variants, see above).

In agreement with our hypothesis, each of these strains could produce CBAD, demonstrating the necessity of cobalt uptake transporters for cobalt chelation (Fig. 2). FH164 had the highest CBAD yield among these three strains. Module 3 was then integrated into the *E. coli* genome and the *endA* gene (encoding DNA-specific endonuclease I) was deleted, resulting in strain FH215. When the plasmids pET28-HBA (Module 1) and pCDF-cobB-BmcobN-his-BmcobS-BmcobT (Module 2) were transformed into FH215, CBAD could be produced in the new strain FH216, which was subsequently used as the CBAD-producing strain for our continued engineering of vitamin B₁₂ production in *E. coli*.

Biosynthesis of (R)-1-Amino-2-propanol O-2-Phosphate in *E. coli*

A pathway for (R)-1-Amino-2-propanol O-2-Phosphate (APP)—an intermediate in cobalamin biosynthesis—has been proposed in *S. typhimurium*: L-threonine undergoes phosphorylation by PduX and decarboxylation by *StCobD* (CobD from *S. typhimurium*) (Fig. 3a). APP is then condensed with AdoCby into AdoCbi-P by CbiB. Although the functions of PduX and *StCobD* have been demonstrated *in vitro*, the supposed APP biosynthetic pathway has not been verified *in vivo*. In *P. denitrificans*, it is recognized

that (R)-1-Amino-2-propanol (AP) is somehow attached to adenosyl-cobyrinic acid catalyzed by a complex comprising CobC, CobD, and the as-yet-unidentified protein α . However, the K_m value for (R)-1-Amino-2-propanol was very high (20 mM²²), suggesting that (R)-1-Amino-2-propanol might not be the true substrate for this AP-synthesizing complex. Interestingly, we found that homologues of the anaerobic pathway genes *pduX*, *cobD*, and *cbiB* from *S. typhimurium* are present in genome of the aerobic-pathway-utilizing *R. capsulatus* as, respectively, the *blue*, *cobC*, and *cobD* genes.

It thus appears likely that the aerobic and anaerobic pathways employ the same biosynthetic reactions to produce APP and AdoCbi-P. To test this hypothesis, *blue* and *cobC* from *R. capsulatus*, which encode potential L-threonine kinase and threonine-O-3-phosphate decarboxylases, respectively, were assayed *in vitro* and *in vivo*. BluE and PduX were initially expressed at a very low level from the pACYCduet-1 plasmid. To minimize locally stabilized mRNA secondary structures and thereby enable efficient translation initiation of the *blue* and *pduX* transcripts, two bicistronic vectors were designed. In the first plasmid (pACYC-MBP-*blue*), a sequence derived from the first 10 amino acids of the maltose binding protein (MBP) was placed located upstream of the second codon-optimized *blue* sequence (Supplementary Fig. 8a, b). In the second plasmid (pACYC-his-*pduX*), a sequence derived from a his tag is located upstream of the second *pduX* sequence (Supplementary Fig. 8c, d). SDS-PAGE analysis showed that the expression level of *blue* and *pduX* were significantly improved in strains carrying this system (Fig. 3b). As BluE degenerated almost completely during purification, crude BluE activity was confirmed via an *in vitro* assay (Supplementary Fig. 9). The activity of RcCobC (CobC from *R. capsulatus*) was also confirmed via *in vitro* assays (Supplementary Fig. 10). The K_m and V_{max} values of RcCobC were measured to be 25.6 μ M/min and 1.89 mM, respectively.

Given that *E. coli* has an endogenous pathway to produce AP (Fig. 3a), we deleted the *gldA* gene from *E. coli* MG1655 (DE3); the resulting strain was designated as FH291. We then designed and introduced an AP pathway into FH291 to confirm the functions of BluE/PduX and RcCobC/StCobD *in vivo* (Fig. 3c, d, e): the new

BluE/PduX strains FH296/FH298 both produced Thr-P (HPLC analysis), demonstrating that both PduX from *S. typhimurium* and BluE from *R. capsulatus* are L -threonine kinases; The new *RcCobC/StCobD* strains FH297/FH300 both produced AP, demonstrating that CobD from *S. typhimurium* and CobC from *R. capsulatus* are L -threonine-O-3-phosphate decarboxylases. These results established that functional BluE/PduX and *RcCobC/StCobD* can be used to synthesize APP in recombinant *E. coli* strains. We also deleted the genes encoding PhoA (an alkaline phosphatase) and AphA (an acid phosphatase) successively in FH297 and FH300. However, as each of these strains still produced AP, the phosphatase that converts APP to AP in *E. coli* is not yet clear.

De novo* biosynthesis of vitamin B₁₂ in *E. coli

AdoCby is formed from CBAD in three steps. In the first step, CBAD is reduced to cob(I)yrinic acid a,c-diamide by an NADH-dependent flavoenzyme that (in aerobic *Pseudomonas denitrificans*) exhibits cob(II)yrinic acid a,c-diamide reductase activity. However, the gene encoding this enzyme has not been identified. The *cobR* gene from aerobic *B. melitensis* has been demonstrated to encode a biosynthetic enzyme with co(II)rrin reductase activity²³. In the next biosynthesis step of the known pathway, cob(I)yrinic acid a,c-diamide is transformed to its adenosyl form by an adenosyltransferase. In some bacteria, identical adenosyltransferases catalyze adenylation reactions on cobinamide in a salvage pathway. Therefore, the adenosyltransferase BtuR of *E. coli* in the salvage pathway might function on the substrate cob(I)yrinic acid a,c-diamide.

To explore this idea, the strain FH216 was used as the host cell for functional testing of a Cbi module (Module 4). Seeking to synthesize the precursor AdoCbi-P, the design of this module included both proteins of known function (CobR from *B. melitensis*, adenosylcobinamide-phosphate synthase from *S. typhimurium*, as well as a L -threonine kinase and a threonine-O-3-phosphate decarboxylase from each of *S. typhimurium* and *R. capsulatus*) and candidate enzymes (BtuR from *E. coli* and an adenosylcobinamide-phosphate synthase from *R. capsulatus*). Note that *E. coli* can

assemble a nucleotide Loop (modified lower axial ligand) using endogenous CobU, CobT, CobS, and CobC, so AdoCbi-P can be transformed into AdoCbl using the endogenous *E. coli* salvage pathway^{10, 24}.

Finally, two recombinant strains FH218 (with *pduX*, *cobD*, and *cbiB* from *S. typhimurium*) and FH219 (with *bluE*, *cobC*, and *cobD* from *R. capsulatus*) harboring Modules 1-5 were obtained (Fig. 4a). We supplemented the CM medium with L-threonine to circumvent the need to engineer the supply of this amino acid in host cells. Our detection of vitamin B₁₂ by LC-MS and comparison of its retention time and spectrum with the chemical reference standard demonstrated the successful assembly of our completed engineered *de novo* vitamin B₁₂ biosynthesis pathway in recombinant strains FH218 and FH219. Beyond this bioengineering result, our successful demonstration of vitamin B₁₂ in this system confirms the scientific fact that APP participates in the biosynthesis of AdoCbi-P in both the aerobic and anaerobic pathways (Fig. 4). However, FH218 and FH219 produced only 2.18 µg/g DCW and 1.22 µg/g DCW vitamin B₁₂, respectively (Supplementary Fig. 11), and it should be noted that HBA was not detected in FH164, a finding which suggested the idea that HBA availability may be a limit to CBAD synthesis.

Increasing the HBA pool increased vitamin B₁₂ production

Vitamin B₁₂ and other tetrapyrrole compounds including heme, siroheme, and coenzyme F₄₃₀ each originate from δ-aminolevulinate (ALA). ALA is synthesized via either a C₄ or C₅ pathway²⁵. In the C₄ pathway, glycine and succinyl-CoA are condensed by ALA synthase (encoded by either *hemA* or *hemO*) to form ALA. In the C₅ pathway, ALA is synthesized from glutamate via a three-step reaction catalyzed successively by the enzymes Gltx, HemA, and HemL²⁶. Then, 8 molecules of ALA undergo condensation, polymerization, and cyclization to form Uro III, which is a precursor for the biosynthesis of vitamin B₁₂, heme, and coenzyme F₄₃₀²⁷.

