Laboratory evolution of *E. coli* with a natural vitamin B_{12} analog reveals roles for cobamide uptake and adenosylation in methionine synthase-dependent growth

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Running title: Lab evolution with pseudocobalamin

Abstract

The majority of bacteria use cobamides as cofactors for methionine synthesis or other diverse metabolic processes. Cobamides are a structurally diverse family of cofactors related to vitamin B_{12} (cobalamin), and most bacteria studied to date grow most robustly with particular cobamides. Because different environments contain varying abundances of distinct cobamides, bacteria are likely to encounter cobamides that do not function efficiently for their metabolism. Here, we performed a laboratory evolution of a cobamide-dependent strain of *Escherichia coli* with pseudocobalamin (pCbl), a cobamide that *E. coli* uses less effectively than cobalamin for MetH-dependent methionine synthesis, to identify genetic adaptations that lead to improved growth with less-preferred cobamides. After propagating and sequencing nine independent lines and validating the results by constructing targeted mutations, we found that increasing expression of the outer membrane cobamide transporter BtuB is beneficial during growth under cobamide-limiting conditions. Unexpectedly, we also found that overexpression of the cobamide adenosyltransferase BtuR confers a specific growth advantage in pCbl. Characterization of this phenotype revealed that BtuR and adenosylated cobamides contribute to optimal MetH-dependent growth. Together, these findings improve our understanding of how bacteria expand their cobamide-dependent metabolic potential.

Importance

In nature, bacteria commonly experience fluctuations in the availability of required nutrients. Thus, their environment often contains nutrients that are insufficient in quantity or that function poorly in their metabolism. Cobamides, the vitamin B_{12} family of cofactors, are ideal for investigating the influence of nutrient quantity and structure on bacterial growth because they must be acquired exogenously by most bacteria and are structurally diverse, with most bacteria having preferences for certain types. We performed a laboratory evolution experiment in *E. coli* with a less-preferred cobamide to examine whether and how bacteria can improve their growth with less ideal nutrients. We found that overexpression of genes for cobamide uptake and modification are genetic adaptations that enable better growth under these conditions. Given that cobamides are key shared metabolites in microbial communities, our results reveal insights into bacterial interactions and competition for nutrients.

Introduction

Cobamides, the vitamin B_{12} family of metabolites, are used by most bacteria as cofactors for diverse metabolic processes including carbon metabolism, synthesis of methionine and deoxyribonucleotides, and natural product biosynthesis (2). They are produced exclusively by prokaryotes, though most bacteria that use cobamides must acquire them exogenously (3, 4). Cobamides are modified tetrapyrroles (corrinoids) with a central cobalt ion that can coordinate to upper and lower axial ligands (4). B_{12} (cobalamin, Cbl) (Fig. 1A) is the most well-studied cobamide due to its importance in human health (5), but nearly 20 other cobamides with structural variability in the lower (α) axial ligand have been described (6-8). Different assortments of cobamides have been found in microbial communities from host-associated and environmental sources, and variability in cobamide abundances has been observed even in samples derived from similar sources (7, 9, 10). Importantly, cobamide structure can profoundly influence microbial growth, largely because cobamide-dependent enzyme function is differentially impacted by

cobamide structure (8, 11-18). Therefore, because microbes are exposed to different cobamides as their environments shift, they encounter cobamides that function at varying levels of effectiveness for their metabolism. Given that cobamide structure and availability impact bacterial fitness, it is important to understand how bacteria are genetically wired to deal with different cobamides.

Many organisms have evolved strategies to cope with the absence of preferred cobamides. Some bacteria and algae carry out cobamide remodeling, whereby non-preferred cobamides are converted into forms that can be used by their cobamide-dependent enzymes (14, 19-22). In addition, organisms can encode cobamide-independent alternative enzymes or pathways, circumventing the need for cobamides for certain processes (23). For example, cobamide-independent methionine synthase (MetE) and ribonucleotide reductases are commonly found in bacteria, even in those that also encode cobamide-dependent counterparts to these enzymes (3, 24). Bacteria can also tailor their genetic response to the cobamides they prefer via selectivity in riboswitches, noncoding RNA elements in the 5' untranslated region (UTR) of mRNA that, upon binding to specific cobamides, typically downregulate expression of cobamide biosynthesis enzymes, transporters, and cobamide-independent enzymes (25-28).

Here, we carried out a laboratory evolution experiment in *E. coli* to investigate whether there are additional genetic strategies microbes may employ to improve their use of less-preferred cobamides. We found that an *E. coli* Δ*metE* mutant, which relies on the cobamide-dependent methionine synthase MetH, can improve its growth with adeninylcobamide (pseudocobalamin, pCbl) (Fig. 1A) via several genetic strategies. Different sets of mutations were found in evolved lines provided with different pCbl concentrations, but a common strategy that emerged was increasing the expression of the outer membrane corrinoid transporter BtuB, which provided a competitive advantage in limiting concentrations of cobamides. We additionally found that evolved lines and engineered strains that overexpress the corrinoid adenosyltransferase BtuR are better adapted for growth on pCbl. As a result, this evolution experiment revealed a previously unknown role for BtuR in MetH-dependent growth.

