Single-enzyme conversion of $FMNH_2$ to 5,6-dimethylbenzimidazole, the lower ligand of B_{12}

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The synthesis of 5,6-dimethylbenzimidazole (DMB), the lower ligand of coenzyme B_{12}, has remained elusive. We report *in vitro* and *in vivo* evidence that the BluB protein of the photosynthetic bacterium *Rhodospirillum rubrum* is necessary and sufficient for catalysis of the O_2-dependent conversion of $FMNH_2$ to DMB. The product of the reaction (DMB) was isolated by using reverse-phase high-pressure liquid chromatography, and its identity was established by UV-visible spectroscopy and MS. No metals were detected in homogeneous preparations of BluB, and the enzyme did not affect DMB synthesis from 4,5-dimethylphenylenediamine and ribose-5-phosphate. The effect of the lack of *bluB* function in *R. rubrum* was reflected by the impaired ability of a Δ*bluB* strain to convert Mg-protoporphyrin IX monomethyl ester (MPE) into protochlorophylide, a reaction of the bacteriochlorophyll biosynthetic pathway catalyzed by the MPE-cyclase enzyme present in this bacterium (BchE, EC 1.14.13.81), a predicted coenzyme B_{12}-dependent enzyme. The growth defect of the Δ*bluB* strain observed under anoxic photoheterotrophic conditions was corrected by the addition of DMB or B_{12} to the culture medium or by introducing into the strain a plasmid encoding the wild-type allele of *bluB*. The findings reported here close an important gap in our understanding of the enzymology of the assembly of coenzyme B_{12}.

B_{12} biosynthesis | B_{12} lower ligand synthesis | oxygenases | vitamin metabolism

Adenosylcobalamin (AdoCbl, coenzyme B_{12}) is a structurally complex cobalt-containing cyclic tetrapyrrolidine/pyrroline synthesized by diverse groups of prokaryotes. Consistent with the complexity of its structure, a great deal of genetic information is dedicated to the assembly of AdoCbl (1). Despite the advances in our understanding of the biosynthesis of AdoCbl, there are steps of the pathway that remain unclear.

Complete corrinoids (cobamides; e.g., AdoCbl) consist of a corrin ring and upper and lower axial ligands. The lower axial ligand of AdoCbl is 5,6-dimethylbenzimidazole (DMB) (2). Our current understanding of the biosynthesis of DMB is limited, because the enzymes involved have not been studied. From labeling studies performed in *Propionibacterium freundenreichii* and *Salmonella enterica*, it was learned that these bacteria synthesize DMB from FMN (3, 4). This conversion was oxygen-dependent (5), with the C-1 carbon of the ribose moiety of FMN becoming the C-2 carbon of DMB (6, 7). Insights into the possible mechanism for the conversion of FMN to DMB were obtained from studies of the nonenzymatic spontaneous synthesis of DMB from 4,5-dimethylphenylenediamine (DMPDA) and ribose-5-phosphate (8). The conclusion from these studies was that the conversion of FMN to DMB was likely to be catalyzed by a single enzyme responsible for triggering a nonenzymatic oxidative cascade yielding DMB (8).

As mentioned above, the identity of the genes encoding the enzymes of the FMN-to-DMB pathway remains unknown. Previous reports of enzymes involved in DMB synthesis proved misleading (9). The first clue to the identity of the DMB biosynthetic genes in any prokaryote was obtained over a decade ago from studies of the photosynthetic purple bacterium *Rhodobacter capsulatus* (10) and more recently from studies of the soil bacterium *Sinorhizobium meliloti* (11). Both studies showed that the function encoded by the *bluB* gene was involved in coenzyme B_{12} synthesis, and Campbell *et al.* (11) showed that B_{12} synthesis in the *S. meliloti bluB* strain was restored by exogenously supplied DMB (10). However, neither one of these studies reported biochemical evidence that the BluB protein catalyzed any of the steps of the DMB biosynthetic pathway, nor did they identify the substrates or products of the BluB reaction.

Cobalamin auxotrophs of *R. capsulatus* accumulate Mg-protoporphyrin IX monomethyl ester (MPE), the substrate of the MPE-cyclase (BchE) enzyme that converts MPE into protochlorophylide, a precursor of bacteriochlorophyll (Bchl) (12). BchE contains a Cbl-binding domain and is believed to require Cbl for catalysis (12). MPE accumulation is readily detectable by its characteristic absorption maximum at 416 nm and can therefore be used to detect a block in Cbl biosynthesis in this bacterium. Purple photosynthetic bacteria (including *R. capsulatus* and *Rhodospirillum rubrum*) synthesize Bchl and grow photoheterotrophically in anaerobic or microaerophilic conditions (13). Anaerobic Bchl synthesis depends on BchE activity in these organisms (14). *R. rubrum* is a well studied bacterium that produces substantial quantities of complete cobamides (15). The genome of this bacterium contains a *bluB* homolog, and genetic tools for the construction of mutant strains are available. We used *R. rubrum* to study the effect of the lack of BluB protein on MPE metabolism as a function of DMB in the culture medium. We report here *in vivo* and *in vitro* evidence that a *R. rubrum bluB* strain is defective in DMB synthesis, and that the BluB enzyme from this bacterium is necessary and sufficient for the O_2-dependent synthesis of DMB from $FMNH_2$ *in vitro*.

