Flexible cobamide metabolism in *Clostridioides* (*Clostridium) difficile* 630 Δ*erm*

Amanda N. Shelton[1], Xun Lyu[2], Michiko E. Taga[1#]

[1]Department of Plant & Microbial Biology and [2]Department of Molecular & Cell Biology, University of California, Berkeley, Berkeley, CA 94720

#Correspondence to taga@berkeley.edu

Running title: B_{12} metabolism in *C. difficile*

Abstract

Clostridioides (Clostridium) difficile is an opportunistic pathogen known for its ability to colonize the human gut under conditions of dysbiosis. Several aspects of its carbon and amino acid metabolism have been investigated, but its cobamide (vitamin B_{12} and related cofactors) metabolism remains largely unexplored. *C. difficile* has seven predicted cobamide-dependent metabolisms encoded in its genome in addition to a nearly complete cobamide biosynthesis pathway and a cobamide uptake system. To address the importance of cobamides to *C. difficile*, we studied *C. difficile* 630 Δ*erm* and mutant derivatives under cobamide-dependent conditions *in vitro*. Our results show that *C. difficile* can use a surprisingly diverse array of cobamides for methionine and deoxyribonucleotide synthesis, and can use alternative metabolites or enzymes, respectively, to bypass these cobamide-dependent processes. *C. difficile* 630 Δ*erm* produces the cobamide pseudocobalamin when provided the early precursor 5-aminolevulinc acid or the late intermediate cobinamide, and produces other cobamides if provided an alternative lower ligand. The ability of *C. difficile* 630 Δ*erm* to take up cobamides and Cbi at micromolar or lower concentrations requires the transporter BtuFCD. Genomic analysis revealed genetic variations in in the *btuFCD* locus of different *C. difficile* strains, which may result in differences in the ability to take up cobamides and Cbi. These results together demonstrate that, like other aspects of its physiology, cobamide metabolism in *C. difficile* is versatile.

Importance

The ability of the opportunistic pathogen *Clostridioides difficile* to cause disease is closely linked to its propensity to adapt to conditions created by dysbiosis of the human gut microbiota. The cobamide (vitamin B_{12}) metabolism of *C. difficile* has been underexplored, though it has seven

metabolic pathways that are predicted to require cobamide-dependent enzymes. Here, we show that *C. difficile* cobamide metabolism is versatile, as it can use a surprisingly wide variety of cobamides and has alternative functions that can bypass some of its cobamide requirements. Furthermore, *C. difficile* does not synthesize cobamides *de novo*, but produces them when given cobamide precursors. Better understanding of *C. difficile* cobamide metabolism may lead to new strategies to treat and prevent *C. difficile*-associated disease.

40 **Introduction**

41 The human gut microbiota is a complex community composed of hundreds to thousands
42 of species of bacteria, archaea, and eukaryotic microbes (1). Members of this community
43 compete for nutrients such as carbon sources, but also release metabolites that benefit other
44 members. The exchange of B vitamins, particularly vitamin B_{12}, is thought to be prevalent in
45 many environments because most bacteria lack the ability to synthesize some of the cofactors
46 they require for enzyme catalysis (2–6), and instead must acquire them from other organisms (7).
47 Such nutrient cross-feeding interactions can influence bacterial metabolism in ways that can
48 affect not only the microbiota, but also host health (8, 9).

49 *Clostridioides (Clostridium) difficile* is a human intestinal pathogen that is among the
50 most common causes of nosocomial infections, with nearly 300,000 healthcare-associated cases
51 per year in the United States (10). *C. difficile* colonization of the gut is correlated with dysbiosis
52 of the gut microbiota (11). Its abilities to germinate from spores, proliferate in the gut, and cause
53 disease are impacted both positively and negatively by ecological and metabolic factors (12–14).
54 The global alteration of the gut metabolome following antibiotic treatment is correlated with
55 increased susceptibility to *C. difficile* infection, and recent work has linked changes in the
56 relative abundance of specific metabolites to changes in the microbiome using model systems
57 (11, 15–17). For example, succinate availability increases after disturbance of the microbiota,
58 allowing *C. difficile* expansion in a mouse model (18). Additionally, specific commensal bacteria
59 have been shown to produce compounds that stimulate *C. difficile* metabolism. In a bi-
60 association, *Bacteroides thetaiotaomicron* can break down host mucin and produce sialic acid,
61 which can be used by *C. difficile* for expansion in the gut (19). *C. difficile* can also induce other

62 members of the microbiota to produce indole, which is thought to create a more favorable
63 environment for the pathogen by inhibiting competing microbes (20).
64 Some interactions with microbiota members have also been shown to be inhibitory to *C.*
65 *difficile*. Co-culturing with certain *Bifidobacterium* spp. on particular carbon sources reduces *C.*
66 *difficile* toxin production relative to monoculture (21). While primary bile acids produced by the
67 host promote *C. difficile* spore germination, *Clostridium scindens* and other 7α-dehydroxylating
68 Clostridia transform these compounds into secondary bile acids, which are inhibitory to *C.*
69 *difficile* (22, 23). The latter example illustrates that compounds in the same class can have
70 different effects on the disease state. Given the complexity of metabolic interactions in the
71 mammalian gut, many additional microbial metabolites likely influence the ability of *C. difficile*
72 to colonize and persist in the gut.
73 One class of metabolites that has not been explored for its ability to affect *C. difficile*
74 growth and virulence is cobamides, the vitamin B_{12} (also called cobalamin) family of cofactors.
75 Cobamides are used in diverse microbial metabolisms including methionine synthesis,
76 deoxyribonucleotide synthesis, acetogenesis, and some carbon catabolism pathways. These
77 reactions are facilitated by fission of the Co-C bond to the cobamide upper ligand, which can be
78 a 5'-deoxyadenosyl group for radical reactions, a methyl group for methyltransferase reactions,
79 or a cyano group in the inactive vitamin form (24) (labeled as "R" in Fig. 1A). Over 80% of all
80 sequenced bacteria (25–27) and 80% of sequenced human gut bacteria (2, 28, 29) have one or
81 more cobamide-dependent enzymes, suggesting that cobamides are widely used cofactors across
82 microbial ecosystems. Strikingly, fewer than 40% of bacterial species are predicted to produce
83 cobamides *de novo* (2, 25–28), and therefore over half of bacteria that use cobamides must
84 acquire them from their environment. Cobamides vary in the structure of the lower ligand (Fig.

85 1A, B), and organisms studied to date are selective in which cobamides they can use (28, 30–37).
86 Seven cobamides in addition to the cobamide precursor cobinamide (Cbi, Fig. 1C) have been
87 detected in the human gut (38). In an environment with plentiful, diverse cobamides and
88 cobamide precursors, a microbial species that requires a particular cobamide can either import
89 that cobamide, synthesize it *de novo,* chemically remodel available cobamides to the preferred
90 structure, or alter its need for the cobamide by using alternative pathways (8, 39).