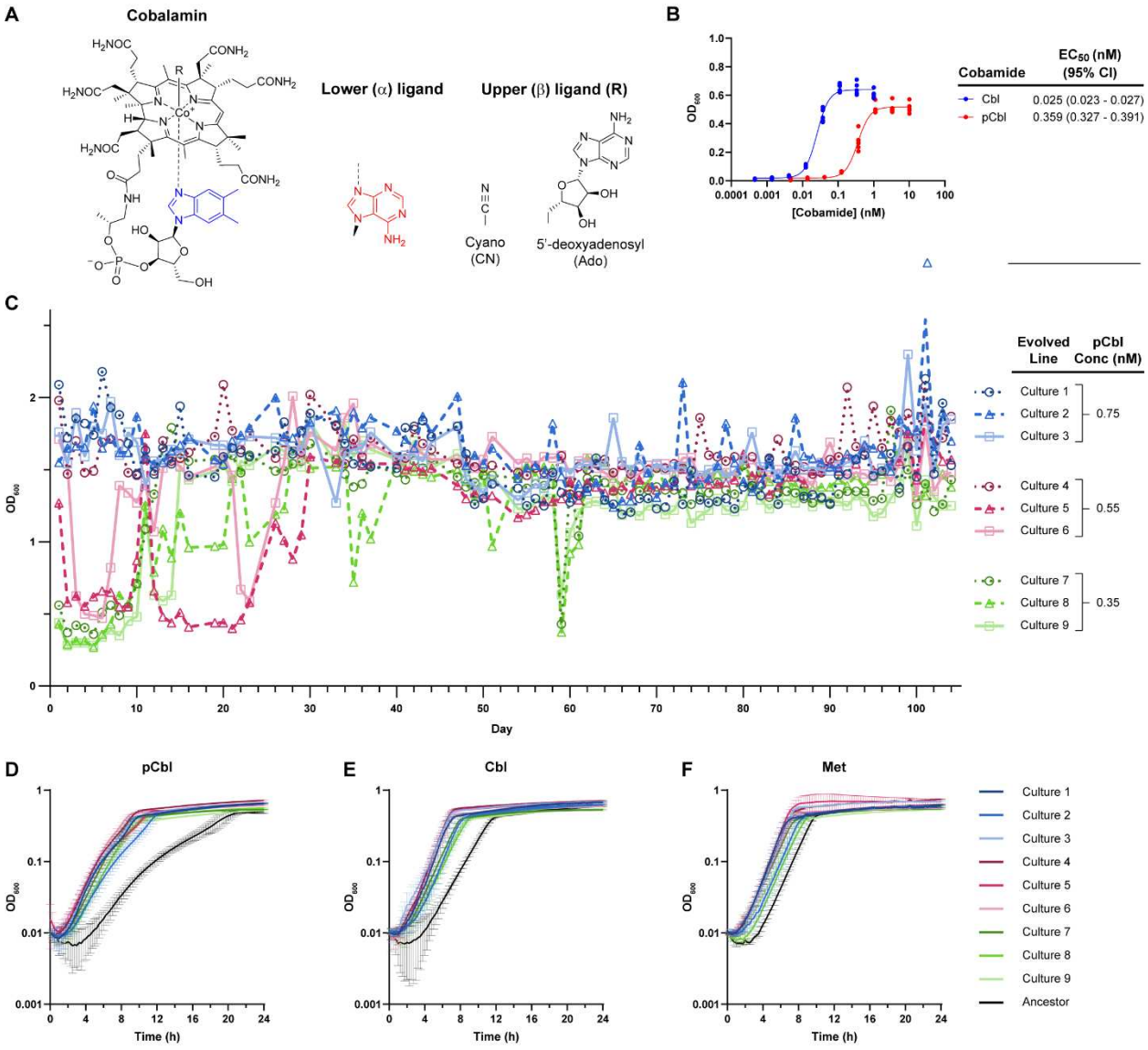

Figure 1. Laboratory evolution of *E. coli* improves its growth with pCbl. A) Structure of cobalamin (Cbl) with its lower ligand 5,6-dimethylbenzimidazole in blue. Pseudocobalamin (pCbl) contains adenine (red) as its lower ligand. The structures of the upper ligands of cobamides used in this study are shown. B) Dose-response curves of *E. coli* Δ*metE* grown in the absence of methionine with various concentrations Cbl or pCbl. OD_{600} was recorded after 22 hours. EC_{50} values and 95% confidence intervals of six biological replicates for each cobamide are shown. C) Growth of *E. coli* Δ*metE* cultures during laboratory evolution. Three biological replicate cultures of *E. coli* Δ*metE* were inoculated into M9 medium supplemented with 0.75, 0.55, or 0.35 nM pCbl and propagated for 104 days. OD_{600} was measured and cultures were passaged 1:100 into fresh medium every 24 hours. D-F) Growth curves of evolved populations (Day 104) and the ancestral Δ*metE* strain with 0.35 nM pCbl (D), 0.35 nM Cbl (E), or 0.1 mg/ml Met (F) are shown. The average of three biological replicates is shown; error bars represent standard deviation.

Results

Laboratory evolution of E. coli *improves use of pCbl during MetH-dependent growth*

E. coli MG1655 has two methionine synthase enzymes, the cobamide-dependent MetH and the cobamide-independent MetE (29). pCbl is less efficient than Cbl in supporting growth of a Δ*metE* strain in minimal medium lacking methionine (Fig. 1B). The concentration of pCbl necessary for half-maximal growth (EC_{50}) of this strain is over 10-fold higher than for Cbl, and the maximal growth yield (OD_{600}) is lower with pCbl. We therefore performed a laboratory evolution experiment in pCbl to determine whether *E. coli* can improve its use of a less-preferred cobamide. Nine independent cultures of the *E. coli* Δ*metE* strain were passaged daily for 104 days and a total of approximately 700 generations in M9 minimal medium containing either 0.75, 0.55, or 0.35 nM pCbl (Fig. 1C). These concentrations encompassed saturating to limiting growth of the ancestral strain (Fig. 1B). Five of the nine cultures had an OD_{600} below 0.6 during the first 10 days, but exceeded an OD_{600} of 1.0 for nearly all passages after day 25, suggesting they had adapted to the limiting pCbl conditions (Fig. 1C). When compared to the ancestor, all nine populations showed improved growth in 0.35 nM pCbl (Fig. 1D). The nine populations also showed improved growth in Cbl (Fig. 1E), suggesting that they had evolved better use of cobamides in general. Growth on Met was modestly improved in the evolved populations, indicating they had adapted to other features of the growth medium (Fig. 1F).

Mutants in one evolved population have a growth advantage specifically with pCbl

We noticed that, when plated on LB agar, some of the colonies from a passaged culture containing 0.35 nM pCbl (Culture 8) were distinctly smaller than the others (Fig. 2A). These small-colony variants first appeared on day 28, and they made up nearly the whole population on day 65 before being almost entirely lost after day 84 (Fig. 2B). Notably, we found that these variants from day 65 persisted only in the presence of pCbl (Fig. 2C). When the day 65 population was grown in media containing Cbl or Met, the small-colony variants were not retained after one week of daily passaging (Fig. 2C).

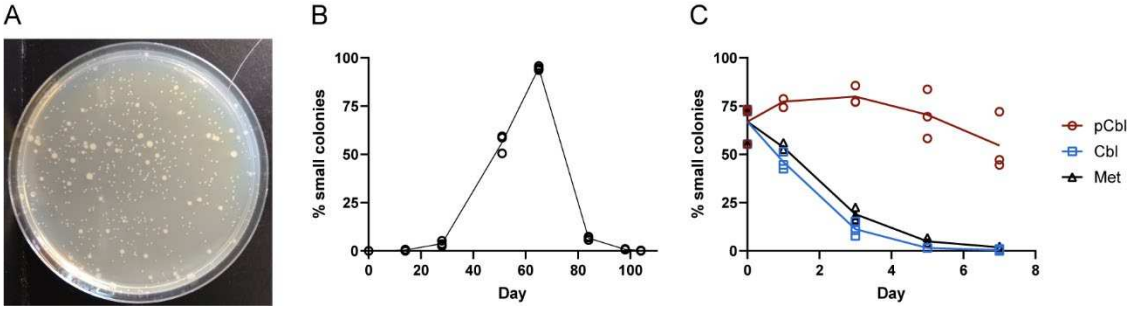

Figure 2. Small colony variants emerge during evolution of Culture 8. A) Plating of the day 65 archive of Culture 8 on LB shows the regular and small colony phenotypes. B) The percentage of total colonies with small size was determined for archived populations of Culture 8 following growth in pCbl. The Δ*metE* ancestor was used for the zero timepoint. C) The percentage of small colonies is plotted for the day 65 archived population of Culture 8 when cultured with 0.35 nM pCbl, 0.35 nM Cbl, or 0.1 mg/ml Met over seven days with daily passaging. Lines connect the means of three biological replicates.

We took advantage of the small colony morphology as a convenient markerless phenotype for further characterizing the pCbl-specific growth advantage. We isolated colonies with different sizes from the day 65 population and individually competed three "small" isolates (S2, S3, and S4) against two "regular" isolates (R1 and R3), as well as the ancestral strain, in media containing either pCbl, Cbl, or Met. All three small isolates had similar phenotypes. When co-cultured, the small isolates outcompeted the ancestor strain in the presence of cobamides, taking over the entire population after a single passage in pCbl and after three passages in Cbl (Fig. 3A, B; Fig. S1 A, B, D, E). In media with methionine, however, the ancestral strain outcompeted the evolved isolates (Fig. 3C; Fig. S1 C, F).