Results

***R. rubrum* Contains a BluB Homolog.** ORF Rru_A3536 in *R. rubrum* is homologous to the *bluB* genes of *R. capsulatus* and *S. meliloti* [supporting information (SI) Fig. 4]. The hypothetical *R. rubrum* BluB protein was 52% identical/65% similar to the one from *R. capsulatus* and 44% identical/55% similar to the one from *S.*

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Abbreviations: DMB, 5,6-dimethylbenzimidazole; DMPDA, 4,5-dimethylphenylenediamine; MPE, Mg-protoporphyrin IX monomethyl ester; Bchl, bacteriochlorophyll; AdoCbl, adenosylcobalamin; Cby, cobyric acid; MN, malate minimal medium; rMN, reduced MN; SMN, supplemented MN; Fre, FMN reductase.

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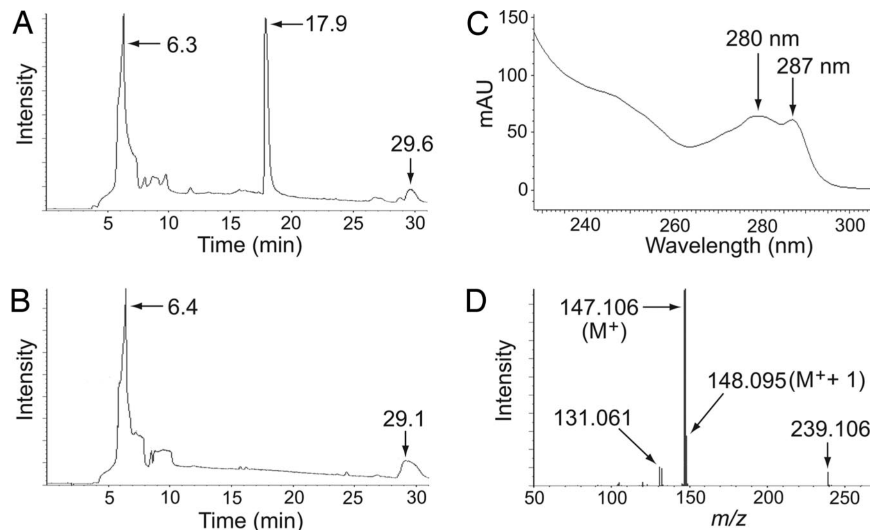

Fig. 1. BluB converts $FMNH_2$ to DMB. Reactions were prepared with 40 μg of $BluB^{WT}$ enzyme/1 μg of Fre enzyme/500 μM FMN/5 mM NADH/100 mM Hepes (pH 8)/50 mM NaCl and incubated for 18 h at 30°C. Products were analyzed by liquid chromatography MS. *A* shows the accumulation of a product eluting at 17.9 min, which is not present in the control reaction lacking BluB, shown in *B*. *C* and *D* show the UV-visible absorbance and mass spectra of the product of the BluB reaction, which were identical to authentic DMB standards (not shown).

meliloti. We used this information to explore the role of the BluB in Cbl biosynthesis *in vivo* and *in vitro*.

BluB Catalyzes the O_2-Dependent Conversion of $FMNH_2$ to DMB. The *bluB*$^+$ gene of *R. rubrum* was cloned and overexpressed in *Escherichia coli*. $BluB^{WT}$ protein was purified to homogeneity (SI Fig. 5) and was used to test the hypothesis that it was solely responsible for the conversion of $FMNH_2$ to DMB. Residue R22, a conserved residue in the putative FMN-binding domain of BluB (SI Fig. 4) was mutated to glutamate in the overexpression vector carrying the *bluB*$^+$ allele. The resulting *bluB2* allele encoded the $BluB^{R22E}$ variant protein that was used as negative control for the *in vitro* experiments described below. H_6-tagged $BluB^{WT}$ and $BluB^{R22E}$ proteins were purified by affinity chromatography and detagged by using rTEV protease; a second chromatographic step yielded protein with only three additional N-terminal residues (Gly-Ala-Ser) and ≥97% homogeneity (SI Fig. 5). Results from gel-filtration analysis showed that $BluB^{WT}$ eluted with a molecular mass of 47 kDa, which suggested that BluB is a homodimer in its native state (SI Fig. 6). Inductively coupled plasma emission spectrometry indicated that there are no metal ions associated with $BluB^{WT}$ (data not shown).