Given that the *cobAIGJMFKLH* operon is driven by a strong T7 promoter, we heterologously expressed the *hemO*, *hemB*, *hemC*, and *hemD* genes (the pathway components from ALA to Uro III) with the aim of converting additional precursor

molecules into HBA. HemO from *R. palustris* and HemB, HemC, and HemD from *S. meliloti* were expressed as a polycistronic product in the FH001 strain, which boosted HBA production from 0.54 mg/g DCW to 6.43 mg/g DCW after 20 h of growth, albeit with reduced overall growth (Supplementary Fig. 12). Our experiments showed that HBA production in the FH001 strain gradually decreased from 8 h to 38 h. However, HBA production of FH168 peaked at 20 h and then gradually decreased, highlighting that the accumulation of precursors from ALA to Uro III does boost HBA production in FH168. We speculate that this apparent gradual decrease in HBA levels from 20 h to 38 may related to HBA stability or perhaps the decreased catalytic function of the heterologous enzymes over time.

In addition, the maximum ALA yields for the FH001 and FH168 strains were 0.10 g/L and 0.67 g/L, respectively, confirming that the expression of heterologous ALA synthase boosted ALA production. Expression of a polycistronic product from a *hemOBCD* gene cluster lead to heme accumulation and was toxic²⁸. To decrease the formation of heme, we used a small RNA strategy²⁹ to knockdown the expression of *hemE*, *hemF*, *hemG*, and *hemH* in FH168 (Supplementary Fig. 13). The FH187 strain (*hemG* knockdown) had the highest HBA yield at 9.34 mg/g DCW, a 45.26% increase over FH168. We then assessed 6 strains that had the expression of two of their heme biosynthesis genes simultaneously knocked down via small RNA (Supplementary Fig. 14). Of particular note, the HBA yield of the strain FH192 (*hemF* and *hemG* knockdown) was 14.09 mg/g DCW, a 50.86% increase over FH187. Collectively, these experiments established that the heterologous expression of a Uro III module (Module 6) combined with the knockdown of heme biosynthetic gene expression can boost HBA production in recombinant *E. coli*.

Next, seeking to reduce the number of plasmids/strain, Module 6 was integrated into the P_{BAD} locus of the *E. coli* genome, resulting in FH236. The plasmid pET28-HBA-antihemFG (Module 1 + dual heme gene knockdown) was transformed into FH236, generating strain FH322, which accumulated 2.00 mg/g DCW HBA. Adding CobB produced strain FH273, which accumulated 0.43 mg/g DCW HBAD. The introduction of plasmids carrying Module 2 and 4 were both transformed into FH322,

generating FH309, which produced 5.72 $\mu\text{g/g}$ DCW vitamin B₁₂ (compare with 2.18 $\mu\text{g/g}$ DCW for FH218 and 1.22 $\mu\text{g/g}$ DCW for FH219)

Genetic modifications of cobalt chelataes and Cbi module increased vitamin B₁₂ production

The gap between the production of HBAD of FH273 and vitamin B₁₂ of FH309 implied that metabolic bottlenecks might exist among cobalt chelataes and Cbi module. In addition, HBAD accumulated in FH309 (Supplementary Fig. 15), suggesting that the cobalt chelation step is indeed a bottleneck. Therefore, recombinant strains expressing cobalt chelataes from *B. melitensis*, *S. meliloti*, or *R. capsulatus* were evaluated for vitamin B₁₂ production (Fig. 5a, b). The strain with the cobalt chelatae from *R. capsulatus* (FH329) had highest vitamin B₁₂ production among these strains, at 21.96 $\mu\text{g/g}$ DCW. In addition, 6 recombinant *E. coli* strains variously expressing combinations of the CobN, CobS, and CobT enzymes from different bacterial species were also evaluated for vitamin B₁₂ production. These strains produced vitamin B₁₂ in amounts ranging from 2.19 $\mu\text{g/g}$ DCW to 11.22 $\mu\text{g/g}$ DCW (Fig. 5c).

A review article has speculated that the enzyme CobW may somehow function in the delivery and presentation of cobalt to CobN³⁰, so we investigated how the expression of CobW might improve vitamin B₁₂ production. Interestingly, vitamin B₁₂ production increased in 5 of the 9 recombinant strains in which we expressed various *cobW* homologs (Fig. 5). The strain FH351 expressing *cobW* from *B. melitensis* achieved the highest vitamin B₁₂ production, at 68.61 $\mu\text{g/g}$ DCW, a 14.9-fold increase over the original strain lacking *cobW*.

After optimizing the cobalt chelataes, we tested a multiple variants of the downstream Cbi module with the aim of further improving vitamin B₁₂ production. Most of the metabolic intermediates between CBAD and AdoCbl are instable and unavailable commercially, which limited our ability to conduct *in vitro* enzyme kinetics assays or to probe each step *in vivo* to identify specific bottlenecks. It is well-established that the expression of enzyme variants can help to increase metabolic flux toward target products³¹. Thus, Cbi module variants comprising enzymes from various bacteria were

examined (Fig. 5), and among the strains expressing CobW, strain FH364 (expressing *cobR* from *B. melitensis*, *StcobA*, *cbiP*, *pduX*, *StcobD* and *cbiB* from *S. typhimurium*) had the highest vitamin B₁₂ production at 171.81 µg/g DCW. It is noteworthy that three strains harboring the *bluE-RccobC-cbiB* expression cassette (FH362, FH363, and FH367) could synthesize vitamin B₁₂, demonstrating that BluE and CobC from *R. capsulatus* perform the same catalytic functions as do PduX and CobD from *S. typhimurium* in the *de novo* vitamin B₁₂ biosynthesis pathway. In addition, the three strains harboring the *pduX-StcobD-RccobD* expression cassette (FH370, FH371, and FH372) could also synthesize vitamin B₁₂, demonstrating that CobD from *R. capsulatus* performs the same catalytic function as does CbiB from *S. typhimurium* in the *de novo* vitamin B₁₂ biosynthesis pathway. These results further highlight that aerobic bacteria and anaerobic bacteria both use the same pathway to synthesize AdoCbi-P from AdoCby.

Optimization of fermentation conditions increased vitamin B₁₂ production

In order to provide an optimal growth medium for the engineered strains, the concentrations of key precursors such as glycine, succinic acid, and betaine were optimized in the medium (Supplementary Table 1 and Supplementary Fig. 16). Vitamin B₁₂ production of FH364 increased to 255.68 µg/g DCW when cultured in the optimized modified CM medium. We then altered growth temperatures and IPTG induction concentrations, as these are known to influence heterologous protein expression (Supplementary Table 2 and Supplementary Fig. 17). We found that growth temperature has a greater influence on vitamin B₁₂ production than IPTG concentrations: low temperature is conducive to the expression of soluble proteins and to correct folding, while high temperature is conducive to bacteria growth. Vitamin B₁₂ production increased from 24 °C to 32 °C, and then declined sharply at 37 °C. Cell growth rates increased slightly from 24 °C to 30 °C, but decreased from 30 °C to 37 °C. Induction at 0.1 mM IPTG resulted in the lowest vitamin B₁₂ production observed for each temperature, and we found that the optimal IPTG concentrations were different for each culture temperature. Collectively, we found that the optimal conditions for is maximal

vitamin B₁₂ production is a growth temperature of 32 °C and an IPTG concentration of 1 mM: under these optimized conditions the vitamin B₁₂ yield of the top-performing FH364 strain increased from 171.81 up to 307.00 µg/g DCW (equal to 0.67 mg/l).

DISCUSSION

The biosynthetic pathway of vitamin B₁₂ is very complex and although most of the biosynthetic pathway steps have been elucidated, the process through which AdoCbi-P is synthesized in the aerobic vitamin B₁₂ biosynthetic pathway remains unknown. Seeking to replace the traditionally used industrial strains for vitamin B₁₂ production, we here chose *E. coli* as the heterologous host to introduce an engineered *de novo* aerobic vitamin B₁₂ biosynthetic pathway. Most of the intermediate molecules of the vitamin B₁₂ biosynthetic pathway are not commercially available, which complicates the verification of the functions of candidate enzymes, so we used a “bottom-up” strategy that integrating genetic and metabolic engineering strategies to develop our engineered vitamin B₁₂-producing *E. coli* strain. Based on rational knowledge of the aerobic pathway, we divided the 32 genes known to be involved in the pathway into 6 separate modules, which facilitated our characterization of each aspect of the design. Ultimately, our efforts led to the first demonstration of complete vitamin B₁₂ production in *E. coli*.