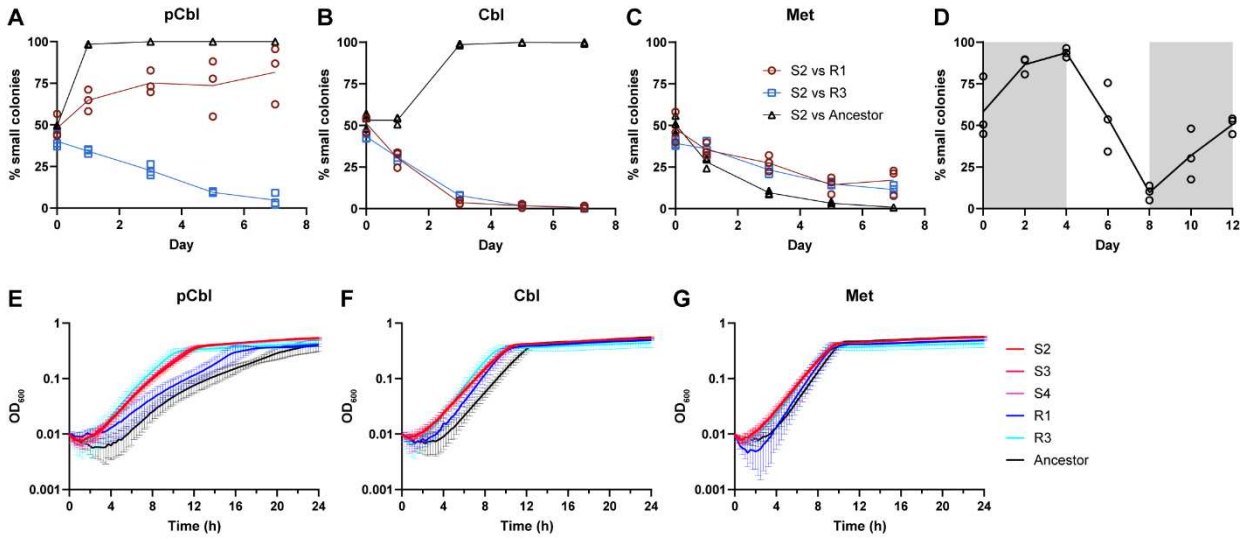

Figure 3. Growth characteristics of isolates S2, R1, and R3 from Culture 8. A-C) Isolate S2 was competed against isolates R1 and R3 and the ancestor strain for seven days with daily passaging in medium containing 0.35 nM pCbl (A), 0.35 nM Cbl (B), or 0.1 mg/ml Met (C). Cultures were diluted and plated on the indicated days to quantify the fraction of small colonies, corresponding to S2 strain abundance. D) Isolates S2 and R1 were competed in medium containing either 0.35 nM pCbl (shaded) or 0.35 nM Cbl (unshaded). Co-cultures were passaged for 12 days and aliquots were plated every two days to quantify the fraction of small colonies in the population. Lines connect the means of three biological replicates. E-G) Growth curves of isolates S2, S3, S4, R1, R3 and the ancestor strain with 0.35 nM pCbl (E), 0.35 nM Cbl (F), or 0.1 mg/ml Met (G). The average of three biological replicates is shown; error bars represent standard deviation.

When the small isolates were competed against the two regular isolates, the small isolates were outcompeted in Cbl and Met, but we observed contrasting phenotypes in pCbl (Fig. 3 A-C, Fig. S1). The small isolates had a competitive advantage over regular isolate R1 but were outcompeted by R3 (Fig. 3 A, Fig. S1 A, D). The competitive advantage of the small isolates in pCbl was further confirmed by co-culturing isolates S2 and R1 in medium supplemented alternately with pCbl and Cbl. When passaged with pCbl for four days, the proportion of S2 increased to over 90%, but after

switching to the Cbl-containing medium, the proportion of S2 decreased to less than 10% after four days. A subsequent return to pCbl resulted in an increase in S2 (Fig. 3D).

The phenotypes of the small and regular isolates that we observed in competition were consistent with their growth characteristics in pure culture (Fig. 3E-G). Isolates S2, S3, and S4, which outcompeted the ancestor in pCbl and Cbl but were outcompeted in Met, grew faster than the ancestor in the presence of cobamides while showing similar growth in Met (Fig. 3E-G). Only isolate R1, which competed poorly in pCbl, had less improved growth than the other isolates, particularly in the medium containing pCbl (Fig. 3E). Isolate R3, which outcompeted the small isolates in all three media conditions, grew similarly to the small isolates in each medium (Fig. 3E-G). Taken together, these results suggest that all of the isolates have acquired one or more mutations that confer a growth advantage with cobamides. Further, based on the growth phenotypes of strains S2, S3, S4 and R3 in pCbl, these strains likely have one or more mutations that confer a specific advantage in pCbl.

Evolved strains have mutations affecting cobamide-related genes

To identify the mutations acquired during the evolution experiment, we performed whole genome sequencing on the isolates from Culture 8. Each isolate has a unique set of mutations, which range from 7 to 14 single nucleotide polymorphisms (SNPs), insertions and deletions (InDels), or structural variants (SVs) (Fig. 4A, Table S1). However, all five isolates have mutations in cobamide-related genes. All of the isolates contain two identical SNPs in the promoter and the ribosome binding site (RBS) of the *btuB-murI* operon, which encodes the outer membrane corrinoid transporter BtuB and the glutamate racemase MurI (Fig. 4A, B). The promoter mutation converts the native -35 sequence to the consensus *E. coli* -35 sequence (Fig. 4B) (30), likely leading to an increase in transcription of the operon. Meanwhile, the RBS mutation may lead to increased translation of the operon by converting it to a sequence closer to the consensus RBS sequence (31). This mutation could also increase operon expression by weakening the translation-inhibiting interaction between the RBS and anti-RBS (A-RBS) in the corrinoid riboswitch located in the 5' UTR (Fig. 4B) (32). Increasing expression of the corrinoid transporter BtuB could explain the improved growth of the isolates with limiting pCbl.

All of the isolates except R1 additionally have mutations in the promoter or 5' UTR of the *yciK-btuR* operon. *yciK* is an uncharacterized gene annotated as a putative oxidoreductase, and *btuR* encodes an adenosyltransferase that installs a 5'-deoxyadenosyl group as the β (upper) ligand of corrinoids (cobamides and biosynthetic precursors). Isolate S3 has a G to A mutation in the -10 element of the promoter that likely increases transcription of the operon (Fig. 4C) (30). Given that all four isolates with mutations in this region have improved growth in pCbl, if the *yciK-btuR* operon is associated with this phenotype, the mutations in S2, S4 and R3 likely increase *yciK-btuR* expression as well.

Sequencing of the archived populations of Culture 8 enabled us to follow the emergence of these mutations during the evolution experiment. The *btuB-murI* mutations were established early and were retained throughout the timecourse (Fig. 4A). Mutations in the -35 element and RBS were present by days 14 and 28, respectively, and their appearances coincided with increases in the OD_{600} of the culture (Fig. 1C).

