Microbes display broad diversity in cobamide preferences

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Abstract

Cobamides, the vitamin B_{12} (cobalamin) family of cofactors, are used by most organisms but produced by only a fraction of prokaryotes, and are thus considered key shared nutrients among microbes. Cobamides are structurally diverse, with multiple different cobamides found in most microbial communities. The ability to use different cobamides has been tested for several bacteria and microalgae, and nearly all show preferences for certain cobamides. This approach is limited by the commercial unavailability of cobamides other than cobalamin. Here, we have extracted and purified seven commercially unavailable cobamides to characterize bacterial cobamide preferences based on growth in specific cobamide-dependent conditions. The tested bacteria include engineered strains of *Escherichia coli*, *Sinorhizobium meliloti*, and *Bacillus subtilis* expressing native or heterologous cobamide-dependent enzymes, cultured under conditions that functionally isolate specific cobamide-dependent processes such as methionine synthesis. Comparison of these results to previous studies of diverse bacteria and microalgae revealed that a broad diversity of cobamide preferences exists not only across different organisms, but also between different cobamide-dependent metabolic pathways within the same organism. The microbes differed in the cobamides that support growth most efficiently, those that do not support growth, and the minimum cobamide concentrations required for growth. The latter differ by up to four orders of magnitude across organisms from different environments and by up to 20-fold between cobamide-dependent enzymes within the same organism. Given that cobamides are shared, required for use of specific growth substrates, and essential for central metabolism in certain organisms, cobamide preferences likely impact community structure and function.

Importance

Nearly all bacteria are found in microbial communities with tens to thousands of other species. Molecular interactions such as metabolic cooperation and competition are key factors underlying community assembly and structure. Cobamides, the vitamin B_{12} family of enzyme cofactors, are one such class of nutrients, produced by only a minority of prokaryotes but required by most microbes. A unique aspect of cobamides is their broad diversity, with nearly 20 structural forms identified in nature. Importantly, this structural diversity impacts growth, as most bacteria that have been tested show preferences for specific cobamide forms. We measured cobamide-dependent growth in several model bacteria and compared the results to previous analyses of cobamide preference. We found that cobamide preferences vary widely across bacteria, showing the importance of characterizing these aspects of cobamide biology to understand the impact of cobamides on microbial communities.

B_{12} and other cobamide cofactors are required by organisms in all domains of life (1). Only certain bacteria and archaea synthesize cobamides (2), while others must acquire them exogenously. Thus, cobamides are considered shared nutrients within microbial communities. A unique aspect of cobamides is their structural diversity. While B_{12} (cobalamin, Cbl) is a well-known vitamin important for human health, nearly 20 cobamides with alternative lower ligands exist in nature (Fig. 1A) (3-5). Though research with alternate cobamides is limited because they are commercially unavailable, cobamide-dependent enzymes and organisms have distinct preferences for different cobamides (6-16). Further, addition of different cobamides to soil or soil-derived enrichment cultures elicited distinct shifts in bacterial abundances, suggesting that cobamide structure influences bacterial growth at the community level (17). Thus, cobamide preference in bacteria is likely important for microbial community structure.

Here, we address four questions about cobamide use and preference: 1) Do different cobamide-dependent enzymes in the same bacterium have the same cobamide requirements? 2) How many, and which, cobamides can support growth? 3) Which cobamides are preferred, and do preferences differ across organisms? 4) How much cobamide do microbes need for growth, and do these requirements vary by enzyme, taxonomy, or environment? We addressed these questions by measuring growth of wild-type and engineered bacteria in different cobamide-dependent conditions with up to eight cobamides at a range of concentrations. Cobamide preference was defined based on the concentration that elicits half-maximal growth (EC_{50}), with lower values corresponding to more preferred cobamides. This *in vivo* assay encompasses the different aspects of cobamide utilization, including uptake, adenosylation, riboswitch-based regulation, and the cobamide-dependent enzymes themselves. We compared these measurements with those from published literature to gain a comprehensive view of cobamide preferences across taxa and environments.