It has been reported that the *cobN*, *cobS*, and *cobT* genes from *B. melitensis* and *P. denitrificans* can produce CBAD production *in vitro*^{3,19}. However, it is understood that the mechanism of cobalt chelation *in vivo* is more complicated than that *in vitro*^{3,19}. We here characterized the role of cobalt transporters in facilitating cobalt chelation. We used *in vitro* assays to successfully confirm the CBAD-production-related functions of CobN, CobS, and CobT enzymes from *B. melitensis*, *R. capsulatus*, and *S. meliloti*, but found that the expression of the genes encoding these enzymes in *E. coli* did not result in CBAD production in cells, ostensibly because these cells lacked the essential substrate cobalt. Consistent with this idea, the expression in these cells of the cobalt uptake transport proteins CbiM, CbiN, CbiQ, and CbiO led to CBAD production, thus

establishing that having a cobalt uptake transport system is essential for *de novo* vitamin B₁₂ biosynthesis using the aerobic pathway in *E. coli*. This may be a requirement for vitamin B₁₂ biosynthesis in all bacteria: consider for example that an ABC-type cobalt transport system (CbtJKL) is essential for growth of *S. meliloti* at trace metal concentrations³² and that the B₁₂ biosynthesis superoperon of *S. typhimurium* includes the *cbiMNQO* gene cassette¹⁷.

Importantly, our study overturned previous assumptions about the aerobic pathway that AP is condensed with AdoCby to form AdoCbi before undergoing a subsequent phosphorylation catalyzed by CobP. Our *in vitro* and *in vivo* assays of BluE and RcCobC demonstrated that these enzymes function as, respectively, a L-threonine kinase and a threonine-O-3-phosphate decarboxylase. Expression of CobD from *R. capsulatus* or CbiB from *S. typhimurium* can both facilitate APP's condensation with AdoCby to form AdoCbi-P. Excitingly, we thus establish that BluE from *R. capsulatus* performs an equivalent function with the unidentified protein α of *P. denitrificans*.

CobU from *S. typhimurium* is a bifunctional enzyme that exhibits both NTP:AdoCbi kinase and GTP: AdoCbi-P guanylyltransferase activities¹³. The kinase activity of CobU is necessary for cobinamide (Cbi) salvage but not the *de novo* synthesis of AdoCbl¹⁰. So a general conclusion is that it is not AdoCbi that undergoes phosphorylation catalyzed by CobU from *S. typhimurium* or by CobP from the aerobic-pathway-utilizing *R. capsulatus*; rather, APP is condensed to AdoCby to yield AdoCbi-P. These results strongly suggest that aerobic bacteria and anaerobic bacteria may use the same *de novo* pathway steps from CBAD to AdoCbl.

Our engineered *de novo* synthetic pathway for vitamin B₁₂ was successfully constructed in *E. coli* by assembling six modules comprising many biosynthetic enzymes and four cobalt uptake transport proteins. Although *E. coli* is a kind of facultative anaerobic bacterium, our study demonstrated that it can be modified to synthesize vitamin B₁₂ using the aerobic pathway. The original recombinant strains produced around 1-2 $\mu\text{g/g}$ DCW of vitamin B₁₂. Subsequently, by strengthening the biosynthesis of precursors and making genetic modifications to cobalt chelatases and Cbi module, we were able to increase the vitamin B₁₂ production to 171.81 $\mu\text{g/g}$ DCW.

It was interesting to note that the expression of *cobW* (strain FH351) increased vitamin B₁₂ productivity by 49.68-fold, highlighting that *cobW* plays an important role in cobalt chelation. Vitamin B₁₂ production in FH364 was finally increased to 307.00 µg/g DCW after optimization culture media recipe, growth temperature, and IPTG concentrations; this final yield was 250.64-fold higher than the original strain bearing our engineered *de novo* vitamin B₁₂ biosynthetic pathway.

Our engineered vitamin B₁₂ producing *E. coli* strain overcomes some major limitations that currently face industrial producers of vitamin B₁₂ like long growth cycles. For example, the present industrial vitamin B₁₂ producing *P. denitrificans* strain needs 180 h to complete fermentation, whereas the recombinant *E. coli* strain constructed here only needs 24 h. Our successful demonstration of the engineering of the complete pathway for vitamin B₁₂ production *E. coli* offers a powerful illustration for how the dozens of genes of a complex heterologous pathway can be successfully introduced into new host organisms. This demonstration of our modular design concept should offer encouragement to other scientists and biotechnologists that are considering similar undertakings.

METHODS

Chemicals. Q5 High-Fidelity DNA polymerase and calf intestinal alkaline phosphatase were purchased from New England Biolabs (USA). Taq PCR Master Mix and DNA ladder were from Tiangen (China). Restriction endonucleases, T4 DNA ligase, and T4 Polynucleotide Kinase (PNK) were purchased from Thermo Fisher Scientific (USA). Chromatography-grade methanol and formic acid were from Thermo Fisher Scientific (USA). ALA, (R)-1-Amino-2-propanol, *L*-threonine O-3-phosphate, and vitamin B₁₂ were from Sigma-Aldrich (USA). Ni Sepharose 6 Fast Flow was from GE Healthcare (USA). DEAE-Sephadex A25 was from Pharmacia (USA).

Medium and growth conditions. For routine purposes, *E. coli* derived strains were grown in liquid LB (Difco) or on LB agar plates with appropriate antibiotics at 37 °C.

For HBA and HBAD production, 5 ml overnight cultures of recombinant *E. coli* strains were inoculated in 500 ml 2×YT medium at 37 °C with shaking (200 rpm). When the OD₆₀₀ of the cultures reached 0.8–1.0, isopropyl- β-D-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM. The cultures were then incubated at 28 °C for 24 h. To produce CBAD and other corrinoids, seed cultures of the recombinant strains were grown in LB medium at 37°C overnight at 220 rpm. The seed cultures were used to inoculate 3 liter shaker flask containing 600 ml TYG medium, a modified medium³³ containing: 5 g/L yeast extract, 10 g/L tryptone, 5 g/L KH₂PO₄, 2 g/L glycine, 10 g/L succinic acid, 20 mg/L CoCl₂•6H₂O, supplemented with 10 g/L glucose and appropriate antibiotics at an inoculation volume of 5% at 37 °C in a rotary shaker (220 rpm) until OD₆₀₀ reached 0.8–1.0; 0.4 mM IPTG was then added to the cultures and the cultures were incubated at 28 °C for 24 h.

For the biosynthesis of (R)-1-Amino-2-propanol O-2-Phosphate, the overnight seed cultures were used to inoculate 50 ml of M9 mineral salt plus 0.5% yeast extract (M9Y medium). at an inoculation volume of 1% in 250 mL flasks at 37 °C until OD₆₀₀ reached 0.6–0.8. At this point, 0.4 mM IPTG and 5 g/L L-threonine were added to the cultures, which were allowed to continue growth at 28 °C for 20 h. For *de novo* biosynthesis of vitamin B₁₂, strains were initially inoculated into LB medium at 37°C overnight at 220 rpm. The seed cultures were used to inoculate 250 ml flasks containing 25 mL of CM medium containing 5 g/L yeast extract, 10 g/L tryptone, 5 g/L KH₂PO₄, 10 g/L glucose, 2 g/L glycine, 10 g/L succinic acid, 10 g/L betaine, 20 mg/L CoCl₂•6H₂O, and 90 mg/L DMBI, pH 6.5 with an starting OD₆₀₀ of 0.15. When the strains were grown at 30 °C and 220 r.p.m. to an OD₆₀₀ of 0.8–1.0, 50 mg/L L-threonine and moderate IPTG was added to the cultures and the cultures were then incubated at 28 °C for 20 h.