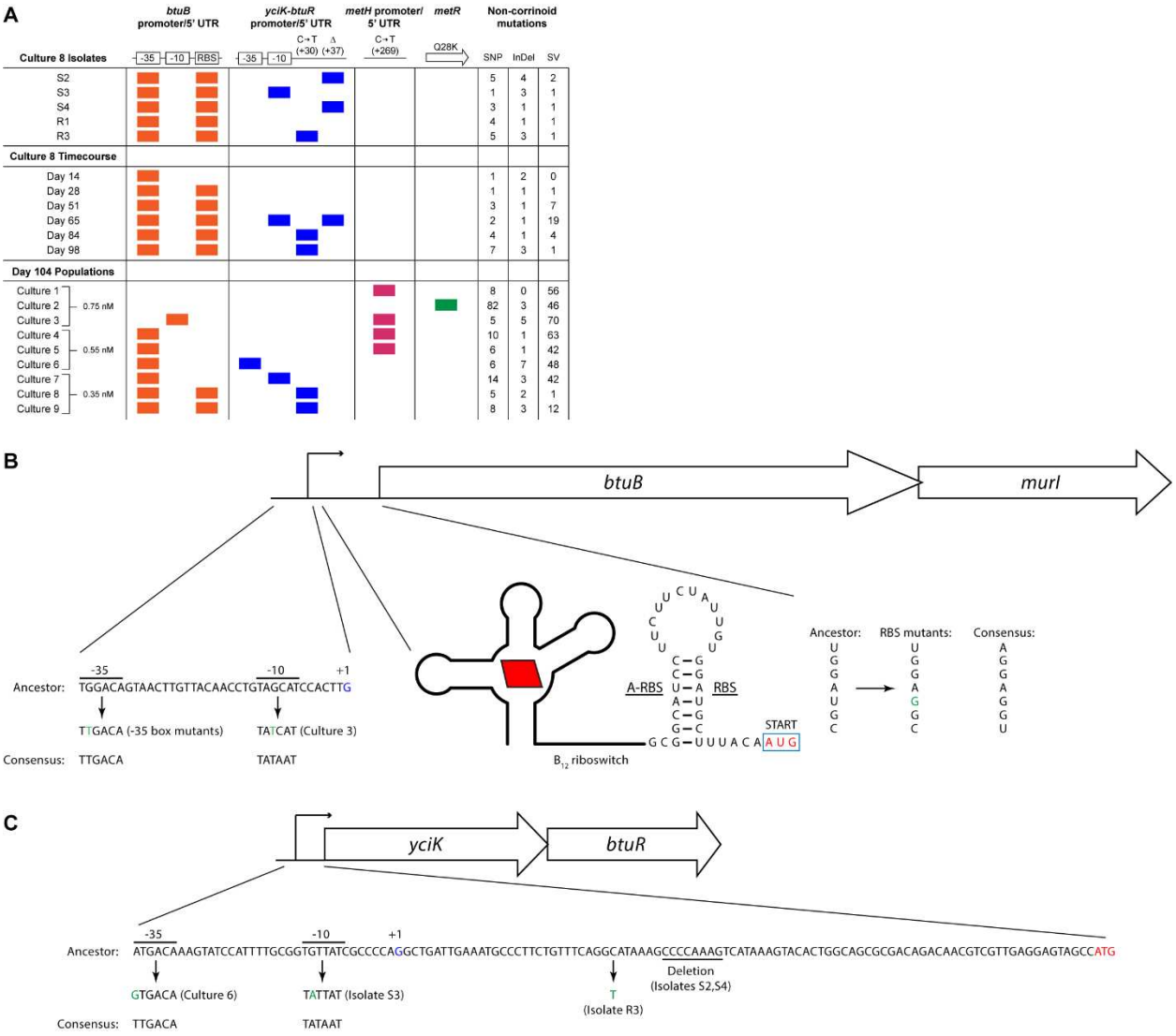

Figure 4. Mutations identified during laboratory evolution. A) Colored boxes show the presence of the indicated mutations affecting corrinoid-related genes in isolates and archived timepoints from Culture 8, and in the endpoint (Day 104) populations of all evolved lines. The concentrations of pCbl in each of the evolved lines (Cultures 1-9) are shown. The numbers of SNPs, InDels, and SVs affecting non-corrinoid-related genes in each sequenced isolate or population are listed (see Table S1). B) Changes in the promoter and 5' UTR (B_{12} riboswitch) of the *btuB-murI* operon found during the evolution. C) Changes in the promoter and 5' UTR of the *yciK-btuR* operon found during the evolution. Identified mutations are shown in green, transcriptional start sites in blue, and start codons in red. The consensus sequences for the σ^{70} promoter -35 and -10 elements are shown for comparison. The promoter for the *yciK-btuR* operon has not been experimentally characterized and was predicted by PromoterHunter (1).

The two *yciK-btuR* mutations found in the small isolates were first detected in the population on day 65, consistent with the small colony variants S2, S3, and S4 dominating the population at this

timepoint (Fig. 2B). At all of the following timepoints, however, only the C to T mutation found in isolate R3 was detected in the population. Given that isolate R3 outcompeted isolates S2, S3 and S4 in pCbl (Fig. 3A, Fig. S1), it is likely that descendants of R3 became dominant in the population after day 65.

E. coli *adapts differently in limiting versus replete pCbl*

Sequencing of the endpoint (Day 104) archives of the nine evolved cultures revealed that mutations in different cobamide-related genes emerged in populations passaged in different concentrations of pCbl (Fig. 4A). Like Culture 8, the two other populations passaged with 0.35 nM pCbl have mutations upstream of both *btuB-murI* and *yciK-btuR* (Fig. 4A, Cultures 7 and 9). In contrast, only one population passaged in 0.75 nM pCbl has a mutation upstream of *btuB-murI*, and one population passaged in 0.55 nM pCbl has a mutation upstream of *yciK-btuR,* though all three populations in 0.55 nM pCbl have a mutation in the *btuB-murI* -35 element. The former two mutations likely increase expression by strengthening their respective promoters (Fig. 4, Cultures 3 and 6, respectively).

All of the evolved populations without mutations affecting *yciK-btuR* have a mutation upstream of *metH* or in the coding sequence of *metR*, a transcriptional activator of *metH* (Fig. 4A, Cultures 1-5) (33). It is unclear how these mutations affect *metH* expression; the *metH* mutation is not located in the promoter, RBS, or MetR binding site (34), while the *metR* mutation is located in its DNA-binding domain (35). Taken together, these results suggest that increasing cobamide uptake and adenosylation are effective strategies for improving growth in limiting to moderate pCbl concentrations, while changing expression of *metH* facilitates adaptation at higher concentrations of pCbl.