The BluB reaction was performed under reduced O_2 levels (see *Materials and Methods*), and $FMNH_2$ was generated *in situ* by using *E. coli*'s NADH-dependent FMN reductase (Fre) enzyme (16); reaction products were analyzed by liquid chromatography MS. After incubation, the complete reaction mixture contained a compound that eluted 17.9 min after injection (Fig. 1*A*). This compound was identified as DMB on the basis of its elution time, its UV-visible spectrum (Fig. 1*C*), and high-resolution MS data (Fig. 1*D*), all of which were identical to authentic DMB standards (data not shown). DMB was absent in a control reaction mixture lacking $BluB^{WT}$ (Fig. 1*B*). The identity of the purified BluB reaction product as DMB was confirmed with a bioassay by using a DMB pseudoauxotrophic *S. enterica cobT* strain (17). Growth of this strain on minimal medium with cobinamide was permitted by addition of either authentic DMB or HPLC-purified BluB reaction product, neither of which restored growth of an *S. enterica cobT cobB* strain [a Cbl auxotroph (17, 18); data not shown].

The rate of $FMNH_2$ conversion to DMB increased as a function of $BluB^{WT}$ concentration. When 20, 30, or 40 μg of $BluB^{WT}$ was present in the reaction mixture, DMB was synthesized at 6, 12, or 25 pmol min^{-1}, respectively. The corresponding calculated specific activities were 285, 410, or 625 pmol DMB $min^{-1}\cdot mg^{-1}$. DMB was not synthesized in the absence of oxygen. To show dependence on $BluB^{WT}$, variant $BluB^{R22E}$ protein was substituted for $BluB^{WT}$ in the reaction mixture. $BluB^{R22E}$ failed to catalyze the conversion of FMN to DMB. Fre and NADH were required for DMB synthesis, indicating that $FMNH_2$, not FMN, was the substrate for $BluB^{WT}$.

$BluB^{WT}$ had no effect on the rate of abiotic DMB synthesis from DMPDA and ribose-5-phosphate (data not shown).

A *R. rubrum* Δ*bluB* Mutant Strain Fails to Synthesize Cobalamin. On the basis of the result described above, we predicted that Cbl synthesis would be blocked in a *R. rubrum* strain lacking *bluB*. To test this idea, an in-frame deletion of the *R. rubrum bluB* gene was constructed (hereafter referred to as Δ*bluB*). The WT and Δ*bluB* strains were grown in minimal medium lacking corrinoids under chemoheterotrophic (oxic) or photoheterotrophic (anoxic) conditions. Corrinoids were extracted from these cultures and analyzed by RP-HPLC. WT *R. rubrum* cells grown in the presence of oxygen contained 1.4 pmol of Cbl per milligram of total protein. The amount of Cbl in aerobically grown Δ*bluB* cells was below the limit of detection (SI Fig. 7). Extracts of aerobically grown Δ*bluB* cells contained a complete cobamide that allowed growth of *S. enterica* strain JE8248 (Δ*cobS*), which lacks cobalamin (5′-P) synthase and is therefore a Cbl auxotroph (data not shown). The cobamide synthesized by the *R. rubrum* Δ*bluB* strain eluted 6.6 min after injection, as compared with 13.6 min for authentic Cbl (SI Fig. 7). Analysis of the MALDI-TOF mass spectrum of this compound identified signals with *m/z* values of 1,370.5, 1,354.5, and 1,332.5 (data not shown). The concentration of this cobamide was calculated at 0.24 pmol per milligram of total protein, and its identity was not pursued.

Cbl was not detected in WT or Δ*bluB R. rubrum* strains grown in the absence of oxygen. However, cell-free extracts of these strains contained small amounts of complete cobamides, as detected by bioassay (data not shown). RP-HPLC retention time and MS data identified cobyric acid (Cby) as the most abundant corrinoid present in *R. rubrum* WT and Δ*bluB* strains grown anoxically (data not shown). The concentration of Cby in WT *R. rubrum* grown