Appropriate antibiotics were added to medium at the following concentrations: Kanamycin, 50 µg/mL; Ampicillin, 100 µg/mL; streptomycin, 50 µg/mL; Chloramphenicol, 34 µg/mL.

Strain and plasmid construction. *E. coli* DH5α was used as the host for cloning. *E.*

coli BL21 (DE3) was used as the host for protein expression and purification. *E. coli* MG1655 (DE3) was used as the host for *de novo* production of vitamin B₁₂ and its intermediates. Plasmids pET28a, pCDFDuet-1, and pACYCDuet-1 were used for plasmid construction and protein expression. The details of the strains and plasmids used are described in Supplementary Data 1. The primers used are listed in Supplementary Data 2.

Construction of Module 1: All genes derived from *R. capsulatus*, *B. melitensis*, and *S. meliloti* were obtained by polymerase chain reaction (PCR) using genomic DNA templates. The HBA pathway was cloned from pET3a-cobAIGJMFKLH to pET28a by digestion with *Xba*I and *Bam*HI, gel purification, and ligation, yielding pET28-HBA. A truncated *cobH* fragment with a C-terminal hexa-his tag and pET28-HBA were both digested by *Bmg*BI and *Bam*HI, purified and ligated with T4 DNA ligase, yielding pET28-HBA-his.

Construction of Module 2: The *cobB* gene was cloned to pCDFDuet-1 at *Bam*HI and *Hind*III site, yielding pCDF-cobB-his. The other *cobB* gene was cloned to pCDFDuet-1 at *Nco*I site via golden gate³⁴ to create pCDF-cobB. The *cobN* genes from *B. melitensis*, *S. meliloti*, and *R. capsulatus* were cloned to pCDF-cobB via Gibson assembly³⁵, yielding pCDF-cobB-BmcobN, pCDF-cobB-SmcobN, pCDF-cobB-RccobN. The plasmids pCDF-cobB-BmcobN-his, pCDF-cobB-SmcobN-his, and pCDF-cobB-RccobN-his were constructed analogously, but with *cobN* with an N-terminal hexa-his tag instead. Amplified *cobS* and *cobT* fragments from *B. melitensis*, *R. capsulatus*, and *S. meliloti* were successively cloned to pACYCDuet-1 via Gibson assembly, yielding, respectively, pACYC-BmcobS-BmcobT, pACYC-SmcobS-SmcobT, and pACYC-RccobS-RccobT. To add a C-terminal hexa-his tag to *cobT* gene, inverse primers were designed to amplify the vectors. The resulting PCR products were phosphorylated by PNK and re-circularized, yielding pACYC-BmcobS-BmcobT-his, pACYC-SmcobS-SmcobT-his and pACYC-RccobS-RccobT-his. pACYC-BmcobS-his-BmcobT-his, pACYC-SmcobS-his-SmcobT-his, and pACYC-RccobS-his-RccobT-his were constructed analogously to add a C-terminal hexa-his tag to the *cobS* gene. The BmcobST pseudo-operon with promoters was amplified from pACYC-BmcobS-

BmcobT and cloned into the *XhoI* site of pCDF-cobB-BmcobN and pCDF-cobB-BmcobN-his via Gibson assembly, yielding , respectively, pCDF-cobB-BmcobN-BmcobS-BmcobT and pCDF-cobB-BmcobN-his-BmcobS-BmcobT Plasmids including pCDF-cobB-SmcobN-SmcobS-SmcobT, pCDF-cobB-SmcobN-his-SmcobS-SmcobT, pCDF-cobB-RccobN-RccobS-RccobT, pCDF-cobB-RccobN-his-RccobS-RccobT, pCDF-cobB-BmcobN-his-SmcobS-SmcobT, pCDF-cobB-BmcobN-his-RccobS-RccobT, pCDF-cobB-SmcobN-his-BmcobS-BmcobT, pCDF-cobB-SmcobN-his-RccobS-RccobT, pCDF-cobB-RccobN-his-BmcobS-BmcobT, and pCDF-cobB-RccobN-his-SmcobS-SmcobT were constructed analogously.

Construction of Module 3: A DNA fragment containing tac, lacUV5 promoters and a bidirectional terminator (Supplementary Fig. 18) was synthesized by GENEWIZ (China). This fragment was ligated via Gibson assembly into an amplified pACYCDuet-1 backbone lacking T7 promoters, multiple cloning sites, or terminators, yielding p15ASI. The *cbiMNQO* operon was amplified from *R. capsulatus* genome and cloned to p15ASI via Gibson assembly, yielding p15ASI-*cbiMNQO*. The codon-optimized gene *cobR* from *B. melitensis* was cloned into vector pACYCDuet-1 via Gibson assembly, yielding pACYC-BmcobR. The genes encoding cob(I)yrinic acid a,c-diamide adenosyltransferase and adenosylcobyrinic acid synthase from *E. coli*, *S. typhimurium*, and *B. melitensis* were cloned into pACYC-BmcobR via Gibson assembly, yielding, respectively, pACYC-BmcobR-BtuRcbiP, pACYC-BmcobR-StcobAcbiP, and pACYC-BmcobROQ.

Construction of Module 4: The gene *pduX* from *S. typhimurium* and codon-optimized *bluE* from *R. capsulatus* were amplified and cloned into *NcoI* and *XhoI* sites, yielding pACYC-*pduX* and pACYC-*bluE*. The plasmids pACYC-his-*pduX* and pACYC-MBP-*bluE* were constructed via site directed mutagenesis³⁶. The genes *cobD* from *S. typhimurium* and *cobC* from *R. capsulatus* were amplified and cloned into the *NdeI* sites of pACYC-his-*pduX* and pACYC-MBP-*bluE*, respectively, yielding pACYC-his-*pduX*-StcobD and pACYC-MBP-*bluE*-RccobC. The gene *cbiB* from *S. typhimurium* was cloned into vector pACYCduet-1 via Gibson assembly, yielding pACYC-*cbiB*. The *cobD* gene from *R. capsulatus* was cloned into vector pET28a,

yielding pET28a-RccobD. The *cobD* expression cassette was cloned into pACYC-MBP-bluE-RccobC and pACYC-his-pduX-StcobD, respectively, yielding pACYC-MBP-bluE-RccobCD and pACYC-his-pduX-StcobD-RccobD. The *cbiB* expression cassette was cloned into pACYC-his-pduX-StcobD and pACYC-MBP-bluE-RccobC, respectively, yielding pACYC-his-pduX-StcobD-cbiB and pACYC-ECB. The *pduX-StcobD-cbiB* expression cassette was cloned into pACYC-BmcobR-BtuRcbiP, pACYC-BmcobR-StcobAcbiP, and pACYC-BmcobROQ, respectively, yielding pACYC-RRPXDB, pACYC-RAPXDB, and pACYC-BMXDB. The *bluE-RccobCD* expression cassette was cloned into pACYC-BmcobR-BtuRcbiP, pACYC-BmcobR-StcobAcbiP, and pACYC-BmcobROQ, yielding, respectively, pACYC-RRPECD, pACYC-RAPECD, and pACYC-BMECD. The *bluE-RccobC-cbiB* expression cassette was cloned into pACYC-BmcobR-BtuRcbiP and pACYC-BmcobR-StcobAcbiP, yielding, respectively, pACYC-RRPECB and pACYC-RAPECB. The *pduX-StcobD-RccobD* expression cassette was cloned into pACYC-BmcobR-BtuRcbiP, pACYC-BmcobR-StcobAcbiP, and pACYC-BmcobROQ, yielding, respectively, pACYC-RRPXDD, pACYC-RAPXDD, and pACYC-BMXDD.