Different cobamide-dependent enzymes in a single organism can have distinct cobamide preferences

First we compared the cobamide requirements of three cobamide-dependent processes in *Sinorhizobium meliloti*. *S. meliloti* produces Cbl for methionine synthase (MetH), methylmalonyl-CoA mutase (MCM), and ribonucleotide reductase (NrdJ), each of which can be tested separately for cobamide preference by altering the genetic background and growth substrates. MetH-dependent growth was supported by all cobamides tested, though EC_{50} values spanned three orders of magnitude (Fig. 1B, Table S1). The concentration requirements and preferences are similar to what we previously observed for MCM-dependent growth (8), except that [2-MeAde]Cba better supported MCM-dependent growth (Fig. 2). NrdJ-dependent growth required 25- and 15-fold higher Cbl and [Bza]Cba concentrations, respectively (Fig. 1B-C, Table S1), which suggests either more cobamide is required for NrdJ function or higher levels of NrdJ enzyme are necessary to support growth. The relative cobamide preferences for MetH- and NrdJ-dependent growth are similar, though phenolyl cobamides did not support NrdJ-dependent growth, consistent with NrdJ being a base-on enzyme (18) and phenolyl cobamides being unable to adopt the base-on conformation (Fig. 1A-C, Fig. 2). Cbl was the preferred cobamide for all three cobamide-dependent conditions, consistent with adaptation to endogenously produced Cbl (Fig. 2).

We used *Escherichia coli* to compare cobamide preferences of ethanolamine ammonia-lyase (EAL) and MetH using engineered strains cultured under different conditions. EAL-dependent growth required 5- to 78-fold higher cobamide concentrations than MetH-dependent growth and showed different cobamide preferences (Fig. 1D-E, Table S1). [2-MeAde]Cba, produced by *E. coli* when provided the precursor cobinamide (19), was most preferred for EAL-dependent growth but less preferred by MetH. In contrast, Cbl was the least preferred cobamide that supports EAL-dependent growth, but most preferred for MetH-dependent growth (Fig. 1D-E). [*p*-Cre]Cba did not support EAL-dependent growth, consistent with EAL being a base-on enzyme (10). Together, these results show that cobamide-dependent enzymes in the same organism can have distinct cobamide preferences and requirements.

Cobamide use in a single process varies across bacteria

We next examined cobamide preferences for MetH-dependent growth in several additional bacteria, including *Bacteroides thetaiotaomicron* and *Ruminococcus gnavus*, which have MetH but lack cobamide-independent methionine synthase MetE, as well as *E. coli* and *Bacillus subtilis* strains heterologously expressing MetH orthologs from *Vibrio cholerae* and *Priestia megaterium*, respectively. In each organism, growth was supported by all or most of the cobamides tested, suggesting there is promiscuity in cobamide uptake, adenosylation, regulation, and use by MetH (Fig. 1E-J). However, EC_{50} values spanned two orders of magnitude among cobamides supporting growth (Fig. 1, 2). While in all cases, either [Ade]Cba or a phenolyl cobamide was least preferred, and Cbl was most preferred except by *B. thetaiotaomicron*, variability in cobamide preference was observed across organisms (Fig. 1F-J). This variability is most apparent when contrasting *B. thetaiotaomicron* and *B. subtilis* expressing *P. megaterium metH*, which did not grow with phenolyl or purinyl cobamides, respectively, at any tested concentration (Fig. 1H-I). The cobamide preferences of the latter strain are largely consistent with prior growth measurements at a single cobamide concentration (20). In contrast, *Akkermansia muciniphila* showed no cobamide preference due to its ability to remodel diverse cobamides to [Ade]Cba (Fig. 2) (21).

Expressing *V. cholerae metH* in *E. coli* afforded the opportunity to compare the cobamide preferences of *E. coli* and *V. cholerae* MetH orthologs in the same intracellular environment. Unlike *E. coli*, wild-type *V. cholerae* cannot use [Ade]Cba for MetH-dependent growth (12). Our results suggest this is due to its poor use by MetH, as the EC_{50} for [Ade]Cba was nearly 300-fold higher for *E. coli* expressing *V. cholerae metH* compared to its native *metH* (Fig. 1F-G, Table S1). Overexpression of *E. coli metH* led to improved growth with the least preferred cobamides (Fig. 1E-F). This suggests MetH is limiting for *E. coli* growth with certain cobamides including [Ade]Cba, and is consistent with our previous observation that mutation of the regulator *metR* or the *metH* 5′ untranslated region improved growth of *E. coli* with [Ade]Cba (22).