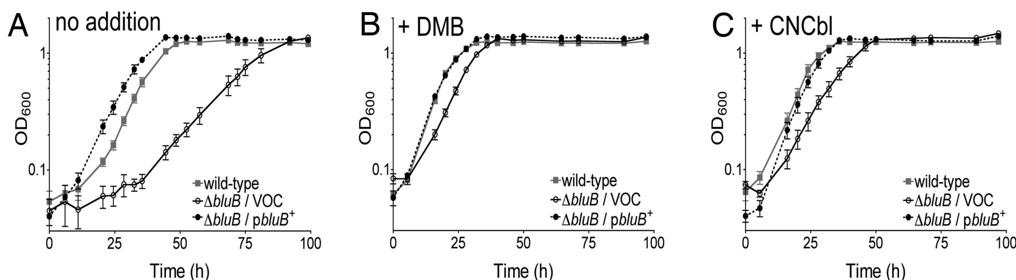

Fig. 2. Effect of the lack of *bluB* function on growth. *R. rubrum* strains were grown under anaerobic phototrophic conditions. Optical density at 600 nm was measured for the WT strain (gray lines), the Δ*bluB* strain carrying an empty cloning vector (VOC, vector-only control) (solid black lines), and the Δ*bluB* strain carrying the cloning vector encoding a $bluB^+$ allele (dashed black lines). *A* shows growth in rMN. *B* and *C* show growth in rMN supplemented with 1 μM DMB or 100 nM cyanocobalamin.

anoxically was ≈3 pmol per milligram total protein, with similar levels detected in the Δ*bluB* strain (data not shown).

A Δ*bluB* Strain of *R. rubrum* Grows Poorly Under Anoxic Photoheterotrophic Conditions in the Absence of DMB. The growth behavior of strains lacking *bluB* function was assessed under anoxic photoheterotrophic conditions in reduced malate (1 g/liter) minimal medium (rMN); WT *R. rubrum* was used as positive control. The Δ*bluB* strain carrying an empty cloning vector grew poorly under these conditions, with an extended lag phase (35 vs. 10 h for the $bluB^+$ strain) and a long doubling time (13 vs. 6 h for the $bluB^+$ strain) (Fig. 2*A*). The growth behavior of a Δ*bluB* strain carrying a plasmid encoding $bluB^+$ was similar to that of the $bluB^+$ strain. Addition of DMB to the culture medium shortened the lag phase of the Δ*bluB* culture and decreased the doubling time of the culture to match those of the $bluB^+$ and Δ*bluB*/p$bluB^+$ strains (Fig. 2*B*). Addition of Cbl had a similar effect (Fig. 2*C*). Together, the above results indicated that Cbl synthesis in *R. rubrum* required *bluB* function. The Δ*bluB* strain did not display any discernable growth defect under oxic growth conditions (data not shown).

Impact of the Lack of *bluB* Function on Chlorophyll Synthesis. Cbl is required for the formation of the isocyclic ring of Bchl under anoxic conditions (12, 14). We predicted that the Δ*bluB* strain would accumulate MPE, an intermediate of Bchl biosynthesis with a diagnostic absorption maximum at 416 nm (14). Analysis of the UV-visible spectra of solvent extracts obtained from an early stationary phase culture of the Δ*bluB* strain grown photoheterotrophically in the absence of oxygen showed the accumulation of a compound with strong absorbance at 416 nm (Fig. 3*A*). This compound was absent in solvent extracts of the $bluB^+$ or Δ*bluB*/p$bluB^+$ strains, suggesting its metabolism depended on the availability of Cbl. Consistent with this idea, this compound was absent in solvent extracts of the Δ*bluB* strain when cells were grown in the presence of DMB (Fig. 3*B*) or Cbl (data not shown).

Discussion

The BluB Protein Is Necessary and Sufficient for the O_2-Dependent Conversion of $FMNH_2$ to DMB. Data reported here (Figs. 1 and 2) strongly support the conclusion that the 24-kDa BluB protein of *R. rubrum* is necessary and sufficient for the O_2-dependent conversion of $FMNH_2$ to DMB. Although one report in the literature suggested another protein is responsible for the synthesis of DMB in bacteria, this study did not show biochemical evidence of such an activity (9, 11), and the observed dependence of B_{12} synthesis on exogenous DMB was later shown to be a pseudoauxotrophy (18).

Our work did not address the mechanism of catalysis of the BluB enzyme. However, previously reported work sheds light onto how the BluB enzyme might work. Elegant studies in *P. freundenreichii* and *S. enterica* showed that an O_2-dependent system converted FMN to DMB in these bacteria, and that the C-2 carbon of DMB was derived from the C-1 carbon of ribose (4, 5, 7, 19–24). Based on the data reported here, we propose a model in which the BluB protein is the oxygenase enzyme that opens the isoalloxazine ring of $FMNH_2$ to yield a phosphoribitylamine intermediate, which proceeds to DMB by facile nonenzymatic oxidative chemistry (8). Abiotic synthesis of DMB from DMPDA and ribose-5-phosphate was not affected by addition of BluB (data not shown). The details of the mechanism of the BluB-catalyzed conversion of $FMNH_2$ to DMB require further investigation.