91 The seven predicted cobamide-dependent enzymes encoded in the *C. difficile* genome are
92 involved in methionine synthesis, nucleotide metabolism, and carbon metabolism (Fig. 2). When
93 grown with amino acids and glucose as carbon and energy sources *in vitro*, *C. difficile* does not
94 require cobalamin supplementation (40). However, in model infection systems, cobamide-
95 dependent metabolism may be important for virulence and growth. For example, access to
96 ethanolamine catabolism may be important in modulating virulence, as deletion of EutA, the
97 reactivating factor required for activity of the cobamide-dependent ethanolamine ammonia lyase
98 (EutBC), in *C. difficile* strain 630 Δ*erm* reduces the mean time to morbidity in a hamster model
99 (41). Additionally, metabolic models and transcriptomics (42, 43) suggest that the cobamide-
100 dependent Wood-Ljungdahl carbon fixation pathway is an important electron sink, and an
101 experimental study suggests that it may be used for autotrophic growth by some *C. difficile*
102 strains (44).

103 The observation that *C. difficile* can grow without added cobamides *in vitro* (40) suggests
104 that it may not require cobamides under those conditions, or that it can biosynthesize cobamides.
105 However, all sequenced strains of *C. difficile* are missing *hemA* and *hemL*, the first two enzymes
106 in the cobamide biosynthesis pathway required for production of the precursor 5-aminolevulinic
107 acid (ALA) (45) (Fig. 1C). Therefore, *C. difficile* is predicted to be able to produce a cobamide

108 only when ALA is available, as has been observed in three other bacteria (25) (Fig. 2). In order
109 to use cobamide-dependent metabolisms, we predict that *C. difficile* requires cobamides or
110 precursors such as ALA from the gut. While ALA is an intermediate made in all tetrapyrrole-
111 producing organisms, including the host, cobamides are only produced by some bacteria and
112 archaea (46).

113 To address the importance of cobamides for *C. difficile* metabolism and to understand
114 how *C. difficile* acquires cobamides, we examined *C. difficile* 630 Δ*erm* and mutant derivatives
115 *in vitro* under cobamide-dependent conditions. We found that the bacterium can use a
116 surprisingly diverse array of cobamides for methionine and deoxyribonucleotide synthesis, and
117 can use alternative nutrient sources or enzymes to fulfill its metabolic needs. In addition to
118 importing and using a variety of cobamides, when provided with ALA or the late intermediate
119 Cbi, *C. difficile* 630 Δ*erm* can produce the cobamide pseudocobalamin, and can produce other
120 cobamides if provided an alternative lower ligand. Together, these results show that *C. difficile* is
121 versatile in its cobamide metabolism.

122

123

124 **Results**

125 ***C. difficile* requires methionine or a cobamide for growth**

126 To investigate cobamide-dependent metabolism in the model *C. difficile* strain 630 Δ*erm*,
127 we sought to culture the organism in conditions that require specific cobamide-dependent
128 enzymes. The *C. difficile* genome encodes the cobalamin-dependent methionine synthase MetH,
129 but does not contain the cobalamin-independent alternative enzyme MetE. The absence of a
130 complete cobamide biosynthesis pathway suggests that *C. difficile* requires either methionine or

131 a cobamide in its growth medium. Previously, methionine was classified as a "growth-
132 enhancing," but not essential, amino acid in a medium containing cyanocobalamin (vitamin B_{12})
133 for seven of eight strains tested (40, 47). To test whether *C. difficile* can use cobamides for
134 methionine synthesis and to identify the specific cobamides that support its MetH-dependent
135 growth, we cultured *C. difficile* in a defined medium lacking methionine with a range of
136 concentrations of cyanocobalamin, Cbi, and eight other cyanylated cobamides that we purified.
137 *C. difficile* was unable to grow in this medium without cobamide or methionine addition (Fig.
138 3A), suggesting that, as predicted, it cannot produce cobamides *de novo* to support the activity of
139 MetH. Remarkably, unlike other bacteria that have been reported to use a limited number of
140 cobamides for methionine synthase activity (28, 48, 49), all of the cobamides and Cbi were able
141 to confer high growth yields to *C. difficile* at concentrations as low as 1 nM (Fig. 3A).
142 Methionine addition also supported growth, though higher concentrations were required than for
143 cobamides (Fig. 3B). We also observed robust growth with addition of ALA (Fig. 3C).
144

145 ***C. difficile* growth with ribonucleotide reductase NrdJ requires a more restricted set of**
146 **cobamides**

147 *C. difficile* genomes encode homologs of the cobalamin-dependent (class II)
148 ribonucleotide reductase (RNR) (*nrdJ,* CDIF630erm_RS07280), as well as two cobalamin-
149 independent RNRs: an oxygen-dependent (class I) RNR (encoded by *nrdE*,
150 CDIF630erm_RS16325, and *nrdF*, CDIF630erm_RS16320) and an oxygen-sensitive (class III)
151 RNR (*nrdD*, CDIF630erm_RS00990 and *nrdG*, CDIF630erm_RS00995). In principle, any of
152 these three isozymes could be used for deoxyribonucleotide synthesis from ribonucleotides,
153 although under anaerobic conditions only the class II and class III RNRs are expected to

154 function. Cobamide addition is not required for anaerobic growth of the parent strain *C. difficile*
155 630 Δ*erm* Δ*pyrE* in a casamino acid medium (CDDM) with glucose, and adding cobamides or
156 cobamide precursors did not affect growth yield (Supplemental Fig. 1), suggesting that the class
157 III RNR, NrdDG, is functional under these conditions. To test whether the class II RNR, NrdJ, is
158 functional, we deleted the *nrdD* and *nrdG* genes while providing exogenous cobalamin, using the
159 allelic exchange system in a Δ*pyrE* background (50). This strain could grow only with cobalamin
160 addition, suggesting that NrdJ is functional and NrdEF is not under these growth conditions (Fig.
161 4A). To determine which cobamides it requires, the Δ*nrdDG* strain was grown with the same
162 cobamides and precursors as in Fig. 3. In contrast to MetH, NrdJ is more selective in the
163 cobamides it can use (Fig. 4A), as expected based on studies with other class II RNRs (33, 36,
164 51, 52). There was little growth with [Cre]Cba, [Phe]Cba, and [5-OHBza]Cba (Fig. 4A).
165 Addition of ALA also supported NrdJ-dependent growth (Fig. 4B).
166
167 ***C. difficile* produces pseudocobalamin from the precursor ALA via the *cbi* genes**
168 The observation that *C. difficile* could grow under cobamide-dependent conditions with
169 ALA or Cbi (Fig. 3A, C, Fig. 4) suggests that it can produce a cobamide from these precursors
170 using the cobamide biosynthetic genes encoded in its genome (25). To test this prediction, the
171 corrinoid fraction, which includes cobamides and late cobamide precursors including Cbi, was
172 extracted from the cell pellets of *C. difficile* 630 Δ*erm* grown with either ALA or Cbi. Consistent
173 with our predictions, HPLC analysis of the extracted corrinoids showed that *C. difficile* produced
174 a cobamide only when ALA or Cbi was added (Fig. 5A). Additionally, as predicted, corrinoid
175 analysis of a strain lacking the corrin ring biosynthesis genes *cbiKLJHGFTEDC* demonstrated
176 that these genes are necessary for cobamide synthesis from ALA, but not Cbi (Fig. 5A). Because