Cobamide requirements in bacteria and microalgae span orders of magnitude and correspond to environment

Comparison of these results to other studies that evaluated cobamide-dependent growth demonstrated that taxonomically diverse microbes from different environments are variable in their relative cobamide preferences (Fig. 2). Further, the lowest EC_{50} values for most soil bacteria

126 and microalgae are 1-2 orders of magnitude lower than those for human gut commensal bacteria
127 (Fig. 2), suggesting the former are adapted to survive at much lower cobamide concentrations. If
128 EC_{50} values are indicative of cobamide concentrations in these environments, the bioavailable
129 cobamide concentration can be estimated as 0.1 to 10 pM in aquatic environments, 1-10 pM in
130 soil, and 10-100 pM in the human gut. Consistent with these values, Cbl has been detected at pM
131 concentrations in aquatic environments (23). 41 nM cobamide has been detected in soil, though
132 soil's robust ability to adsorb cobamides suggests not all may be bioavailable (17). Cobamide
133 concentrations in the gut regions that harbor the bacteria in this study have not been measured.

134 Together, these results demonstrate that cobamide preferences of enzymes in the same organism
135 and of cobamide-dependent growth across taxonomically diverse microbes are variable.
136 Furthermore, cobamide concentrations required for growth vary by orders of magnitude across
137 environments, suggesting microbes are adapted to cobamide levels in their environment, likely by
138 tuning the sensitivity of cobamide uptake, adenosylation, regulation, and use by cobamide-
139 dependent enzymes. The characterization of cobamide abundances, bioavailability, requirements,
140 and preferences are therefore necessary to understand the role of cobamide metabolism within
141 microbial communities.