Some dioxygenases depend on transition metals or organic cofactors to activate O_2. We did not detect any metals in our preparations of BluB protein. A family of cofactor-free dioxygenases involved in the degradation of *N*-heteroaromatic compounds has been described (25), but these enzymes have no homology to BluB, and their mechanism of catalysis is not well understood.

Rate of the BluB Reaction. The BluB reaction was slow *in vitro* (25 pmol·min^{-1}). DMB synthesis is a rate-limiting step in Cbl synthesis, and many other Cbl synthetic enzymes also have low specific activities (1). *In vitro* DMB-forming reactions were performed

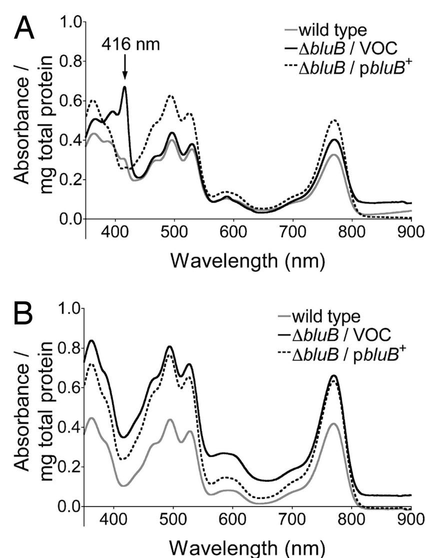

Fig. 3. MPE accumulation in the Δ*bluB* strain. (*A*) The absorbance spectra of pigment extracts prepared from early stationary phase anoxic photoheterotrophic cultures of the WT strain (gray lines), the Δ*bluB* strain carrying an empty cloning vector (VOC, vector-only control) (solid black lines), and the Δ*bluB* strain carrying the cloning vector encoding a $bluB^+$ allele (dashed black lines). (*B*) The absorbance spectra of the same strains grown in the presence of 1 μM DMB.

under low O_2 partial pressures to strike a balance between its role as substrate and its reactivity with $FMNH_2$. Because of the reactivity of O_2 and $FMNH_2$, we did not measure exact concentrations of substrates in the reaction, and we explain the slow rate of the reaction as the result of the technical difficulty of providing saturating levels of O_2 without depleting $FMNH_2$. Although not optimal, these conditions were efficient enough to demonstrate BluB, $FMNH_2$, and O_2 dependence of the reaction.

In vivo, DMB synthesis may occur more efficiently. The BluB reaction could be accelerated if BluB were in close proximity to the cell membrane where the concentration of O_2 would be higher. This localization could be afforded by interactions with integral membrane proteins of the Cbl synthesis pathway, like cobalamin (5′-P) synthase and cobinamide-P synthase (ref. 26; C. L. Zayas and J.C.E.-S., unpublished results). Another way of accelerating the BluB reaction would be to facilitate product (DMB) removal. We note that genome sequences of some actinomycetes (e.g., *Streptomyces coelicolor*, *Thermobifida fusca*) contain gene fusions of the *bluB* and *cobT* genes. The CobT enzyme consumes DMB during the synthesis of α -ribazole-5′-phosphate from nicotinate mononucleotide or nicotinamide adenine dinucleotide (27, 28). It is possible that the BluB-CobT fusion protein catalyzes the synthesis of DMB more efficiently.

In *R. rubrum*, Lack of *bluB* Function Impairs Bchl Synthesis. The genome of *R. rubrum* encodes all of the genes known to be necessary for the late-cobalt-insertion (aerobic) Cbl biosynthetic pathway (1). This bacterium synthesizes Cbl only aerobically (SI Fig. 7). The Δ*bluB* strain displayed a growth defect correctable with either DMB or Cbl (Fig. 2). This defect was observed only under conditions that demanded Bchl synthesis, which depends on the activity of BchE, an oxygen-sensitive MPE cyclase enzyme thought to require coenzyme B_{12} for function (12, 14). *R. rubrum* lacks a homolog of *acsF*, the gene encoding the oxygen-tolerant Cbl-independent MPE cyclase enzyme present in other photosynthetic bacteria (14, 29). Our data support the prediction that a block in DMB synthesis in *R. rubrum* would reduce the amount of Cbl accumulated during aerobic growth of the inoculum. This reduction in the Cbl level would lead to a defect in Bchl synthesis after the shift to anoxic conditions, hence result in poor photosynthetic growth. Growth was not abolished in the Δ*bluB* strain (Fig. 2*A*), because this bacterium synthesizes small amounts of an alternative cobamide (SI Fig. 7). Synthesis of an alternative cobamide was not surprising, because the CobT enzyme is known to have low specificity for its base substrate (30), resulting in a variety of naturally occurring cobamides with lower ligands other than DMB (reviewed in refs. 7 and 31).