177 *C. difficile* lacks all known genes for biosynthesis of benzimidazoles and attachment of phenolic
178 lower ligands, it is predicted to be incapable of producing benzimidazolyl or phenolyl
179 cobamides, but may produce a purinyl cobamide (49, 53–59). Indeed, the major cobamide
180 present in *C. difficile* corrinoid extracts co-eluted with the purinyl cobamide pseudocobalamin
181 (Fig. 5A). The UV-Vis spectrum of the major cobamide was consistent with a pseudocobalamin
182 standard (Supplemental Fig. 2C). Mass spectrometry analysis verified that the major cobamide
183 extracted from cultures grown with ALA is pseudocobalamin (Supplemental Fig. 2A, B).
184

185 ***C. difficile* can perform guided biosynthesis but does not remodel cobamides**

186 Some bacteria can perform guided biosynthesis, a process in which an exogenously
187 provided, non-native lower ligand base is incorporated into a cobamide (32, 36, 48, 60, 61). To
188 test if *C. difficile* is capable of guided biosynthesis to produce cobamides other than its native
189 pseudocobalamin, either DMB (the lower ligand of cobalamin, Fig. 1A) or a related compound,
190 benzimidazole (Bza, Fig. 1B) was added to cultures containing either ALA or Cbi. Analysis of
191 corrinoid extracts showed that *C. difficile* could attach either of these exogenous lower ligands to
192 form cobalamin and [Bza]Cba, respectively, with both precursors (Fig. 5B). A small amount of
193 pseudocobalamin was also recovered in cultures containing Cbi with Bza (Fig. 5B).

194 Some bacteria and archaea are able to remodel cobamides by removing the lower ligand
195 and nucleotide loop with the amidohydrolase enzyme CbiZ and rebuilding the cobamide with a
196 different lower ligand (31, 62–64). We were unable to identify a *cbiZ* homolog in the *C. difficile*
197 genome, and accordingly, we did not observe evidence of remodeling; when cobalamin, [2-
198 MeAde]Cba, or [Cre]Cba was provided to *C. difficile*, the same cobamides were recovered from
199 the cells (Fig. 6A).

200

201 ***C. difficile* requires *btuFCD* for efficient uptake of cobamides and Cbi**

202 The presence of cobamides in the cellular fraction of cultures grown with either Cbi or a
203 cobamide at nanomolar concentrations (Fig. 5, 6A) suggested that *C. difficile* takes up Cbi and
204 cobamides via an active transporter. We identified a candidate cobalamin uptake operon
205 (*btuFCD*) downstream of a sequence annotated as a cobalamin riboswitch, suggesting that these
206 genes function in corrinoid import (27, 28, 65–70). No corrinoids could be detected in the
207 cellular fraction of the Δ*btuFCD* mutant grown with 10 nM Cbi or cobalamin (Fig. 6A). In
208 contrast, ALA uptake is apparently unaffected in the Δ*btuFCD* mutant, as pseudocobalamin can
209 be recovered from the cellular fraction when ALA is provided (Fig. 6A). Furthermore, the
210 Δ*btuFCD* mutant grew poorly in methionine-free medium even when Cbi or cobalamin was
211 added at concentrations 10^3 to 10^4-fold higher than required for growth of the parental strain
212 (Fig. 6B). The ability of methionine or ALA to support growth remained unaffected by the
213 Δ*btuFCD* mutation (Fig. 6B). Interestingly, genomic analysis identified strains of *C. difficile* that
214 contain a *tlpB* transposon insertion in *btuC*, likely rendering the BtuFCD transporter
215 nonfunctional (Supplemental Fig. 3) (71). Of the genomes analyzed, the *tlpB* insertion in this
216 locus appears to be restricted to strains in the PCR-ribotype 027 (RT027) clade, including the
217 hypervirulent strain R20291, based on a multi-locus sequence typing (MLST) tree of *C. difficile*
218 strains (Supplemental Fig. 3, red labels). This observation suggests that unlike strain 630 Δ*erm*
219 examined in this study, members of the RT027 clade may be unable to take up cobamides and
220 Cbi efficiently.

221

222

223 **Discussion**

224 The potential of *C. difficile* to cause disease is closely linked to its ability to fill
225 ecological niches made available by gut microbiota dysbiosis (13), using a suite of metabolic
226 pathways to make use of newly available nutrient sources. *C. difficile* has an unusually high
227 number of cobamide-dependent metabolisms encoded in its genome (25), but their functions
228 have been underexplored. Here, we show that *C. difficile* is able to use many cobamides and
229 cobamide precursors in two of its seven cobamide-dependent pathways. The promiscuous use of
230 cobamides and the ability to bypass these cobamide-dependent metabolisms highlights the
231 metabolic flexibility of *C. difficile.*

232 The cobalamin-dependent methionine synthase, MetH, is the most abundant cobamide-
233 dependent enzyme in bacterial genomes (25) and is found in numerous organisms in all three
234 domains of life, including humans (24). Compared to the majority of other MetH homologs that
235 have been studied, our results indicate that the *C. difficile* MetH homolog is unusually
236 promiscuous in its cobamide selectivity. For example, several eukaryotic algae grew robustly
237 under MetH-dependent conditions with cobalamin, but did not grow with pseudocobalamin at
238 the same concentrations (33). The human gut commensal bacterium *Bacteroides*
239 *thetaiotaomicron* could use benzimidazolyl and purinyl cobamides for MetH-dependent growth,
240 but could not use phenolyl cobamides (28). An example of MetH selectivity *in vitro* was in
241 *Spirulina platensis,* where the purified enzyme bound its native cobamide, pseudocobalamin,
242 with a higher affinity than cobalamin (72). An exception to this observed selectivity is another
243 gut pathogen, *Salmonella enterica*, which can use its native cobamide, pseudocobalamin, in
244 addition to cobalamin, [Phe]Cba, and [Cre]Cba for MetH-dependent growth, although other