Module 5 comprised four native genes from *E. coli*: *cobU*, *cobT*, *cobS*, and *cobC*. Construction of Module 6: A fragment containing the Pr promoter, MicC Scaffold, B0015 double terminator, and BioBrick cloning sites was synthesized and cloned into pUC57 by GENEWIZ, yielding pKDG. This plasmid was used as the basis for gene inhibition via small RNA²⁹. The plasmid pKDG was amplified using inverse primers containing respective around 24 bp N terminal sequences for the *hemE*, *hemF*, *hemG*, and *hemH* genes; amplified gene fragments were then phosphorylated by PNK and re-circularized, yielding plasmids pKDG-anti-hemE, pKDG-anti-hemF, pKDG-anti-hemG, and pKDG-anti-hemH. A fragment containing the *hemF* regulation module (around 300 bp from Pr promoter to B0015 double terminator) amplified from the pKDG-anti-hemF plasmid was digested by *XbaI* and *PstI*, and then purified and ligated with *SpeI*- and *PstI*-digested pKDG-anti-hemE, yielding pKDG-anti-hemEF. The other plasmids including pKDG-anti-hemEG, pKDG-anti-hemEH, pKDG-anti-hemFG, pKDG-anti-hemFH, and pKDG-anti-hemGH were constructed analogously. The *hemO*

gene from *Rhodopseudomonas palustris* and the *hemB*, *hemC*, and *hemD* genes from *S. meliloti* were cloned together into p15ASI via Gibson assembly, yielding p15ASI-hemOBCD. The respective regulation modules of small RNA were amplified from pKDG-anti-hemE, pKDG-anti-hemF, pKDG-anti-hemG, pKDG-anti-hemH, pKDG-anti-hemEF, pKDG-anti-hemEG, pKDG-anti-hemEH, pKDG-anti-hemFG, pKDG-anti-hemFH, and pKDG-anti-hemGH were cloned into the *Bam*HI site of pET28-HBA, yielding pET28-HBA-anti-hemE, pET28-HBA-anti-hemF, pET28-HBA-anti-hemG, pET28-HBA-anti-hemH, pET28-HBA-anti-hemEF, pET28-HBA-anti-hemEG, pET28-HBA-anti-hemEH, pET28-HBA-anti-hemFG, pET28-HBA-anti-hemFH, and pET28-HBA-anti-hemGH. The *hemOBCD* operon with *tac* promoter was amplified and cloned into the *Sac*I site of pET28-HBA-anti-hemFG, yielding pET28-HBA-anti-hemFG-hemOBCD.

Construction of *E. coli* recombinant strains: The T7 RNA polymerase expression cassette was amplified from the *E. coli* BL21 (DE3) genome and integrated into the *lacZ* locus of *E. coli* MG1655 via λ /red recombination³⁷, resulting in MG1655 (DE3). Briefly, a *kanamycin-sacB* cassette with upstream and downstream homologous arms was transformed into *E. coli* MG1655 harboring pKD46. The recombinant strains were initially screened on LB agar plates containing 30 mg/L kanamycin. The T7 RNA polymerase expression cassette with upstream and downstream homologous arms was transformed into the positive strain by electrotransformation. The second round of selection was carried out using sucrose resistance. Strains selected on LB agar plates containing 10% sucrose were confirmed by PCR, and then loss of pKD46 was induced by culturing at 37 °C. The *ackA* and *pta* were also deleted analogously. Module 3 and module 6 were integrated into the *ldhA* locus and the arabinose promoter locus, respectively using CRISPR/Cas9³⁸. The other recombinant strains were constructed by transformation of corresponding plasmids (listed in Supplementary Data 1).

Corrinoids biosynthesis and purification. For routine separation and purification, corrinoids were extracted from recombinant *E. coli* strains harboring corresponding plasmids via ion exchange chromatography. Briefly, cells were harvested by

centrifugation at $5,000 \times g$ for 20 min and re-suspended in IEX-buffer A containing 20 mM Tris-HCl (pH 7.4) and 100 mM NaCl. The cell suspension was disrupted twice using a JN-3000 Plus homogenizer at 1,200 v and centrifuged at $11,000 \times g$ for 1 h. The supernatant was passed through a DEAE-Sephadex A25 column. The column was washed with five column volumes of IEX-buffer B containing 20 mM Tris-HCl (pH 7.4) and 300 mM NaCl. Corrinoids were eluted with IEX-buffer C containing 20 mM Tris-HCl (pH 7.4) and 2 M NaCl. Samples were then concentrated and filtered using $0.22 \mu\text{m}$ filters prior to HPLC analysis.

HBA and HBAD were extracted via a modified enzyme-trap method⁵. Briefly, cells were resuspended in enzyme-trap buffer A (100 mM NaCl, 5 mM imidazole, 20 mM HEPES, pH 7.5) and disrupted twice using a JN-3000 Plus homogenizer at 1,200 v and centrifuged at $11,000 \times g$ for 1 h. The supernatant was filtered using a $0.22 \mu\text{m}$ filter. The filtrate was then loaded onto an equilibrated Ni Sepharose column (GE Healthcare). After washing with 10 column volumes of enzyme-trap buffer B (100 mM NaCl, 60 mM imidazole, 20 mM HEPES, pH 7.5), the proteins were eluted using enzyme-trap buffer C (100 mM NaCl, 400 mM imidazole, 20 mM HEPES, pH 7.5).

Gene expression and protein production and purification. Purified proteins were used for enzyme assays. For the production of recombinant proteins, *E. coli* BL21 (DE3) cells carrying recombinant plasmids were pre-inoculated into 5 mL LB liquid media and overnight cultures were inoculated into 500 mL of fresh LB media containing appropriate antibiotics. The cultures were left to grow at 37°C until the OD_{600} reached 0.6 and were then induced with 0.5 mM IPTG at 30°C for 12 h. Protein purification was performed as previously described, with some modifications³⁹. Briefly, cells were harvested by centrifugation at $5,000 \times g$ for 20 min and re-suspended in buffer A (20 mM sodium dihydrogen phosphate, 500 mM sodium chloride, 30 mM imidazole, pH 7.4). The cell suspension was disrupted using a JN-3000 Plus homogenizer at 1,200 v and centrifuged at $11,000 \times g$ for 1 h. The supernatant was filtered using a $0.22 \mu\text{m}$ filter. The filtrate was then loaded onto an equilibrated Ni Sepharose column (GE Healthcare). After washing three times with 10 column volumes of buffer B (20 mM sodium

dihydrogen phosphate, 500 mM sodium chloride, 100 mM imidazole, pH 7.4), the proteins were eluted using buffer C (20 mM sodium dihydrogen phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 7.4). The protein storage buffer was then exchanged for buffer I (50 mM Tris-HCl, pH 7.5, 50 mM sodium chloride, 50% (v/v) glycerol) through ultrafiltration using Millipore's Amicon® Ultra-15 centrifugal filters and stored at -20°C. The protein content of the samples was analyzed by SDS-PAGE. Proteins were quantified using a Bradford Protein Assay Kit (Beijing Solarbio Science & Technology Co., Ltd) using bovine serum albumin as a standard.

***In vitro* enzyme assay of CobB.** CobB was assayed as reported previously⁷. Briefly, 5 μM HBA, 0.1 M Tris hydrochloride (pH 7.6), 1 mM ATP, 2.5 mM magnesium chloride, and 1 mM glutamine in a total volume of 250 μL were combined and incubated at 30°C for 60 min. HBAD production was monitored with liquid chromatography/mass spectrometry (LC-MS).

***In vitro* enzyme assay of the CobNST proteins.** The CobNST proteins were assayed based on a previously described assay³. The reaction mixture consisted of 0.1 M Tris hydrochloride (pH 8.0), 100 μM cobalt chloride, 7.5 mM ATP, 6.5 mM magnesium chloride, and purified CobST in a 12.4×12.4×45 mm quartz micro-cuvette. The reaction was initiated by addition of cobN-HBAD purified using the enzyme-trap method (above), and absorbance was measured once each min for 20 min at 305-600 nm at 30°C. The control lacked cobalt chloride in the reaction mixture. Cobyric acid a,c-diamide production was monitored using LC-MS.

***In vitro* enzyme assay of BluE.** Standard assay mixtures for BluE were modified from a previous report¹²: 15 mM HEPES, pH 7.4, 20 mM NaCl, 10 mM MgCl₂, 200 μM ATP, and 500 μM L-threonine were combined in a total volume of 200 μL. The reaction was initiated by the addition of 10 μg crude BluE. The reaction was stopped by incubation at 100 °C for 10 min, and L-threonine phosphate was monitored using HPLC.