Effect of the Lack of *bluB* Function on *de Novo* Corrin Ring Biosynthesis. As reported by Campbell *et al.* (11), the lack of *bluB* function in *S. meliloti* causes the accumulation of cobinamide-GDP, the immediate precursor of Cbl. In contrast, the lack of *bluB* function in *R. rubrum* leads to the accumulation of Cby, the penultimate intermediate of the *de novo* corrin biosynthetic branch of the pathway (1). This finding suggests that flux of Cby through the remaining steps of the pathway may be coupled to the activity of the cobalamin (5′-P) synthase enzyme. How this coupling may occur remains an open question. We note that Cby also accumulated in anoxically grown WT cells, consistent with the hypothesis that DMB synthesis occurs in *R. rubrum* only in the presence of molecular oxygen.

Materials and Methods

Bacterial Strains and Growth Conditions. All strains and plasmids used in this study are listed in SI Table 1. All *R. rubrum* strains were derived from WT strain UR2, which is resistant to nalidixic acid and streptomycin (32, 33). *R. rubrum* was grown at 30°C in supplemented malate minimal medium (SMN) (33), MN (34), or reduced malate MN (rMN) (1 g/liter). Anaerobic growth curves of *R. rubrum* were performed in rMN to accurately measure optical density. MN and rMN media were supplemented with $CoCl_2$ (1 mg/liter) to ensure efficient Cbl synthesis (15). For growth of *R. rubrum* in MN or rMN, starter cultures were grown aerobically for 3 days in SMN containing appropriate antibiotics. Cells were rinsed twice with sterile MN and used to inoculate fresh medium (1% vol/vol inoculum). Protocols for preparing anoxic media were as described (35). Aerobic cultures of *R. rubrum* (500 ml in a 4-liter flask) were grown in the dark with shaking at 200 rpm in an orbital shaker. *E. coli* was grown at 37°C in lysogenic broth (Difco, Sparks, MD) (36, 37). *Salmonella enterica* sv. *Typhimurium* (hereafter *S. enterica*) was grown at 37°C in nutrient broth (Difco), LB, or no-carbon E minimal medium (38) containing $MgSO_4$ (1 mM) and glucose (11 mM) or glycerol (22 mM). When used, nalidixic acid was at 20 μg/ml, ampicillin was at 100 μg/ml, kanamycin at 10 μg/ml, and tetracycline was at 10 μg/ml (for *E. coli* or *S. enterica*; 1 μg/ml for *R. rubrum*). When added, cyanocorrinoids were present at 100 nM. Chemicals were purchased from Sigma (St. Louis, MO).

Genetic and Molecular Techniques. DNA manipulations were performed by using described methodology (39). Restriction and modification enzymes were purchased from Fermentas (Ontario, Canada) Promega (Madison, WI) and used according to the manufacturer's instructions. All DNA manipulations were performed in *E. coli* DH5α. Plasmid DNA was isolated by using the Wizard Plus SV Plasmid Miniprep kit (Promega). PCR products were purified by using the QiaQuick PCR purification kit (Qiagen, Valencia, CA). DNA sequencing reactions were performed by using nonradioactive BigDye protocols (ABI PRISM; Applied Biosystems, Foster City, CA) and resolved at the Biotechnology Center of the University of Wisconsin, Madison. Plasmids derived from plasmids pRK404 (40) and pUX19 (41) were conjugated into *R. rubrum* as described (42).

Construction of the *R. rubrum* Δ*bluB* Mutant. The sequence of all primers used in this work can be found in SI Table 2. Primers [1] and [2] were used to amplify a 3,021-bp fragment of *R. rubrum* chromosomal DNA containing *bluB* and 2,385 bp of flanking sequence (1,185 bp upstream and 1,200 bp downstream). This fragment was cloned into the KpnI and BamHI sites of plasmid pUX19 to yield plasmid pBLUB16.

Primers [3] and [4] were used with plasmid pBLUB16 as template to amplify a 6.3-kbp DNA fragment, which was cut with SacI and ligated to yield plasmid pBLUB17, which contained an in-frame deletion of *bluB* in which bases 28–564 of *bluB* were replaced by a 6-bp SacI restriction site. The hypothetical gene product encoded by this Δ*bluB* (*bluB1*) allele would be a 32-aa peptide.

Plasmid pBLUB17 was conjugated into WT *R. rubrum*. Kanamycin-resistant transconjugants were picked, grown for ≈50 generations in SMN broth containing nalidixic acid and screened for kanamycin sensitive variants on SMN agar plates. The presence of the in-frame deletion in *bluB* was confirmed by amplifying and sequencing the *bluB* region by using primers [5] and [6].