245 cobamides were not tested (48, 49). The versatility of *C. difficile*'s cobamide use is notable given
246 the diversity of cobamides that have been detected in the gut (38).
247 In contrast to MetH, our growth experiments indicate that the selectivity of *C. difficile*'s
248 NrdJ is more similar to that of other organisms that rely on NrdJ for growth. For example,
249 *Sinorhizobium meliloti* was unable to grow with [Cre]Cba and grew poorly with
250 pseudocobalamin relative to its native cobamide, cobalamin (36); *Lactobacillus leichmannii*
251 could only use benzimidazolyl or purinyl cobamides (51); and *Euglena gracilis* grew well with
252 cobalamin and [Bza]Cba and poorly with pseudocobalamin, [5-OHBza]Cba, and [Cre]Cba (33,
253 52). Unlike MetH, the NrdJ enzyme requires cobamides that can adopt the "base-on"
254 configuration in which the lower ligand base is coordinated to the cobalt ion throughout the
255 catalytic cycle (24). Phenolyl cobamides are unable to adopt the base-on configuration, so their
256 inability to support growth in the NrdJ-dependent condition was expected. *C. difficile* 630 Δ*erm*
257 also contains an active class III cobamide-independent RNR, NrdDG, which may be an
258 important strategy to maintain deoxyribonucleotide synthesis when cobamides are scarce.
259 However, in other species, under certain conditions the class II RNR provides an advantage over
260 other RNR classes, such as during oxidative stress (73), although the conditions where NrdJ
261 would provide an advantage for *C. difficile* have yet to be uncovered.
262 Seven different cobamides and the precursor Cbi have been detected in human feces (38).
263 In stool samples of individuals not taking cobalamin supplements, the average total corrinoid
264 present is approximately 1300 ng per gram feces, roughly equivalent to 1 μM (38). Cbi is found
265 at tens of ng per gram (38). Growth experiments under MetH and NrdJ-dependent conditions
266 showed that *C. difficile* 630 Δ*erm* reached maximum growth yield with as little as 1 nM
267 cobamide or Cbi (Fig 3, 4). Based on the absence of corrinoids in the cellular fraction of a 630

268 Δ*erm* Δ*pyrE* Δ*btuFCD* strain (Fig. 6), we infer that strains with an insertion in *btuC*
269 (Supplemental Fig. 3), including the hypervirulent R20291 and CD196 strains (71), would
270 require cobamides or Cbi at extracellular concentrations higher than 100 μM if relying on
271 cobamide-dependent enzymes. This suggests that these strains may not be able to use the
272 cobamides or Cbi present in the gut.

273 Our results show that not only is *C. difficile* able to use multiple cobamides to support its
274 metabolism, but it can also use the early precursor ALA to produce pseudocobalamin. The ability
275 to use ALA to produce a cobamide, and thus not strictly rely on cobamide or Cbi uptake, could
276 be important to strains with a transposon insertion in the *btuC* gene (70, 74). ALA concentrations
277 in the human gut have not been reported. However, we speculate that, similar to cobamides and
278 Cbi, ALA and possibly other early cobamide precursors could be provided by other members of
279 the microbiota. Alternatively, ALA could be provided by the host either through the diet or via
280 biosynthesis of heme, which also uses ALA as a precursor. Other commensal gut microbiota
281 have been reported to be able to salvage ALA (25), suggesting that ALA could be available in
282 the gut.

283 *C. difficile* is also able to incorporate non-native lower ligands to form benzimidazolyl
284 cobamides (guided biosynthesis). Free benzimidazole bases have been found in animal
285 gastrointestinal tracts such as rumen fluid and termite guts (75), but benzimidazole levels in the
286 human gut have not been measured. The cobamides used by *C. difficile* could therefore also vary
287 with the presence of different benzimidazole-producing organisms in the microbiota. Our results
288 show that pseudocobalamin and most benzimidazolyl cobamides support growth of *C. difficile*
289 equally for the two metabolisms we investigated in this study, but the cobamide preferences of
290 the other five cobamide-dependent metabolisms have not been investigated.

291 We have identified cobamides and precursors that *C. difficile* can use *in vitro,* but which
292 cobamides or cobamide precursors it predominantly uses in the gut remains to be discovered.
293 Evidence from transcriptomics is ambiguous with respect to expression of genes encoding
294 cobamide-dependent enzymes or cobamide biosynthesis during infection, likely due to
295 differences in study design (15, 43, 76–78). Since both diet and the microbiota can contribute to
296 the cobamide profile in the gut (38, 79, 80), the availability of cobamides may vary significantly
297 across infection systems and affect the expression and use of cobamide biosynthesis and
298 cobamide-dependent pathways by *C. difficile*. In one study, *hemB*, which encodes the enzyme
299 that converts ALA to the next intermediate, porphobilinogen, was among the most highly
300 expressed genes in *C. difficile* strain VPI 104363 in a mouse model (43), suggesting that *C.*
301 *difficile* produces cobamides from ALA in the gut. How the cobamide content in the gut
302 environment changes during *C. difficile* infection is unknown, but since much of the cobamide
303 content in the lower gastrointestinal tract is produced by resident gut microbes (79, 80), it is
304 possible that cobamide abundances change during dysbiosis. Further *in vivo* studies are needed to
305 determine the extent to which cobamide metabolism is important to *C. difficile* associated
306 disease.
307
308
309 **Materials and Methods**
310 **Bacterial strains and growth conditions**
311 *C. difficile* 630 Δ*erm*, an erythromycin-sensitive derivative of the isolate 630 (81), and *C.*
312 *difficile* 630 Δ*erm* Δ*pyrE*, a derivative of 630 Δ*erm* with a uracil auxotrophy (50), were streaked
313 from frozen stocks onto BHIS agar (82) before being transferred to *Clostridium difficile* defined

314 medium (CDDM) containing casamino acids (83) and 8 g/L glucose. Agar plates and 96-well
315 plates containing liquid cultures were incubated at 37ºC in an anaerobic chamber (Coy Labs)
316 containing 10% H_2, 10% CO_2, and 80% N_2. For *C. difficile* 630 Δ*erm* Δ*pyrE* and derived strains,
317 5 µg/ml uracil was included in all defined media. For corrinoid extractions and NrdJ phenotype
318 experiments, strains were cultured in CDDM plus 8 g/L glucose. For MetH phenotype
319 experiments, CDDMK medium plus 8 g/L glucose without methionine was used. CDDMK
320 contains the same salts, trace metals, and vitamins as CDDM, but the casamino acids, tryptophan
321 and cysteine are replaced with the individual amino acids as follows: 100 mg/L histidine, 100
322 mg/L tryptophan, 100 mg/L glycine, 100 mg/L tyrosine, 200 mg/L arginine, 200 mg/L
323 phenylalanine, 200 mg/L threonine, 200 mg/L alanine, 300 mg/L lysine, 300 mg/L serine, 300
324 mg/L valine, 300 mg/L isoleucine, 300 mg/L aspartic acid, 400 mg/L leucine, 500 mg/L
325 cysteine, 600 mg/L proline, 900 mg/L glutamic acid (40). All liquid defined media were
326 prepared by boiling under 80% N_2/20% CO_2 gas. After the pH stabilized between 6.8 and 7.2,
327 the medium was dispensed into stoppered tubes and autoclaved. Filter-sterilized glucose and
328 vitamins were added after autoclaving. Cultures in stoppered tubes were incubated at 37ºC.
329 For MetH phenotype assays, *C. difficile* 630 Δ*erm* was grown in CDDM, then washed
330 twice in CDDMK without methionine prior to inoculation in CDDMK at an optical density
331 ($O.D._{600}$) of 0.01 in a 96-well plate. For NrdJ phenotype assays, *C. difficile* 630 Δ*erm* Δ*pyrE*
332 Δ*nrdDG* was grown in CDDM with 5 µg/ml uracil and 10 nM cyanocobalamin, and washed
333 three times in CDDM medium without cyanocobalamin prior to inoculation in CDDM at an
334 $O.D._{600}$ of 0.01 in a 96 well plate. $O.D._{600}$ was measured on a BioTek Synergy 2 plate reader after
335 23 to 24 hours of growth.