Overexpression of the corrinoid uptake gene btuB *is advantageous at limiting cobamide concentrations*

To confirm that the mutations that commonly arose in our evolution experiment indeed impact growth in pCbl, we constructed strains overexpressing the affected genes. Since the glutamate racemase MurI has no known function in cobamide metabolism (36), we tested the hypothesis that phenotypes associated with the mutations upstream of the *btuB-murI* operon are due to an increase in the expression of *btuB*. We constructed a strain that overexpresses *btuB* by inserting a second copy of the gene into the chromosome, with its promoter containing the G to T mutation found in the -35 element of cultures 4-9. In a Δ*metE* background, we competed this strain against one containing only the wild type *btuB* locus, with each strain expressing either CFP or YFP to monitor their abundances in co-culture. We found that overexpression of *btuB* conferred a competitive advantage in 1 nM pCbl, but not in 1 nM Cbl or in Met (Fig. 5 A-C). However, varying the cobamide concentration showed that *btuB* overexpression is beneficial in both pCbl and Cbl at concentrations at which the cobamide is limiting, namely 1 nM and less for pCbl, and under 0.25 nM for Cbl (Fig. 5D, E). Thus, the *btuB* mutations that arose during passaging in limiting pCbl presumably improved *E. coli*'s ability to import cobamides to support MetH-dependent growth.

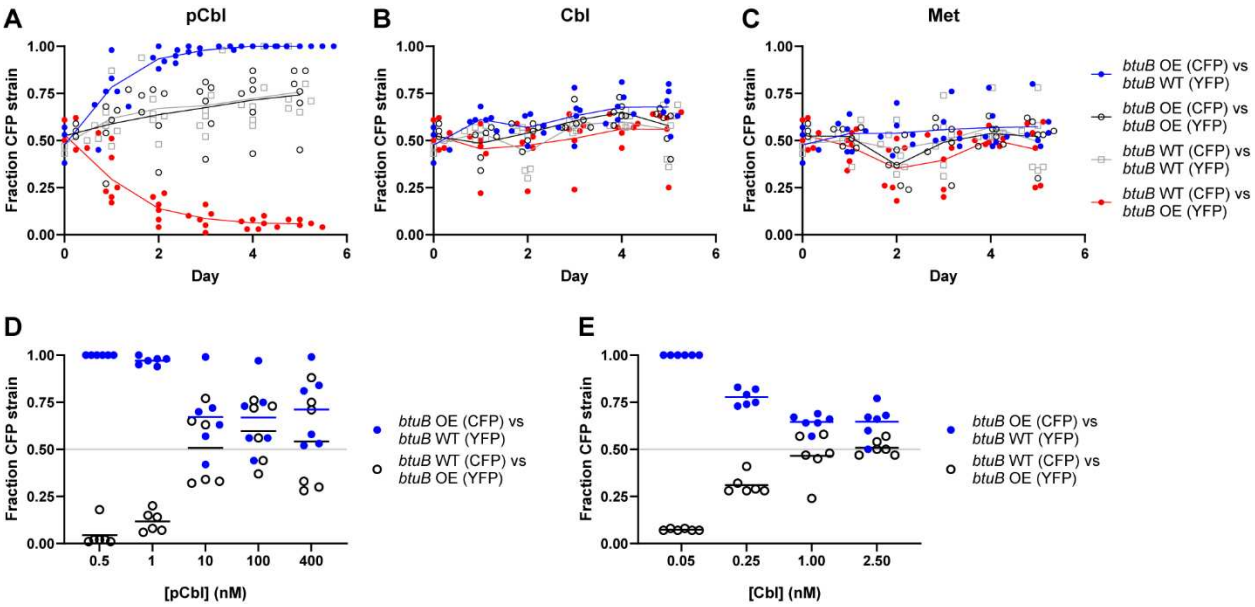

Figure 5. Overexpression of *btuB* confers a competitive advantage at limiting cobamide concentrations. A-C) *E. coli* Δ*metE* strains overexpressing *btuB* (OE) or producing native levels of *btuB* (WT) were competed in co-culture for five days with daily passaging in medium containing either 1 nM pCbl (A), 1 nM Cbl (B), or 0.1 mg/ml Met (C). The fraction of the CFP-containing strain in each co-culture is shown. Control co-cultures containing CFP- and YFP-expressing strains in the same genetic background (black and gray) were included to rule out a growth disadvantage caused by the fluorescent proteins. D-E) The CFP- and YFP-expressing strains that overexpress *btuB* (OE) or produce native levels of *btuB* (WT) were competed in co-culture at different concentrations of pCbl (D) or Cbl (E). Fluorescence measurements were taken on day 3 following daily passaging. Lines represent the means of six biological replicates.

The corrinoid adenosyltransferase gene btuR *is required for optimal MetH-dependent growth*

Next, we assessed whether *btuR* expression levels impact MetH-dependent growth by overexpressing *btuR* on a plasmid. We found that, similar to the results with *btuB*, a strain overexpressing *btuR* outcompeted a strain with wild type *btuR* levels when grown with pCbl, but not with Cbl or Met (Fig. 6 A-C). Though it is in an operon with *yciK*, *btuR* alone was responsible for this phenotype, as overexpression of *yciK* did not confer a growth advantage in pCbl and co-expression of *yciK* with *btuR* did not influence the effect of overexpression of *btuR* alone (Fig. S2). However, unlike *btuB*, overexpression of *btuR* remained beneficial even at concentrations of up to 400 nM pCbl, and failed to confer a competitive advantage at any concentration of Cbl tested (Fig. 6 D, E).

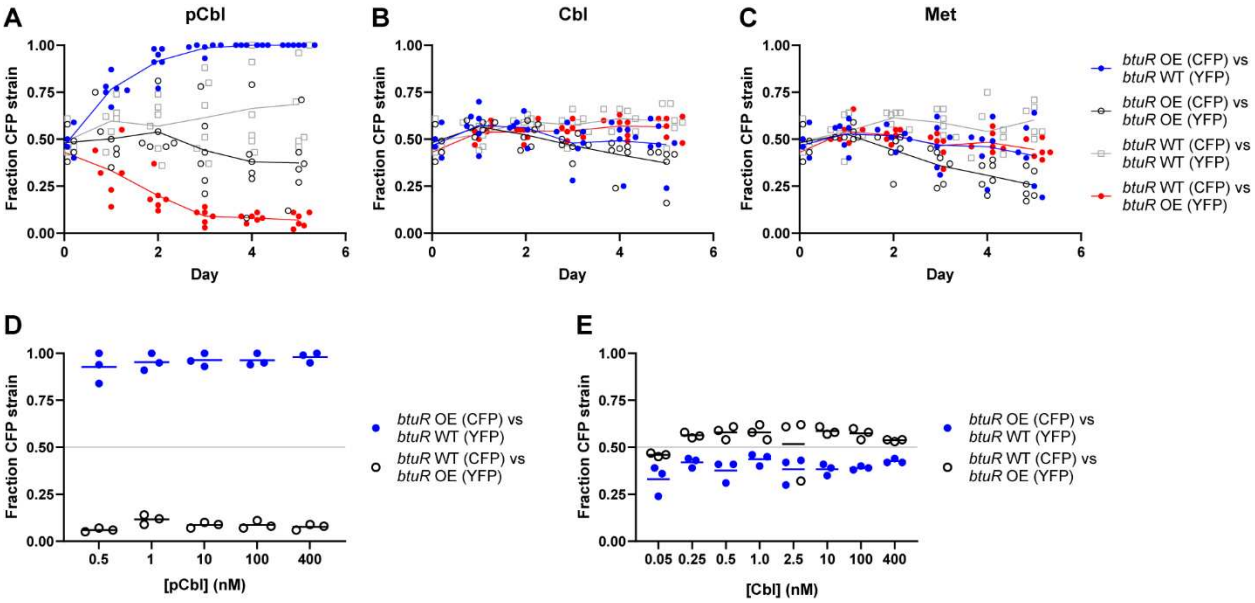

Figure 6. Overexpression of *btuR* confers a growth advantage only during growth with pCbl. A-C) *E. coli* Δ*metE* strains overexpressing *btuR* (OE) or producing native levels of *btuR* (WT) were competed in co-culture for five days with daily passaging in medium containing either 1 nM pCbl (A), 1 nM Cbl (B), or 0.1 mg/ml Met (C). The fraction of the CFP-containing strain in each co-culture is shown. Control co-cultures containing CFP- and YFP-expressing strains in the same genetic background (black and gray) were included to rule out a growth disadvantage caused by the fluorescent proteins. D-E) The CFP- and YFP-expressing strains that overexpress *btuR* (OE) or produce native levels of *btuR* (WT) were competed in co-culture at different concentrations of pCbl (D) or Cbl (E). Fluorescence measurements were taken on day 3 following daily passaging. Lines represent the means of six biological replicates.