***In vitro* enzyme assay of L-threonine-O-3-phosphate decarboxylase.** The L-threonine-O-3-phosphate decarboxylase assay was conducted as described previously with modification¹¹. Specifically, reactions had a 100 μ l final volume and contained 50 mM PIPES, pH 6.8, 500 μ M L-threonine O-3-phosphate, 500 μ M PLP; the reaction was initiated by the addition of 100 μ g purified CobC. After incubation at 37 °C for 90 min, 178 μ L of the reaction mixture was diluted to 200 μ L with 20 μ L NEB CutSmart buffer and 2 μ L calf intestinal alkaline phosphatase (CIP). The mixture was incubated at 37 °C for 30 min, and the reaction was subsequently stopped by incubation at 100 °C for 10 min. (R)-1-Amino-2-propanol was detected by HPLC.

Analytical methods. HBA and HBAD were measured using an Agilent 1260 High Performance Liquid Chromatography (HPLC) system; equipped with a diode array detector and a SB-Aq column (4.6 \times 150 mm, 5 μ m, Agilent); samples were analyzed at 30 °C and signals were monitored at 329 nm. Samples containing HBA were filtered using a 0.22 μ m filter, and a 20 μ L portion was directed analyzed via HPLC. The mobile phase was water (solvent A) and methanol (solvent B) (both contain 0.1% formic acid) at a flow rate of 1 mL/min. Elution gradients were: 0-25% B (0-2 min), 25-34% B (2-4 min), 34-70% B (4-12 min), 70-100% B (12-17 min), 100% B (17-23 min), 0% B (23-25 min), and 0% B (25-27 min). The molar extinction coefficient of HBAD is available. To determine the HBA concentration of extracted HBA, it was completely converted to HBAD by CobB enzyme from *R. capsulatus in vitro*. Then HBA attained by enzyme-trap method was used as a reference to quantify HBA production of recombinant strains. HBA in samples were identified and quantified by comparison to standard HBA attained from FH-HBA, a strain harboring pET28-HBA-his (Supplementary Data 1) via an enzyme-trap method, with some modifications⁵. Corrinoids were extracted for LC-MS analysis from 600 mL of cell culture via ion exchange chromatography, and 35 μ L of concentrated sample was injected for analysis. The reaction mixtures from *in vitro* assays were filtered using 0.22 μ m filters and then directly injected for analysis. LC-MS analysis was carried out using an Agilent 1260 HPLC equipped with a TC C18 column (4.6 \times 250 mm, 5 μ m, Agilent) and a Bruker microQ-TOF II mass spectrometer

equipped with an ESI ionization source. The mobile phase was water (solvent A) and methanol (solvent B) (both contain 0.1% formic acid) at a flow rate of 0.7 mL/min. HPLC program was as follows: 25% B (0-5 min), 25- 34% B (5-15 min), 34-100% B (15-19 min), 100% B (19-24 min), 100-25% B (24-25 min), 25% B (25-35 min).

L-threonine, L-threonine phosphate and (R)-1-Amino-2-propanol in the supernatant of the fermentation culture were detected by HPLC (Agilent 1260) with a Zorbax Eclipse-AAA column (4.6×150 mm, 5 µm particle size, Agilent) at 40 °C monitored at 338 nm after automated online derivatization using o-phthalaldehyde (OPA). The elution was performed using a gradient of solvent A (40 mM NaH₂PO₄•2H₂O, pH 7.8) and solvent B (methanol/acetonitrile/water = 45:45:10, by vol.) at 2 ml/min. Elution gradients were: 0 to 1.9 min, 0% B; 18.1 min, 57% B; 18.6 min, 100% B; 22.3 min, 100% B; 23.2 min, 0% B; 26 min, 0% B.

Vitamin B₁₂ was prepared as follows: The bacterial pellet from 20 ml fermentation broth was collected and resuspended with ddH₂O to a final volume of 1.3 ml; and then 130 µL of NaNO₂ 8% (w/v), 130 µL of glacial acetic acid and 10 µL of NaCN 1% (w/v) were added; thereafter, the mixture was boiled for 30 min and centrifuged at 13,000 × g for 10 min. The supernatant was resolved on a reverse phase C-18 column (4.6×250 mm, 5 µm, Agilent) by HPLC (Agilent 1260) operating at 30 °C monitored at 361 nm. The mobile phase consisted of 30% methanol at a flow rate of 0.8 ml min⁻¹ for 15 min.

Data availability. All data generated in the present study and included in this article and its supplementary information files are available from the authors upon reasonable request.

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Author contributions

H.F. and D.Z. conceived and designed the experiments; H.F., D.L., J.K., and P.J. performed the experiments; H.F. and D.Z. analysed the experimental data; H.F., J.S., and D.Z. prepared the manuscript.

Competing interests

This work described here has been included in a patent application by the Tianjin Institute of Industrial Biotechnology of the Chinese Academy of Sciences.

Additional information

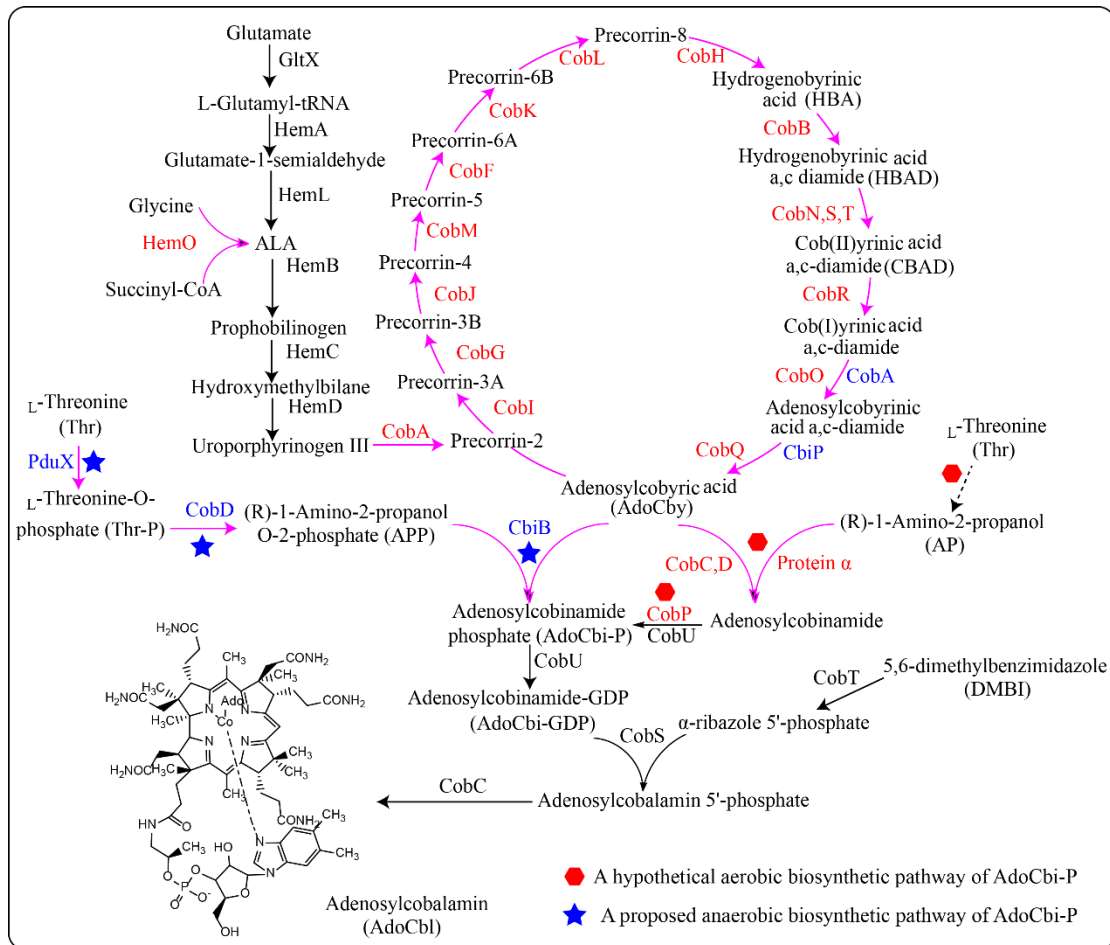


Fig. 1 Biosynthetic pathway of adenosylcobalamin. Endogenous enzymes from *E. coli* are shown in black. Enzymes from aerobic bacteria such as *B. melitensis*, *R. capsulatus*, *S. meliloti* and *R. palustris* are shown in red. Enzymes from *S. typhimurium* are shown in blue.