336 ALA, Cbi and cyanocobalamin were purchased from Sigma Aldrich. Other cobamides
337 were purified from bacterial cultures as described in Men *et al*. (84)
338
339 **Strain and plasmid construction**
340 The allelic coupled exchange (ACE) system of Ng *et al*. was used for construction of *C.*
341 *difficile* mutant strains (50). Briefly, 500-1000 bp sequences flanking the target gene(s) (arms of
342 homology) in the *C. difficile* 630 Δ*erm* genome (CP016318,
343 https://www.ncbi.nlm.nih.gov/nuccore/CP016318.1/) were amplified by PCR (Supplemental
344 Table 1) and then were cloned into pMTL-YN3 (Chain Biotech) by Gibson assembly (85) in *E.*
345 *coli* XL1-Blue. Plasmid inserts were sequenced by Sanger sequencing before transformation of
346 the plasmid into *E. coli* CA434 (Chain Biotech). Conjugation of *E. coli* CA434 and *C. difficile*
347 630 Δ*erm* Δ*pyrE* was performed as described (86), except that *C. difficile* and *E. coli* were each
348 cultured for 5-8 hours prior to pelleting *E. coli* and mixing with the *C. difficile* recipient. After 16
349 hours growth on BHIS agar, the mixed cells were resuspended in 1 ml PBS, and 100 µl of the
350 suspension was plated on each of 5-7 plates of BHIS agar with added 10 µg/ml thiamphenicol,
351 250 µg/ml D-cycloserine, and 16 µg/ml cefoxitin. Colonies were purified at least twice by
352 streaking onto BHIS with 15 µg/ml thiamphenicol, 250 µg/ml D-cycloserine, and 16 µg/ml
353 cefoxitin, before counterselection on CDDM agar supplemented with 2 mg/L 5-fluoroorotic acid
354 (5-FOA) and 5 µg/ml uracil. The resulting colonies were purified by streaking at least twice on
355 the counterselection medium prior to screening by colony PCR for the deletion and the presence
356 of the *C. difficile* toxin gene *tcdB* (86). For the deletion of *nrdDG,* 10 nM cobalamin was added
357 to all media during the ACE procedure.
358
359 **Corrinoid extraction and analysis**

360 *C. difficile* was grown in 50 ml CDDM plus 8 g/L glucose under 80% N_2/20% CO_2
361 headspace for 16-22 hours at 37ºC prior to corrinoid extraction. Two cultures were combined for
362 each condition for a total volume of 100 ml for each extraction. Corrinoid extractions were
363 performed as described (31), except that cell pellets were autoclaved for 35 minutes and cooled
364 prior to addition of methanol and potassium cyanide. Two or more biological replicates were
365 performed for each condition.
366 High-performance liquid chromatography (HPLC) analysis was performed with an
367 Agilent Series 1200 system (Agilent Technologies, Santa Clara, CA) equipped with a diode array
368 detector with detection wavelengths set at 360 and 525 nm. For Fig. 5B and 6A samples were
369 injected onto an Agilent Zorbax SB-Aq column (5 µm, 4.6 × 150 mm) at 30°C, with 1 mL/min
370 flow rate. Compounds in the samples were separated with a gradient of 25 to 34% acidified
371 methanol in acidified water (containing 0.1% formic acid) over 11 min, followed by a 34 to 50%
372 gradient over 2 min, and 50 to 75% over 9 min. For Fig. 5A, samples were injected onto an
373 Agilent Eclipse Plus C18 column (5 µm, 9.6 × 250 mm) at 30 ºC, with 2 mL/min flow rate.
374 Compounds in the samples were separated with a gradient of 10 to 42% acidified methanol in
375 acidified water over 20 min. The amount of standards that were injected were as follows: Cbi (**1**),
376 200 pmol; pseudocobalamin (**2**), 225 pmol; cobalamin (**3**), 50 pmol; [Bza]Cba (**4**), 114 pmol; [2-
377 MeAde]Cba (**5**), 114 pmol; [Cre]Cba (**6**), 151 pmol. 5% to 20% by volume *C. difficile* samples
378 were injected.
379
380 **Supplemental material**
381 Supplemental methods, figures, and tables are provided.
382

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Author Contributions

A.N.S. performed growth experiments, corrinoid extractions, and phylogenetic analysis. X.L. and A.N.S. created the mutant strains. A.N.S. and M.E.T. wrote the manuscript.

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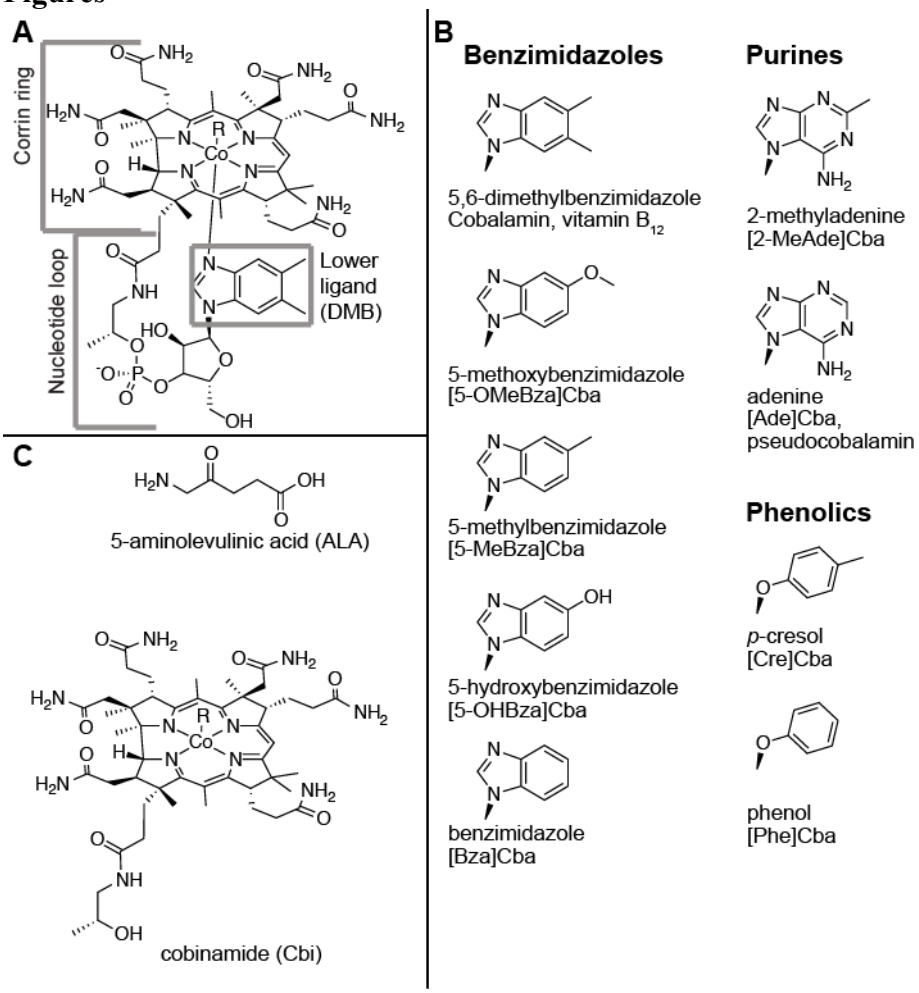
642 **Figures**