In the Δ*metE* mutant, which relies on MetH activity for growth, cobamides are used by the MetH enzyme to transfer methyl groups from methyltetrahydrofolate to homocysteine by alternately methylating and demethylating the cobamide at the β position. It was therefore puzzling to find that overexpression of BtuR, which adenosylates cobamides at the β position, improves MetH-dependent growth. To further explore the role of BtuR in MetH-dependent growth, we deleted *btuR* and performed growth assays with pCbl or Cbl with either cyano (CN, as in Fig. 1-3, 5, 6) or adenosyl (Ado) β ligands (Fig. 1A). Growth measurements with these cobamides showed that a Δ*btuR* Δ*metE* strain has impaired growth in cyanopseudocobalamin (CNpCbl), with a lower maximum OD_{600} and an EC_{50} over 25-fold higher than the Δ*metE* strain (Fig. 7A). Growth with adenosylpseudocobalamin (AdopCbl) led to a higher maximum OD_{600} and lower EC_{50} of the Δ*btuR* Δ*metE* strain, though growth was still considerably impaired compared to the Δ*metE* strain (Fig. 7A). A similar trend was observed when these strains were cultured with cyanated versus adenosylated forms of Cbl, though the growth impairment of the Δ*btuR* Δ*metE* strain was more modest (Fig. 7B). Together, these results confirm that *btuR* impacts MetH-dependent growth in *E. coli.*

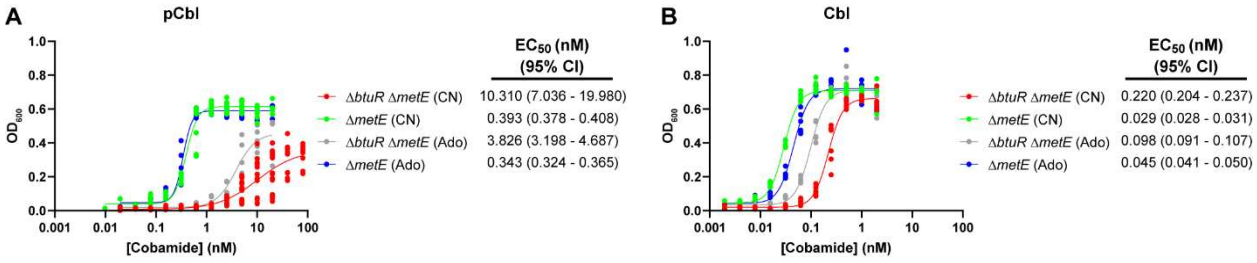

Figure 7. **Deletion of *btuR* causes poorer growth with pCbl and Cbl.** A-B) Cobamide dose-response curves are shown for *E. coli* Δ*btuR*::*kan*^{R} Δ*metE* and Δ*metE* strains grown in the absence of methionine and with various concentrations of CNpCbl and AdopCbl (A), or CNCbl and AdoCbl (B). OD_{600} was recorded after 22 hours of growth. EC_{50} values and 95% confidence intervals were calculated from 6-18 biological replicates for each cobamide.

The growth defect of the Δ*btuR* Δ*metE* strain and partial rescue by adenosylated cobamides could be due to differences in cobamide uptake or retention. To examine this, we cultured the Δ*btuR* Δ*metE* and Δ*metE* strains with each of the four cobamides tested in Figure 7 (AdopCbl, CNpCbl, AdoCbl, CNCbl) and measured the amount of cobamide in the supernatant and cell pellet fractions using a quantitative corrinoid bioassay. *E. coli* strains lacking *btuR* had approximately twofold less AdopCbl and CNpCbl in the cell pellet fraction (Fig. S3A); this result was validated by HPLC analysis of corrinoid extractions (Fig. S3D). No differences in the levels of either AdoCbl or CNCbl was observed between the Δ*btuR* Δ*metE* and Δ*metE* strains, but less AdoCbl was found in the cell pellets than CNCbl (Fig. S3 B, C). The twofold differences in intracellular pCbl levels between the strains likely only partially explains the 10- to 25-fold differences in EC_{50} and differences in maximal OD_{600} observed in the dose-response assays (Fig. 7A). Thus, it is possible that the adenosylated forms of cobamides are beneficial for MetH activity via a previously unknown mechanism.

Discussion

Cobamides are considered key shared nutrients because they function as cofactors for numerous microbial processes but are only produced by a subset of prokaryotes. They have been detected in diverse microbial communities, both environmental and host-associated, and a wide range in cobamide abundances has been observed across these ecosystems, with some dominated by one or two cobamides, while others contain up to eight different types. These differences in cobamide diversity across environments are noteworthy in light of the observation that many bacteria have preferences for particular cobamides. This raises the question of how bacteria adapt in the presence of non-preferred cobamides. We addressed this question here by using a cobamide-dependent mutant of *E. coli* as a model in a laboratory evolution experiment. We found that *E. coli* is indeed capable of improving its growth with pCbl, and it uses differing strategies depending on the availability of the nutrient. Competition experiments and genetic analyses revealed regulation of corrinoid uptake as a limiting factor in *E. coli* and a previously unappreciated role for the corrinoid adenosyltransferase BtuR in MetH-dependent growth.

We previously showed that the cobamide-dependent enzyme methylmalonyl-CoA mutase (MCM) has different binding affinities for different cobamides, and that these cobamide-binding affinities largely mirror MCM-dependent growth with different cobamides in *Sinorhizobium meliloti* (16). Other studies have shown that MetH orthologs from different organisms display distinct preferences for different cobamides (14, 17, 21). Given that pCbl is less preferred by *E. coli* than Cbl (Fig. 1B), we hypothesized that passaging in pCbl would lead to the accumulation of mutations in the MetH enzyme that improved its ability to use pCbl. Though we observed mutations that presumably impact *metH* expression, no mutations were found in the *metH* coding sequence. Altering expression of corrinoid-related genes was the general outcome of our evolution experiment, suggesting that modifying the regulation of cobamide metabolism may be a more readily accessible mechanism of adaptation than changes to the specificity of the dependent enzyme, particularly in our experimental timeframe. Changes to gene expression are routinely seen in laboratory evolution experiments, including the targeting of global regulators (37, 38).