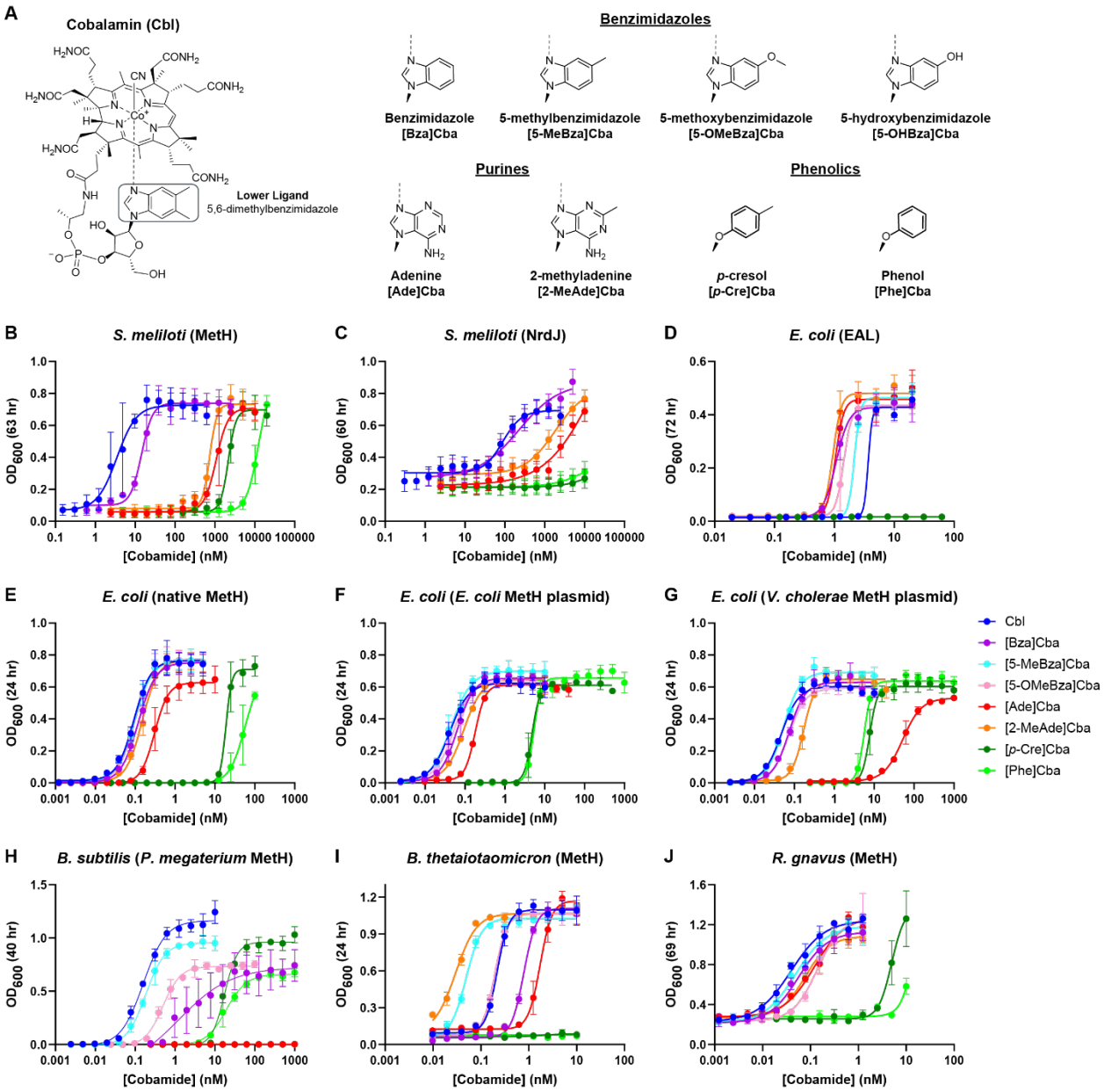

Figure 1. Cobamide structures and cobamide-dependent growth. A) Structure of B_{12} (cobalamin), which contains the lower ligand 5,6-dimethylbenzimidazole, is shown in the base-on conformation in which the ring nitrogen is coordinated to the cobalt ion (dashed line) (left). Alternative lower ligands of cobamides in this study (right) are shown with the names of the cobamides given below each structure. (B-J) Cobamide dose-dependent growth assays showing OD600 measured at the indicated times for (B) MetH-dependent growth of *S. meliloti*, (C) NrdJ-dependent growth of *S. meliloti*, (D) EAL-dependent growth of *E. coli*, (E) MetH-dependent growth of *E. coli*, (F) MetH-dependent growth of *E. coli* expressing *E. coli metH* on a plasmid, (G) MetH-dependent growth of *E. coli* expressing *V. cholerae metH* on a plasmid, (H) MetH-dependent growth of *B. subtilis* expressing *P. megaterium metH*, (I) MetH-dependent growth of *B. thetaiotaomicron*, and (J) MetH-dependent growth of *R. gnavus.* The EC_{50} values calculated from the curves in B-J and genotypes of the engineered strains in B-H are shown in Table S1. OD_{600} values for [Bza]Cba-supplemented cultures of *B. subtilis* were recorded after 72 hr because growth was not observed until after 45 hr. Points represent the means of 3-6 biological replicates; error bars represent standard deviation.