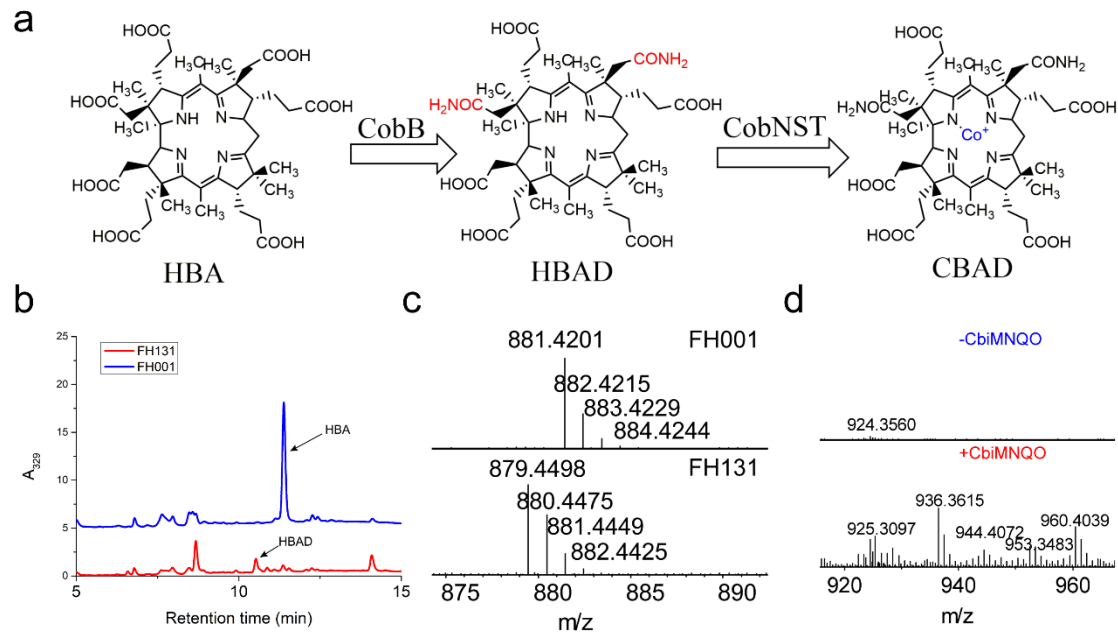


Fig. 2 The CBAD biosynthetic pathway and LC-MS detection of biosynthetic intermediates. **a** HBA is converted to CBAD by CobB and the CobN, S, T enzymes. **b** The strain FH131 expressing *cobB* converted HBA to HBAD, as assessed by HPLC. **c** Comparison of mass spectra for HBAD from the FH001 and FH131 stains. **d** Comparison of mass spectra for CBAD from recombinant *E. coli* stains with or without the expression of the *chiM, N, Q, O* proteins.

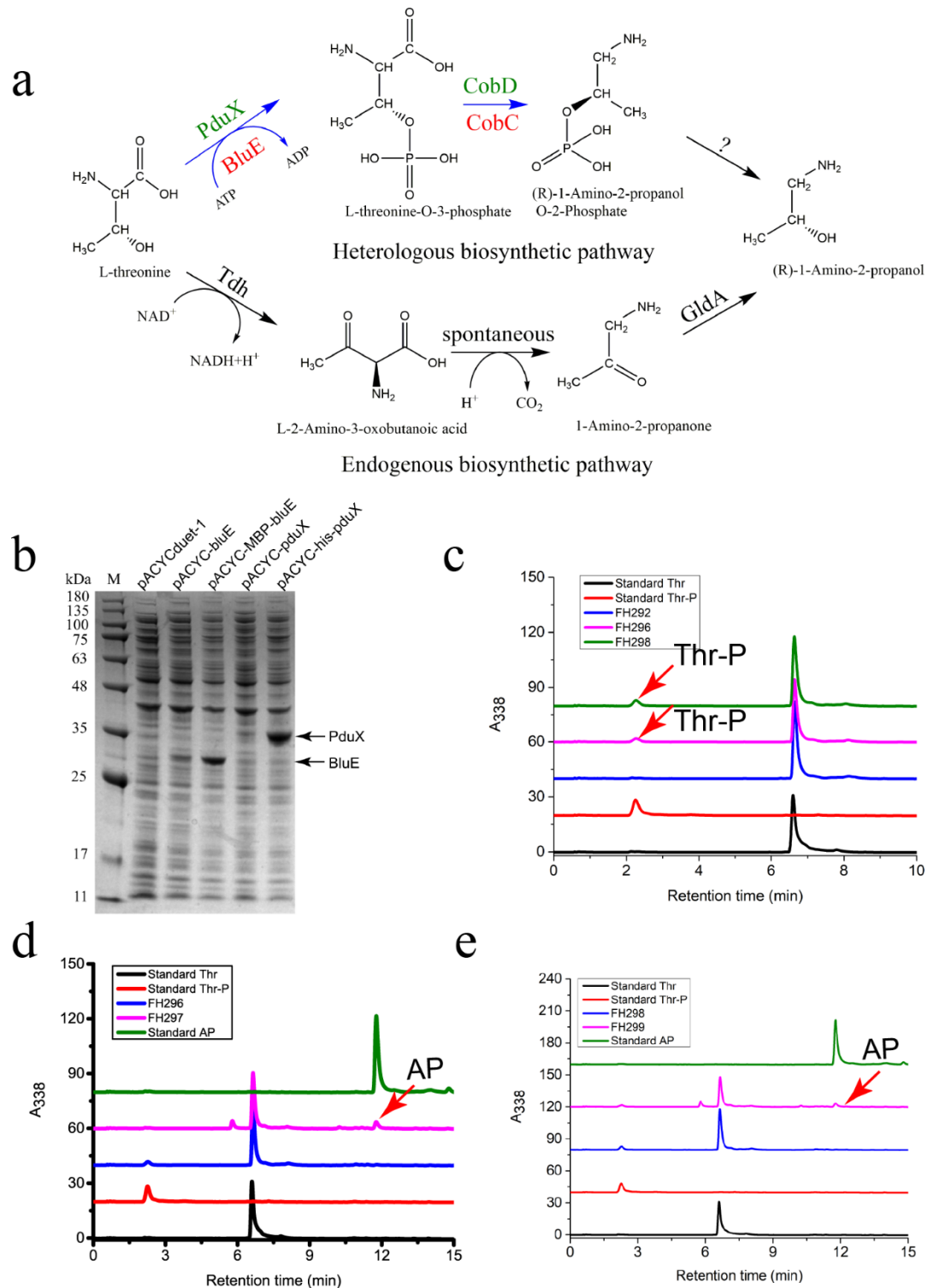


Fig. 3 Metabolic engineering of the heterologous APP biosynthetic pathway in *E. coli*. **a** Endogenous and heterologous APP biosynthetic pathway in *E. coli*. **b** Optimization of the expression of the *blue* and *pduX* genes via a bicistronic design that included an MBP tag upstream of the *blueE* gene and a his tag upstream of the *pduX* gene. **c** Confirmation of the enzymatic functions of PduX and BluE in *E. coli*. **d** Confirmation

of function of CobD from *S. typhimurium* in *E. coli*. **e** Confirmation of the function of CobC from *R. capsulatus* in *E. coli*. Note that Calf intestinal alkaline phosphatase can transform APP to AP. Owing to the lack of a commercially available APP standard, we indirectly monitored the APP level by detecting conversion to AP using the endogenous phosphatase of *E. coli*.