While mutations in *metH* and its transcriptional activator *metR* were found at the higher concentration of pCbl, mutations upregulating the outer membrane corrinoid transporter BtuB arose primarily when pCbl was limiting, consistent with its role in corrinoid uptake. Indeed, we previously showed that overexpression of the corrinoid transport machinery in *Bacillus subtilis* increases the amount of cobamide imported (28), and here, overexpression of *btuB* enables *E. coli* to compete more effectively when cobamides, both pCbl and Cbl, are limiting. In the human gut commensal bacterium *Bacteroides thetaiotaomicron*, cobamide uptake is critical for colonization in a mouse model (39). *B. thetaiotaomicron* and other *Bacteroides* species encode multiple corrinoid transport systems (40), which include high-affinity corrinoid binding proteins absent from *E. coli* , and it is thought that these systems enable *Bacteroides* to outcompete other microbes for corrinoids, allowing for successful gut colonization (41, 42). Based on our observation that corrinoid uptake and competitiveness in *E. coli* can readily be improved via mutations in the *btuB* promoter or RBS, we speculate that *E. coli* is not evolved to maximize corrinoid uptake, despite its being a member of the gut microbiota like *B. thetaiotaomicron*. This is notable given that the purinyl cobamides, which include pCbl, were found to be the dominant fecal corrinoids in the majority of human subjects (7). *E. coli* could be under less selective pressure to maximize corrinoid uptake because, unlike *B. thetaiotaomicron*, *E. coli* has the cobamide-independent methionine synthase *metE* as well as *metH*, rendering it less dependent on exogenous cobamides. In addition, BtuB is a phage receptor in *E. coli*, so increased expression of *btuB* may not always be beneficial in natural settings (43).

The BtuR corrinoid adenosyltransferase is responsible for installing a 5'-deoxyadenosine moiety as the β ligand of cobamides to produce adenosylcobamides (44), which are required for the subset of cobamide-dependent enzymes, such as MCM, that carry out radical-based reactions (45). However, no role for adenosylcobamides has been proposed for methyltransferases such as MetH, which use methylcobamides – cobamides with a methyl group as the β ligand – to shuttle methyl groups from a methyl donor to a substrate. Therefore, it was surprising to find that, in MetH-dependent *E. coli*, overexpression of *btuR* provides a competitive advantage in pCbl, deletion of *btuR* impairs growth in both pCbl and Cbl, and supplementation of the Δ*btuR* mutant with adenosylcobamides did not completely rescue the phenotype. These results suggest that adenosylcobamides, and perhaps the BtuR protein itself, could have previously unknown roles in MetH function. Some cobamide-dependent enzymes such as MCM require a corrinoid adenosyltransferase and other accessory proteins to load the cobamide cofactor into the enzyme

(46-48). It is possible that BtuR fulfills such a role for MetH in *E. coli*, particularly for cobamides that function poorly as a cofactor for MetH. Alternatively, adenosylcobamides and/or BtuR could facilitate SAM-dependent cobamide reactivation, a step required approximately every 2,000 turnovers for Cbl following spontaneous cofactor oxidation (49-51). Until recently, studies of *E. coli* MetH have been unable to address the cofactor loading step because the enzyme is stable only when pre-loaded with Cbl during purification. Future *in vitro* studies with a newly identified MetH homolog that is stable in its apo form will facilitate analysis of the cofactor loading step (52). Because pCbl functions more poorly than Cbl in *E. coli* MetH-dependent growth, our evolution experiment may have fortuitously uncovered a role for adenosylated cobamides in corrinoid-dependent physiology. Future work will be aimed at understanding the molecular mechanisms underlying these observations.

Material and Methods

Media and growth conditions

E. coli MG1655 Δ*metE* evolution was performed at 37°C with aeration in M9 glycerol minimal medium with the indicated concentrations of pCbl (53). 20 ml cultures were grown in glass test tubes with 0.2 ml transferred into fresh media every 24 hours. A sample of each population was archived on days 14, 28, 51, 65, 84, 98, and 104 in 25% glycerol and stored at -80°C. Before the start of the evolution experiment, the three replicate cultures were passaged daily for 16 days with a saturating concentration of pCbl (5 or 2.5 nM) to facilitate identifying the appropriate concentrations for the evolution experiment.

M9 medium was supplemented with 0.1 mg/ml L-methionine (Met) when indicated. LB agar was used as solid medium. For experiments with the *E. coli* Δ*metE* ancestor or evolved isolates from Culture 8, M9 medium was inoculated with individual colonies grown on LB agar. Pre-culturing of populations and strains in M9 medium was performed at 37°C with aeration.

Strain construction

All strains used for evolution and mutant validation are derivatives of wild type K12 strain MG1655. *E. coli* strains were cultured at 37°C with aeration in LB medium. Media were supplemented with antibiotics at the following concentrations when necessary: kanamycin, 25 mg/liter (pKIKO, pETmini); carbenicillin, 100 mg/liter (pCP20); chloramphenicol, 10-20 mg/liter (pACYCDuet-1). pKIKO*arsB*Km plasmids were propagated in *E. coli* DH5α containing λ*pir*.

The Δ*metE*::kan^R and Δ*btuR*::kan^R mutations from the Keio collection were introduced by P1 transduction into *E. coli* strain MG1655 (54, 55). The kanamycin resistance cassette was removed by introducing the plasmid pCP20 as described, leaving the FRT site in place of the *metE* coding sequence (56).

An *E. coli* strain overexpressing *btuB* was created by integrating an additional copy of the gene at the *arsB* (arsenite transporter) locus using the KIKO system as described (57). pKIKO*arsB*Km was a gift from Lars Nielsen & Claudia Vickers (Addgene plasmid # 46766; http://n2t.net/addgene:46766; RRID:Addgene_46766). *E. coli btuB* with its promoter and riboswitch was cloned into pKIKO*arsB*Km, with the promoter containing the -35 element mutation (TTGACA) found in evolved populations and isolates. The construct also contained a

synonymous mutation in codon 581 encoding a valine (GTT to GTA). The *btuB* construct was integrated at the *arsB* locus using the PCR-based method. For a control strain, we integrated the *arsB*-flanked kanamycin resistance cassette without an insert. The constructs were first integrated into MG1655 and subsequently transduced via P1 into the Δ*metE* strain. Finally, the kanamycin resistance cassette was removed using pCP20. Constructs were confirmed by PCR and Sanger sequencing.

The *btuR* and *yciK* genes were overexpressed in a pACYCDuet-1 plasmid in which the T7 promoters were replaced with the *lac* promoter and operator (pACYCDuet-1-pLac). This allowed for repression of gene expression in the presence of glucose (0.02% in LB, 0.2% in M9) and expression in the absence of glucose due to the leakiness of the *lac* promoter. *E. coli btuR*, *yciK*, and the *yciK-btuR* operon were each cloned downstream of the *lac* promoter in pACYCDuet-1-pLac. mCerulean and mCitrine genes from pSG013 and pSG015 (with J23100 promoter and B0034 RBS) were inserted between the chloramphenicol resistance cassette and p15A origin in each of these plasmid constructs to enable tracking of strains by fluorescence measurements (58).