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645 **Figure 1. Structures of cobamides and cobamide precursors. A.** Structure of cobalamin (B_{12}). The
646 corrin ring, nucleotide loop, and lower ligand are labeled. **B.** Lower ligands of cobamides analyzed in this
647 study, with the three structural classes labeled. The lower ligand name, abbreviation for the cobamide
648 containing the lower ligand, and alternative names of the cobamide (when applicable) are indicated. **C**.
649 Cobamide precursors used in this study. R, upper ligand (-CN, -OH, $-CH_3$ or 5'-deoxyadenosyl).
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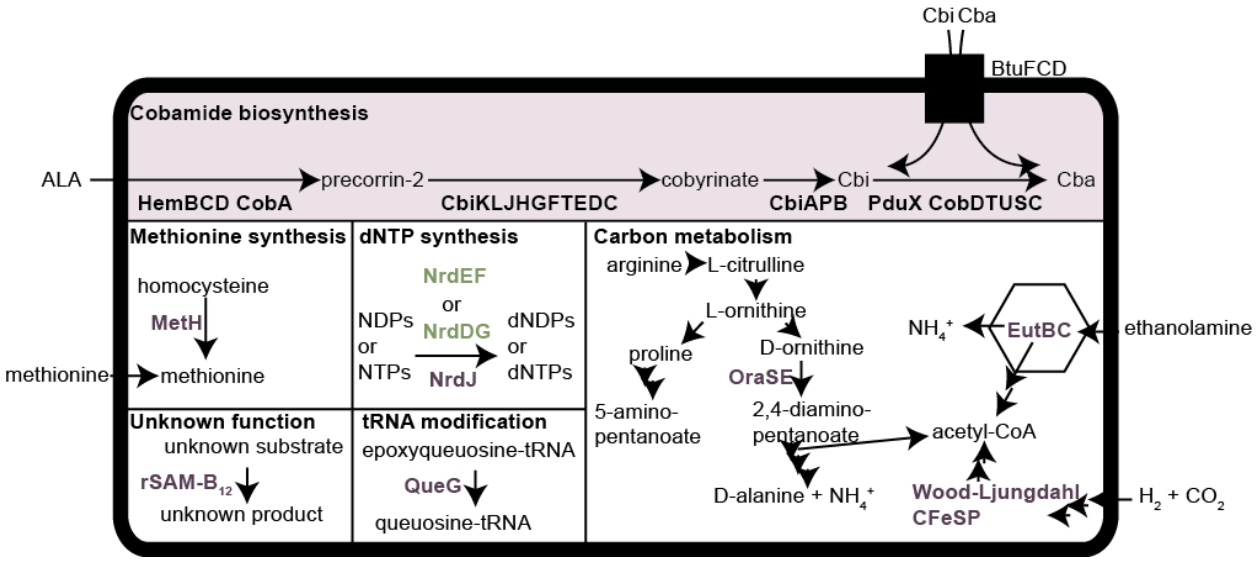

Figure 2. Predicted cobamide metabolism in *C. difficile* 630 Δ*erm*. The cobamide biosynthesis pathway is shown with a purple background, homologs of cobalamin-dependent enzymes in purple text, cobalamin-independent isozymes in green text, and the transporter BtuFCD as a black rectangle. Abbreviations: Cba, cobamide; Cbi, cobinamide; ALA, 5-aminolevulinic acid; rSAM, radical *S*-adenosylmethionine; NDPs, ribonucleoside diphosphates; NTPs, ribonucleoside triphosphates; dNDPs, deoxyribonucleoside diphosphates; dNTPs, deoxyribonucleoside triphosphates. Enzymes: MetH, cobalamin-dependent methionine synthase; NrdEF, cobalamin-independent, aerobic (oxygen-requiring, class I) ribonucleotide reductase (RNR); NrdDG, cobalamin-independent, anaerobic (oxygen-sensitive, class III) RNR; NrdJ, cobalamin-dependent (class II) RNR; QueG, epoxyqueuosine reductase; EutBC, ethanolamine ammonia lyase; CFeSP, corrinoid iron-sulfur protein; OraSE, D-ornithine 4,5-aminomutase.

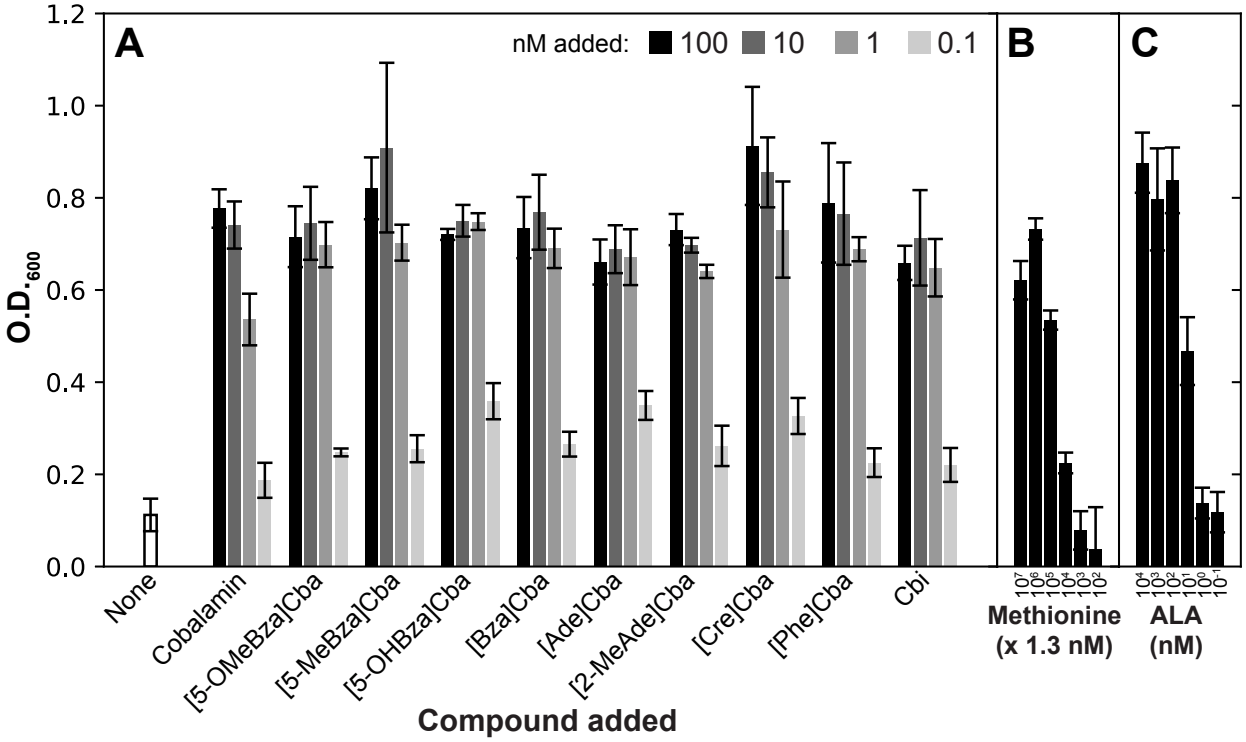

Figure 3. *C. difficile* can use a broad range of cobamides for MetH-dependent growth. The $O.D._{600}$ of *C. difficile* 630 Δ*erm* cultures grown to saturation (22.5 hours) in CDDMK medium plus glucose without methionine with the addition of **A.** cobamides or Cbi, **B.** methionine, and **C.** ALA is shown. The mean and standard deviation of four biological replicates are shown in the bars and error bars, respectively.