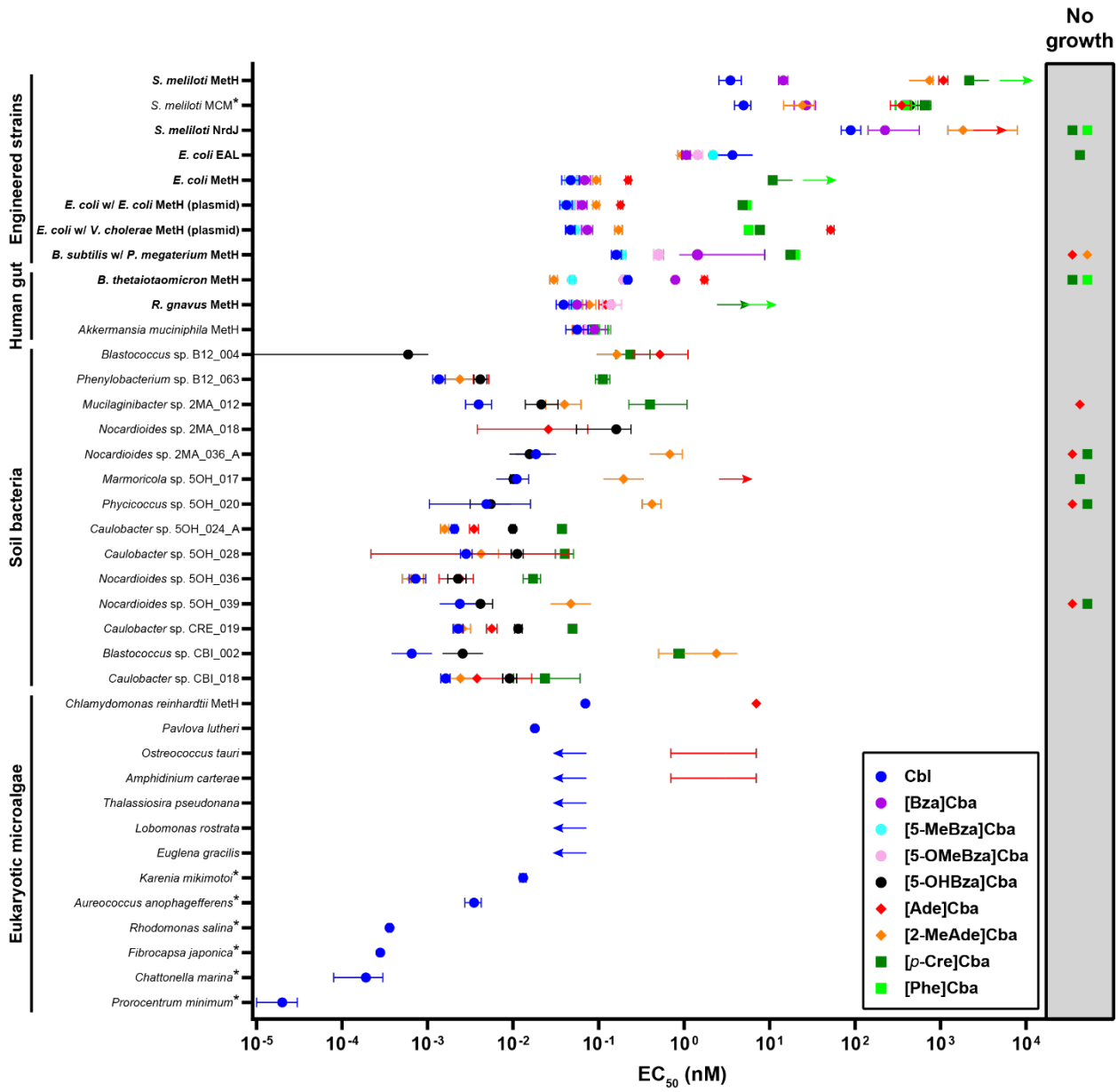

Figure 2. Comparison of EC_{50} values for cobamide-dependent growth. Organisms examined in the current study (bold) are compared with those from previous studies (8, 13, 21, 24, 25). *S. meliloti* Rm1021 Δ*nrdJ cobD*::*gus* Gm^R *metH*::Tn*5* pMSO3-*nrdAB*(*E. coli*) was used for *S. meliloti* MCM-dependent growth (8). EC_{50} values for *S. meliloti* are higher than for the other tested microbes likely because wild-type *S. meliloti* synthesizes Cbl *de novo* and lacks a high-affinity cobamide uptake system. MetH-dependent growth of *C. reinhardtii* was tested in a *metE* mutant (13). Symbols show the mean EC_{50} values. Capped bars represent 95% confidence intervals, except with organisms labeled with *, which indicates error as standard deviation. Error of *C. reinhardtii* and *P. lutheri* EC_{50} values were not reported (13). Bars are uncapped on the left or right when lower or upper bounds for 95% confidence intervals could not be determined, respectively. The lower bound for *Blastococcus* sp. B12_004 grown with [5-OHBza]Cba is 10^{-7} nM (25). The base of the leftward and rightward arrows represents maximal and minimal concentrations for EC_{50} from dose-response assays in which lack of growth or saturating growth was not reached, respectively. For *O. tauri* and *A. carterae*, EC_{50} values could not be calculated, but the capped bars for [Ade]Cba show the upper and lower bounds (13). Symbols in the shaded region on the right represent cobamides that were unable to support growth at any concentration tested.