Construction of *bluB*$^+$ Plasmid for Complementation Studies. The *bluB*$^+$ coding sequence plus 158 bp of 5′ sequence was amplified by using primers [7] and [8], and the resulting product was cloned into the HindIII site of plasmid pRK404 to yield plasmid pBLUB12.

Construction of Plasmids for the Overproduction of BluB^{WT} and BluB^{R22E} Proteins. The *bluB*$^+$ coding sequence was amplified with primers [9] and [10] and cloned into the NheI and EcoRI sites of plasmid pTEV5 to yield plasmid pBLUB10. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used with primers [11] and [12] and plasmid pBLUB10 to

construct plasmid pBLUB13, which carried a *bluB* allele (*bluB2*) encoding the variant $BluB^{R22E}$ protein.

Purification of BluB Proteins. $BluB^{WT}$ or $BluB^{R22E}$ protein fused to an rTEV protease cleavable N-terminal H_6 tag was overproduced by using plasmids pBLUB10 (*bluB*$^+$) or pBLUB13 (*bluB2*). One milliliter of an overnight culture of the overexpressing strain was inoculated into 2 liters of lysogenic broth containing ampicillin. Cultures were grown at 37°C with shaking to a cell density of OD_{600} = 0.6, isopropyl-β-D-thiogalactopyranoside added to 0.3 mM, then incubated for 18 h at 15°C with shaking. Cells were harvested by centrifugation for 15 min at 5,000 × *g* at 4°C, resuspended in 20 ml of 20 mM Tris-HCl buffer (pH 7.9 at 4°C) containing NaCl (1 M) and imidazole (10 mM), and broken with a French press (1.6×10^5 kPa, 4°C). Cell lysate was cleared by centrifugation for 1 h at 14,000 × *g* at 4°C and filtered through a 0.45-μm syringe filter (Nalge Nunc, Rochester, NY). Tagged BluB proteins were purified by using His-Bind resin (Novagen, San Diego, CA). The H_6 tag was removed after 48 h at 4°C with rTEV protease (43) present in the buffer at a 1:100 ratio of BluB:rTEV protease. H_6-rTEV protease was resolved from de-tagged BluB proteins by passing the protein mixture over His-Bind resin. Purity was monitored by SDS/PAGE (44) and Coomassie blue staining (45). Fractions containing detagged BluB protein were pooled, dialyzed (M_r cutoff = 10,000 membrane; Pierce, Rockford, IL) at 4°C against 1 liter of Tris·HCl buffer (20 mM, pH 7.9 at 4°C) containing NaCl (50 mM) and glycerol (10% vol/vol), and stored at −80°C after flash freezing in liquid N_2. Protein purity was assessed by using the TotalLab software package (Nonlinear Dynamics, Durham, NC).

Metal Analysis of BluB. $BluB^{WT}$ was analyzed for bound metal ions by inductively coupled plasma emission spectrometry (ICPES; Chemical Analysis Laboratory, University of Georgia, Athens), by using a Thermo Jarrell-Ash 965 (New Port Richey, FL) inductively coupled argon plasma spectrophotometer.

Purification of Fre. H_6-tagged Fre (*E. coli* flavin reductase) was purified as described (16).

BluB Activity Assays. DMB synthesis was performed in 200-μl reaction mixtures containing 20–40 μg of $BluB^{WT}$ or $BluB^{R22E}$ protein; 1 μg of Fre protein; 100 mM Hepes buffer, pH 8; 50 mM NaCl; 500 μM FMN; and 5 mM NADH. Reactions were incubated for 0, 2, or 4 h at 30°C in the dark and stopped by boiling for 15 min. Precipitated protein was removed by filtration. Assay mixtures were prepared aerobically in 0.8-ml Eppendorf tubes and overlaid with 100 μl of mineral oil to limit diffusion of oxygen into the reaction. Further reduced O_2 conditions were obtained by sparging the buffer containing FMN and NADH with N_2 gas for 15 min at 15 $ml \cdot s^{-1}$. Enzymes were dispensed into 2-ml serum vials (Wheaton, Millville, NJ) on ice and sealed, and the overhead space was flushed with N_2 gas for 2 min at 15 $ml \cdot s^{-1}$. Buffer mixture was added anoxically to each serum vial with a syringe. Anoxic reactions were prepared as above but passing N_2 gas over a freshly regenerated copper catalyst, and buffer/FMN/NADH mixtures were sparged for 30 min with O_2-free N_2 at 15 $ml \cdot s^{-1}$.