Cobamide reagents

CNCbl and AdoCbl were purchased from MilliporeSigma. CNpCbl was extracted from *Propionibacterium acidi-propionici* strain DSM 20273 and purified as described (59, 60). AdopCbl was chemically synthesized from CNpCbl and purified as described (16). Cobamides were quantified spectrophotometrically (16, 22). Cbl and pCbl were used in their cyano forms (CNCbl and CNpCbl) unless otherwise indicated.

Growth assays and competition experiments

To quantify the percentage of small colonies present during the evolution of Culture 8, archived populations were cultured overnight in M9 glycerol medium supplemented with 0.35 nM pCbl, diluted, and plated on LB agar.

Growth assays and competition experiments were performed in 200 µl cultures in 96-well plates (Corning, 3598). For growth curves, populations or isolates were pre-cultured in M9 glycerol medium supplemented with 0.35 nM pCbl, while cultures for cobamide dose-response assays were supplemented with Met. Cells from saturated cultures were collected by centrifugation, resuspended in M9 glycerol medium, and OD_{600} was measured. Each population or strain was then inoculated at a starting OD_{600} of 0.01 in M9 glycerol medium with the indicated supplement. 96-well plates were sealed with Breathe-Easy (Diversified Biotech). Growth assays were performed in a BioTek Synergy 2 microplate reader with shaking at medium speed at 37°C and OD_{600} recorded every 15 min for 24 hours. OD_{600} for cobamide dose-response assays was measured with the BioTek Synergy 2 microplate reader following 22 h growth at 37°C with shaking in either the plate reader or a heated benchtop microplate shaker (1,200 rpm, Southwest Science). Preparation of cultures containing adenosylcobamides was done under red light and the plates were incubated in the dark. EC_{50} values were calculated using Graphpad Prism (Dose-response – Stimulation; [Agonist] vs. response – Variable slope (four parameters)).

For competition experiments involving evolved populations or strains, cells were pre-cultured in M9 glycerol medium supplemented with Met. Cells were pelleted, washed twice with 0.85%

saline, and resuspended in M9 glycerol medium, with the exception of the experiments shown in Fig. 2C and 3D, in which the cells were pelleted and resuspended in M9 glycerol medium without washing. OD_{600} was measured and the population or an equal ratio of two strains was inoculated at a starting OD_{600} of 0.01 in 200 µl M9 glycerol medium containing the indicated supplement. A dilution of the culture was plated on LB agar to establish the percentage of small colonies at time 0. The plate was sealed and incubated at 37°C in a benchtop microplate shaker at 1,200 rpm. 2 µl of each culture was transferred into 198 µl fresh medium every 24 h. On the indicated days, dilutions from the cultures were plated on LB agar to determine the percentage of small colonies in the population.

Competition experiments involving the *btuB*-overexpression strain were tracked by fluorescence (58). Strains were pre-cultured in M9 glycerol medium supplemented with Met. Cells were pelleted, washed twice with 0.85% saline, and resuspended in M9 glycerol medium. OD_{600} was measured and each sample was adjusted to an OD_{600} of 0.25. Co-cultures were prepared by mixing an equal volume of each strain. 100 µl of each co-culture was transferred to a 96-well glass bottom plate (P96-1.5P, Cellvis) and cyan and yellow fluorescence were measured on a multiwell plate reader (Tecan Spark) as described (58). Separately, 8 µl of each mono- and co-culture were added to 192 µl of M9 glycerol medium (starting OD_{600} of 0.01) containing the specified amendment in 96-well plates. Plates were sealed and incubated at 37°C in a benchtop microplate shaker (1,200 rpm). 2 µl of each culture was transferred into 198 µl fresh medium every 24 h. At the specified timepoints, aliquots were diluted in M9 medium and CFP and YFP values were measured. Standard curves for normalization of fluorescence to OD_{600} were generated from the overnight cultures grown in tubes (for t = 0 readings only) or mono-culture controls grown in 96-well plates (after 1 day). Competition experiments with pACYCDuet-1-pLac plasmids expressing *btuR* and/or *yciK* were performed similarly except that strains were pre-cultured in M9 glucose (0.2%) medium with Met.

Whole genome sequencing and analysis

Evolved populations were grown in M9 medium supplemented with 0.35 nM pCbl, while evolved isolates and Δ*metE* ancestor were cultured in M9 medium supplemented with Met. Genomic DNA was isolated with a DNeasy Blood and Tissue Kit (Qiagen) and submitted to Novogene (Sacramento, CA, USA) for library preparation and whole genome sequencing using an Illumina NovaSeq 6000.

Identification of mutations was performed by Novogene by comparison to the *E. coli* MG1655 reference genome (accession PRJNA57779). SNPs and InDels were detected using SAMtools with the parameter 'mpileup -m 2 -F 0.002 -d 1000' and annotated using ANNOVAR (61, 62). The results were filtered such that the number of support reads for each SNP/InDel was greater than 4 and the mapping quality of each SNP/InDel was higher than 20. SVs were detected by BreakDancer and annotated by ANNOVAR (63). SVs were filtered by removing those with fewer than 2 supporting PE reads. A comparison to the Δ*metE* ancestor was made to eliminate mutations present prior to the laboratory evolution.

Corrinoid bioassay to assess cobamide uptake and retention

E. coli Δ*btuR* Δ*metE* and Δ*metE* strains were pre-cultured in M9 glycerol medium supplemented with Met. The strains were then inoculated at an OD_{600} of 0.01 in 1 ml M9 glycerol medium supplemented with AdopCbl, CNpCbl, AdoCbl, or CNCbl. The medium was also supplemented with 0.02 mg/ml Met, a concentration that ensured saturating growth of the pCbl cultures but did not affect growth of the Δ*metE* strain in the subsequent bioassay. Cultures were incubated at 37°C with aeration for 22 hours. Cultures containing adenosylcobamides were prepared under red light and incubated in the dark. 750 µl of each culture was centrifuged for 5 min at 6,000 x g to pellet cells. 600 µl of the supernatant was passed through a 0.22 µm filter. The cell pellet was washed twice with 0.85% saline and resuspended in 750 µl saline. All samples were then incubated at 100°C for 20 min. Samples containing the pellet fraction were centrifuged for 5 min at 6,000 x g and 600 µl of supernatant was removed to use as the cell lysate. The *E. coli* bioassay was performed in 96 well plates as described (60), except M9 glycerol was used as the growth medium and plates were incubated at 37°C in a microplate shaker for 22 hours prior to measuring OD_{600}. The concentration of cobamides in each sample was determined using standard curves generated with CNpCbl and CNCbl.

Corrinoid extraction and analysis

E. coli Δ*btuR* Δ*metE* was pre-cultured in M9 glycerol medium supplemented with Met. OD_{600} was measured and cells were inoculated into 250 ml M9 glycerol medium containing 1 nM pCbl or Cbl at an OD_{600} of 0.01. The medium with 1 nM pCbl was supplemented with Met to enable growth of *E. coli* Δ*btuR* Δ*metE*. Cultures were grown at 37°C with aeration for 22 h. Cells were collected by centrifugation and washed twice with saline. Corrinoids were extracted with KCN as described (60). Extractions were analyzed on an Agilent Zorbax SB-Aq column (5 µm, 4.6 x 150 mm) with an Agilent 1200 series HPLC equipped with a diode array detector using Method 2 (11). Cobamides in each sample were quantified using standard curves generated with CNpCbl and CNCbl.

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