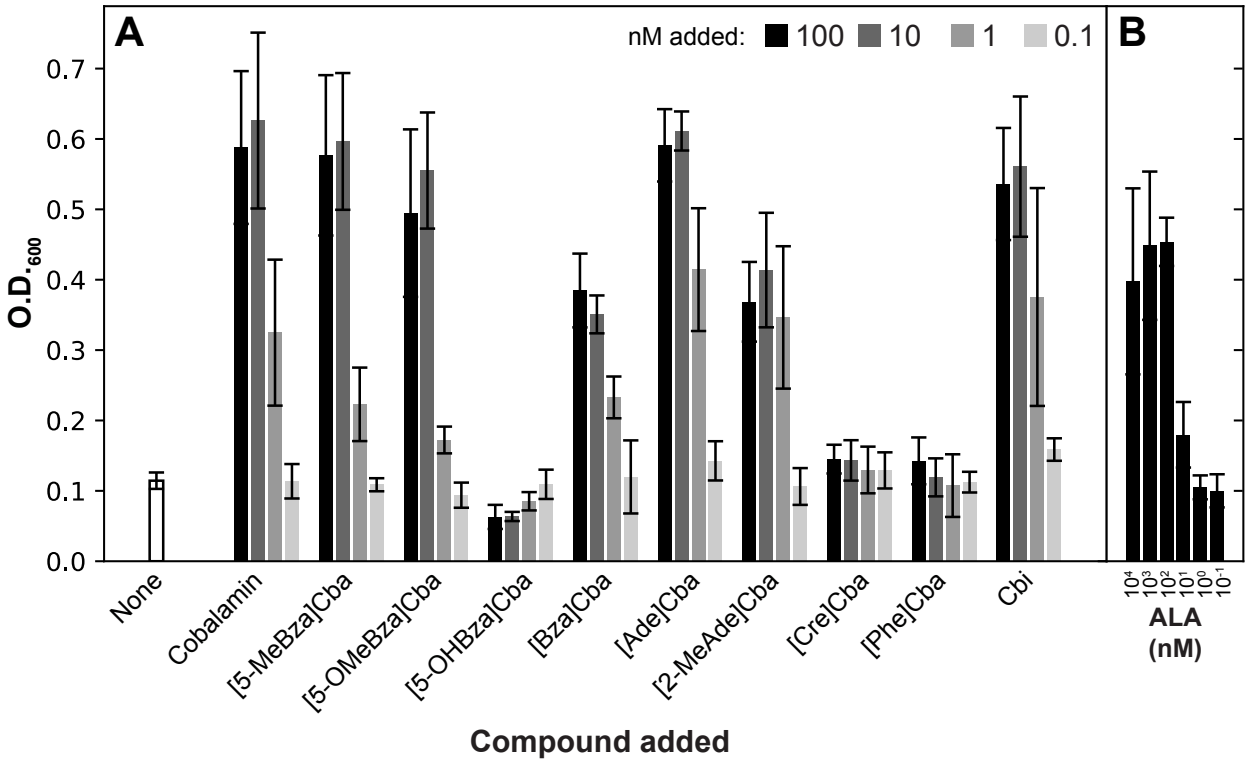

Figure 4. *C. difficile* is selective in which cobamides it can use for NrdJ-dependent growth. The O.D.$_{600}$ of *C. difficile* 630 Δ*erm* Δ*pyrE* Δ*nrdDG* cultures grown to saturation (22.5 hours) in CDDM with added uracil and glucose is shown for **A.** cobamides and Cbi, and **B.** ALA added. The mean and standard deviation of three biological replicates are shown in the bars and error bars, respectively.