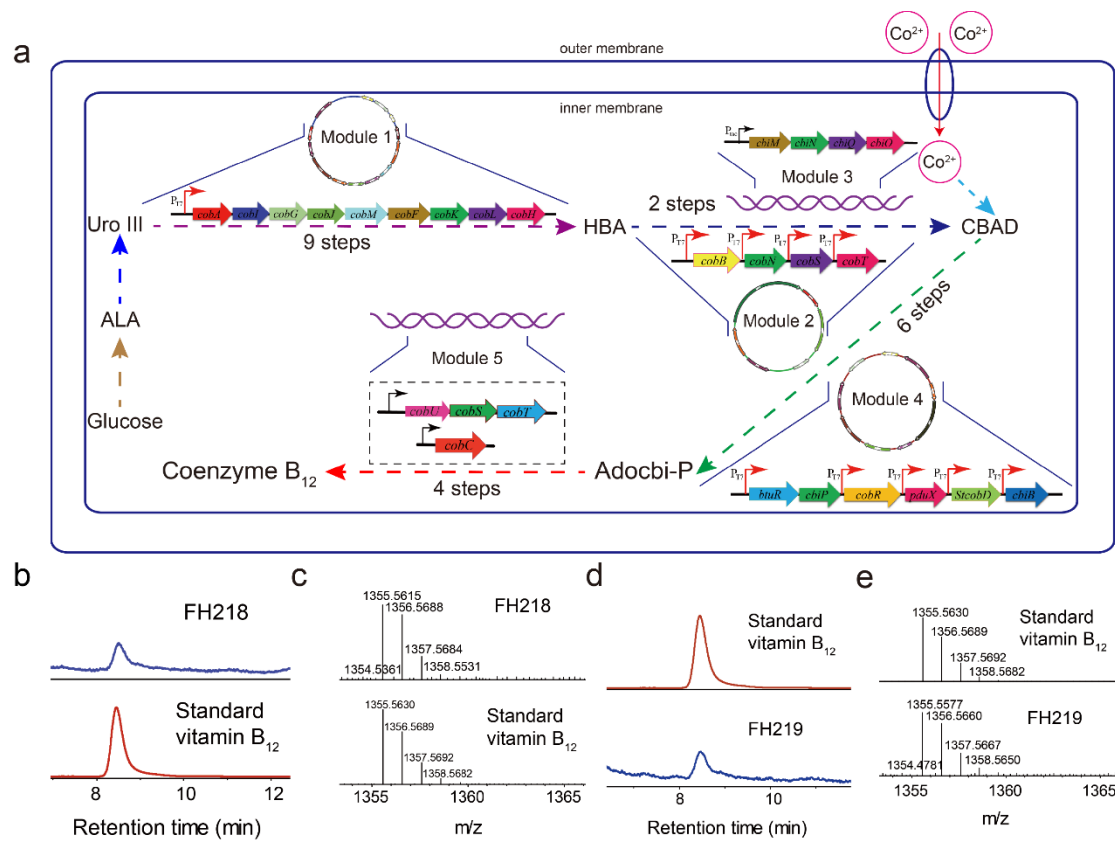


Fig. 4 Metabolic engineering of vitamin B₁₂ production in *E. coli*. **a** Metabolic engineering in *E. coli* of a *de novo* vitamin B₁₂ biosynthetic pathway. Five modules are used to synthesize vitamin B₁₂ from glucose in *E. coli*. **b** HPLC analysis and **c** mass spectrometry analysis of vitamin B₁₂ produced by the FH218 strain. **d** HPLC analysis and **e** mass spectrometry analysis of vitamin B₁₂ produced by FH219. Adenosylcobalamin was converted to vitamin B₁₂ for detection.

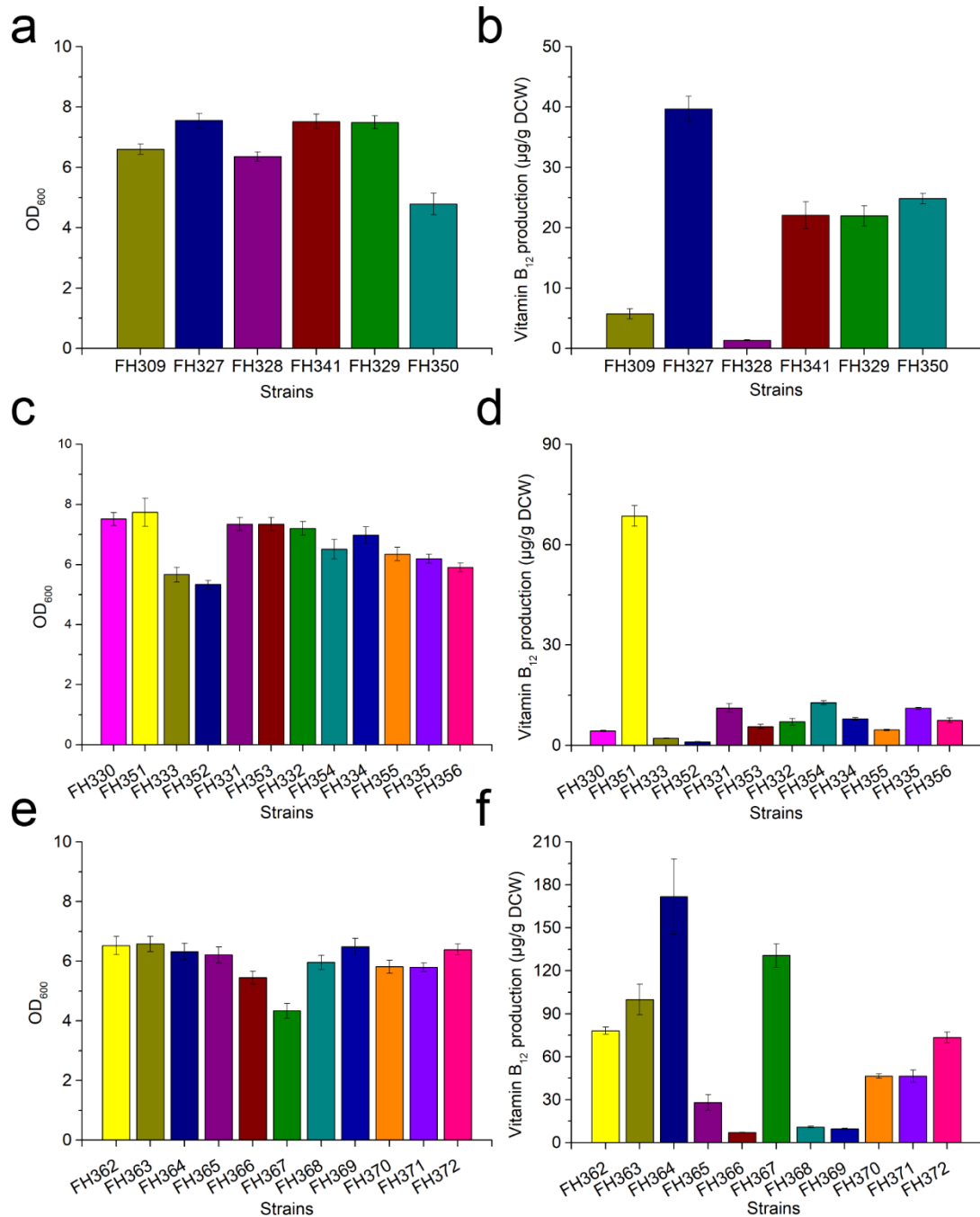


Fig. 5 Comparison of growth and vitamin B₁₂ production in *E. coli* strains expressing Module 4 and 5. **a** Growth and **b** vitamin B₁₂ production of *E. coli* expressing the *cobN,S,T* genes from a single species (*B. melitensis*, *S. meliloti* or *R. capsulatus*) as well as the *cobW* gene from *B. melitensis*, *S. meliloti*, or *R. capsulatus*. **c** Growth and **d** vitamin B₁₂ production of *E. coli* strains expressing various combinations of the *cobN*, *cobS*, *cobT*, and *cobW* genes from *B. melitensis*, *S. meliloti*, or *R. capsulatus*. **e** Growth and **f** vitamin B₁₂ production of *E. coli* expressing different variant forms of Module 4.

Error bars indicate standard deviations from triplicate biological replicates. All strains were cultured in CM medium for vitamin B₁₂ production.