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	Cbl	[Bza]Cba	[5-MeBza]Cba	[5-OMeBza]Cba	[Ade]Cba	[2-MeAde]Cba	[*p*-Cre]Cba	[Phe]Cba
***S. meliloti* (MetH)** (Δ*nrdJ cobD*::*gus bhbA*::Tn*5* pMSO3-*nrdAB*(*E. coli*))	3.481 (2.548 - 4.672)	14.47 (12.76 - 16.32)			1077 (949 - 1216)	740.4 (< 808.5)	2159 (> 1971)	> 5000
***S. meliloti* (NrdJ)** (Δ*metH cobD*::*gus, bhbA*::Tn*5*)	88.69 (68.58 - 116.2)	223.2 (142.3 - 564.3)			> 2500	1830 (1223 - 7914)	NG	NG
***E. coli* (EAL)** (Δ*metH*)	3.676 (ND)	1.069 (0.9621 - 1.182)	2.176 (> 2.006)	1.442 (< 1.634)	1.042 (> 0.937)	0.946 (> 0.847)	NG	
***E. coli* (native MetH)** (Δ*metE*)	0.047 (0.037 - 0.059)	0.069 (0.060 - 0.080)	0.048 (0.040 - 0.056)	0.058 (0.050 - 0.066)	0.222 (0.208 - 0.237)	0.094 (0.085 - 0.105)	10.87 (> 10.01)	> 25
***E. coli* (*E. coli* MetH plasmid)** (Δ*metE* Δ*metH* pETmini-*metH*(*E. coli*)	0.042 (0.035 - 0.049)	0.064 (0.057 - 0.073)	0.044 (0.037 - 0.052)	0.052 (0.046 - 0.059)	0.180 (0.168 - 0.193)	0.094 (0.086 - 0.102)	4.816 (4.414 - 5.270)	5.382 (4.723 - 6.113)
***E. coli* (*V. cholerae* MetH plasmid)** (Δ*metE* Δ*metH* pETmini-*metH*(*V. cholerae*)	0.047 (0.041 - 0.053)	0.074 (0.063 - 0.085)	0.053 (0.048 - 0.059)	0.072 (0.065 - 0.080)	51.75 (47.11 - 56.92)	0.171 (0.154 - 0.188)	7.715 (7.020 - 8.568)	5.672 (5.206 - 6.177)
***B. subtilis* (*P. megaterium* MetH)** (Δ*queG*::Pveg-*btuFCDR* Δ*metE*::*loxP* *amyE*::Pveg-*metH*(*P. megaterium*))	0.161 (0.141 - 0.184)	1.273 (< 6.495)	0.187 (0.169 - 0.208)	0.496 (0.441 - 0.557)	NG	NG	17.49 (15.99 - 19.21)	19.18 (16.52 - 22.35)
***B. thetaiotaomicron* (MetH)**	0.219 (0.205 - 0.234)	0.788 (0.755 - 0.823)	0.049 (0.046 - 0.053)	0.195 (0.184 - 0.207)	1.728 (1.604 - 1.861)	0.030 (0.027 - 0.033)	NG	NG
***R. gnavus* (MetH)**	0.039 (0.032 - 0.048)	0.056 (0.045 - 0.072)	0.049 (0.039 - 0.063)	0.139 (0.112 - 0.186)	0.121 (0.101 - 0.147)	0.078 (0.065 - 0.093)	> 2.5	> 5

Table S1. EC_{50} values calculated from data in Figure 1 B-J. EC_{50} values (nM) and 95% confidence intervals (in parentheses) are given for each growth condition shown in Figure 1B-J (four-parameter non-linear fit in GraphPad Prism (v9.5.1)). Genotypes are given for engineered strains. NG, no growth; empty, not measured. A greater than (>) value for EC_{50} is an estimate for cultures that failed to reach saturation at any cobamide concentration tested. Greater than (>) and less than (<) symbols in confidence intervals were used when upper or lower bounds could not be determined, respectively; ND represents a confidence interval in which both upper and lower bounds could not be determined.