Identification of the Product of the BluB Reaction. A 200-μl reduced-O_2 reaction containing 40 μg of $BluB^{WT}$ was incubated for 18 h at 30°C; a control reaction lacked BluB protein. Removal of proteins by filtration stopped the reaction. Reaction products were identified by liquid chromatography MS (University of Wisconsin Biotechnology Center, Madison, WI) by using an Agilent Technologies (Palo Alto, CA) LC/MS ESI-TOF with a mass accuracy of >3 ppm. Separation was performed with an Agilent 1100 LC with a 2.1 × 100-mm Insertsil C18 column by using a 90-min gradient of 100% solvent A (0.1% formic acid in water) to 100% solvent B (0.1% formic acid in acetonitrile). Reference masses of 121.05087 and 922.0098 atomic mass units from the Agilent API TOF reference mass solution kit were used as a lock mass standard.

Bioassay of BluB Reaction Products. The identity of the BluB reaction product was confirmed by means of a bioassay. A 6-ml reaction mixture containing 1.2 mg of $BluB^{WT}$ was incubated for 18 h at 30°C; a control reaction lacked BluB protein. Reactions were stopped by boiling for 15 min, and reaction products were purified by RP-HPLC, dried under vacuum by using a Savant concentrator (ThermoElectron, San Jose, CA), desalted by using a 1-ml C18 Sep-Pak cartridge (Waters, Milford, MA), and resuspended in 5 μl of DMSO. *S. enterica* strains JE1244 (*cobT*) and JE2501 (*cobT cobB*) were used as indicator strains in an overlay on glucose minimal medium containing 15 nM cobinamide. A 5-μl sample of purified BluB reaction product (92 nmol; calculated by using the $\varepsilon = 5{,}260\ M^{-1} \cdot cm^{-1}$ for DMB in methanol)/90 nmol of authentic DMB (positive control)/2 pmol of Cbl were spotted onto the agar overlay (positive control). Plates were incubated aerobically at 37°C. *S. enterica cobT* mutants are DMB pseudoauxotrophs because of the activity of the alternative gene *cobB* (17). An *S. enterica* strain lacking both *cobT* and *cobB* is a Cbl auxotroph unresponsive to DMB (17, 18).

HPLC Analysis of BluB Reaction Products. The products of the BluB reaction were resolved by using a Beckman Coulter (Fullerton, CA) System Gold 126 HPLC system equipped with a Beckman Coulter System Gold 508 autosampler and a 150 × 4.6-mm Alltima HP C18 AQ column (Alltech, Jerome, ID). Details of the protocol used have been described (46). Five minutes after injection, the column was developed for 5 min with a linear gradient to a composition of 41.2% solvent A (15% methanol/5 mM ammonium acetate)/58.8% solvent B (100% methanol). A second, 6-min linear gradient developed the column to 23.5% A/76.5% B. A final linear gradient developed the column to 100% B over 5 min. Products were detected with a photodiode array detector. DMB was identified by its absorbance spectrum and by comparison with an authentic DMB standard; the detection limit was 0.5 nmol.

Effect of BluB on Abiotic DMB Synthesis. DMB synthesis from DMPDA and ribose-5-phosphate was performed in 200-μl reaction mixtures containing 100 mM Hepes buffer (pH 8), 50 mM NaCl, 500 μM DMPDA, and 500 μM ribose-5-phosphate, with and without 40 μg of $BluB^{WT}$. Assay mixtures were prepared aerobically in 0.8-ml Eppendorf tubes and overlaid with 100 μl of mineral oil. Reactions were incubated at 30°C in the dark and stopped by addition of 20 μl of 75% (wt/vol) trichloroacetic acid. Precipitated protein was removed by filtration. Reaction products were identified by RP-HPLC as described above.

Corrinoid Extraction and Analysis. The WT and Δ*bluB* mutant strains were grown in MN broth either anaerobically with light or aerobically in the dark. Corrins were extracted with methanol and purified by RP-HPLC (see *SI Methods*). Identity of corrins was determined by means of a bioassay and by MALDI-TOF MS (see *SI Methods*). The detection limit for Cbl was 0.02 pmol of Cbl per milligram of total cell protein.

Pigment Extraction and Analysis. *R. rubrum* was grown phototrophically in rMN medium. Samples (100 μl) of each culture taken in early stationary phase were lysed with BugBuster (Novagen, San Diego, CA), and total protein content was determined by using the Bradford assay (Bio-Rad, Hercules, CA) (47). Cells were harvested by centrifugation at 2,000 × *g* at

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4°C for 15 min, pigments were extracted with 2 ml of ice-cold 7:2 (vol/vol) acetone/methanol (14), and cell debris was discarded. Absorption spectra were determined by using a Lambda 45 UV/Vis spectrophotomer (PerkinElmer, Wellesley, MA) and normalized to milligram of total protein.

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