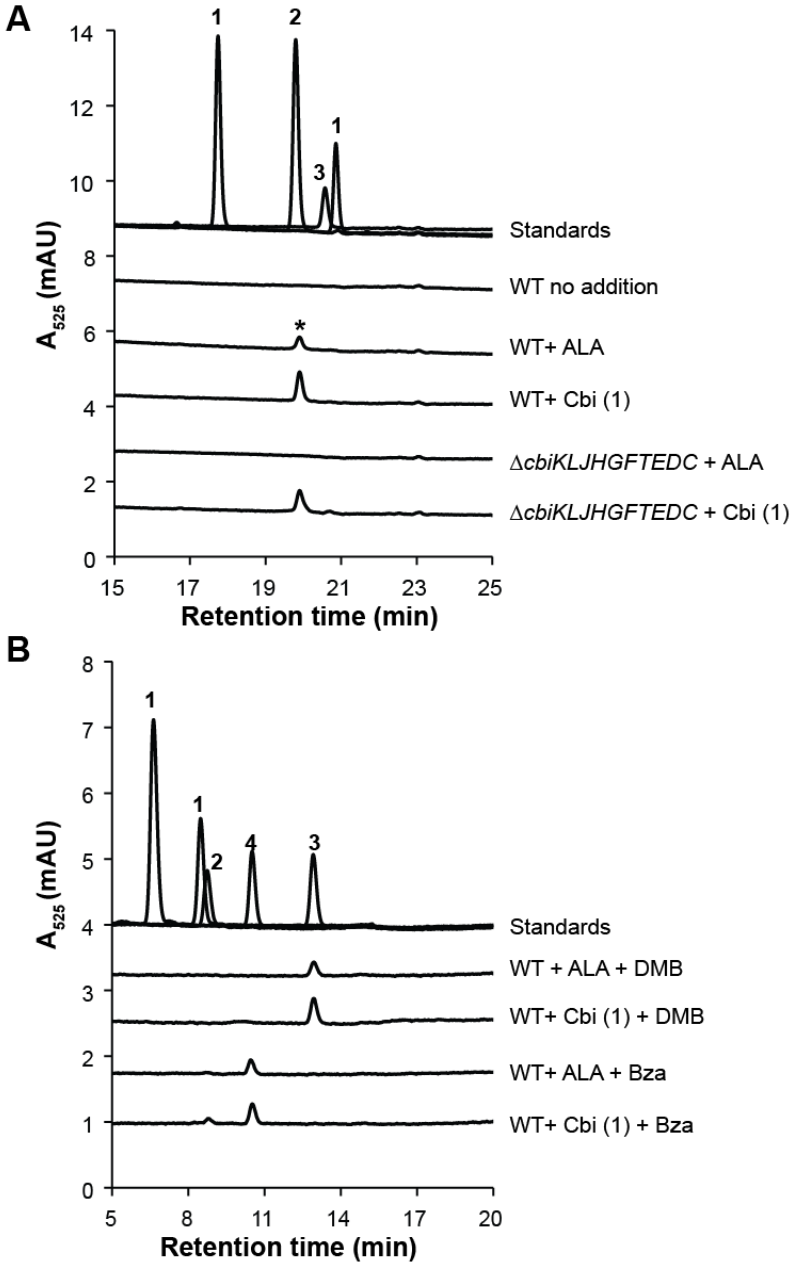

Figure 5. HPLC analysis of corrinoid extracts from *C. difficile* cultures. A. HPLC analysis of corrinoid extracts from cell pellets of *C. difficile* 630 Δ*erm* (WT) and 630 Δ*erm* Δ*pyrE* Δ*cbiKLJHGFTEDC* grown to saturation in CDDM with glucose with either 100 nM ALA or 10 nM dicyanocobinamide (Cbi) added. An asterisk (*) indicates the corrinoid peak validated by mass spectrometry (Supplemental Fig. 2). **B**. HPLC analysis of corrinoid extracts of *C. difficile* 630 Δ*erm* grown with either 100 nM ALA or 10 nM Cbi and 100 nM lower ligand bases DMB or Bza. An Agilent Eclipse Plus C18 column and an Agilent Zorbax SB-Aq column were used to separate corrinoid extractions in panels A and B, respectively. Cbi (1), pseudocobalamin (2), cobalamin (3), and [Bza]Cba (4) are shown as standards.

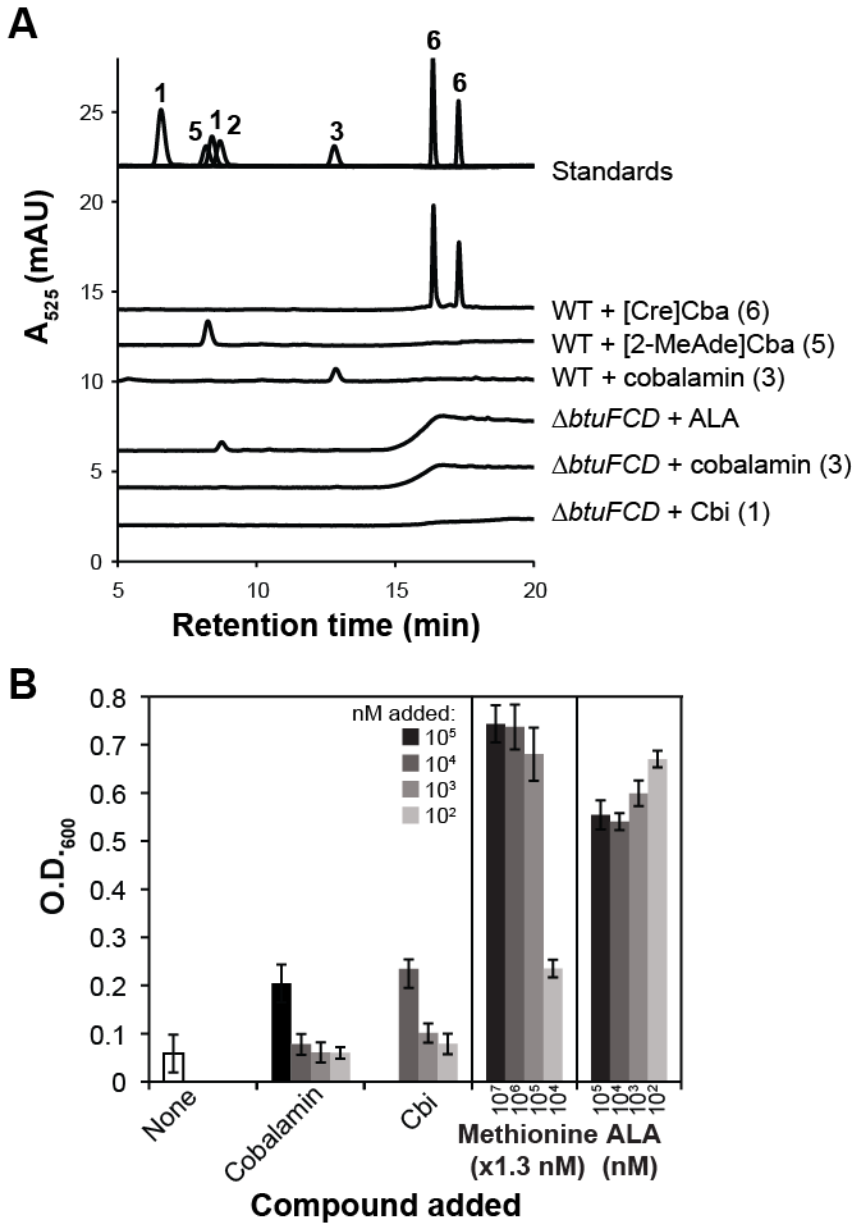

Figure 6. *C. difficile* Δ*btuFCD* mutant is impaired in cobamide and Cbi uptake. A. HPLC analysis of corrinoid extracts from cell pellets of *C. difficile* 630 Δ*erm* (WT) and *C. difficile* 630 Δ*erm* Δ*pyrE* Δ*btuFCD* grown with 10 nM cobamides or 100 nM ALA. Cbi (1), pseudocobalamin (2), cobalamin (3), [2-MeAde]Cba (5), [Cre]Cba (6) are shown as standards. **B.** Growth of *C. difficile* 630 Δ*erm* Δ*pyrE* Δ*btuFCD* in MetH-dependent conditions. The $O.D._{600}$ of saturated cultures (23.5 hours) in CDDMK without methionine plus glucose and uracil is plotted as a function of the amount of compound added. Bars and error bars are the mean and standard deviation of three biological replicates.

704 **Table 1: Bacterial strains and plasmids**

Strain or plasmid	Description	Source
Strains		
Escherichia coli XL1-Blue		QB3 MacroLab
Escherichia coli CA434	hsd20(rB-, mB-, recA13, rpsL20, leu, proA2, with IncPb conjugative plasmid R702	Chain Biotech(87)
Clostridioides difficile		
630 Δ*erm*	Erythromycin sensitive strain	(81)
630 Δ*erm* Δ*pyrE*	Strain CRG1496	(50)
630 Δ*erm* Δ*pyrE* Δ*btuFCD*		This study
630 Δ*erm* Δ*pyrE* Δ*cbiKLJHGFTEDC*		This study
630 Δ*erm* Δ*pyrE* Δ*nrdDG*		This study
Plasmids		
R702	Conjugation helper plasmid	(87)
pMTL-YN3	Allelic exchange vector	(50)
pXL001	pMTL-YN3 containing *btuFCD* deletion construct	This study
pXL002	pMTL-YN3 containing *cbiKLJHGFTEDC* deletion construct	This study
pXL003	pMTL-YN3 containing *nrdDG* deletion construct	This study

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