Regulating vitamin B12 biosynthesis via the cbiMCb1 riboswitch in Propionibacterium strain UF1

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Vitamin B12 (VB12), also known as cobalamin, is uniquely synthesized by few bacteria and archaea bacteria and is a crucial cofactor for critical enzymes catalyzing numerous transmethylation and biochemical reactions (1, 2). Structurally, VB12 consists of a corrin ring in which the cobalt is positioned centrally and coordinated with upper and lower ligands composed of 5, 6-dimethylbenzimidazole (DMB) (1, 3, 4). In both bacterial and mammalian cells, methionine synthase for biosynthesis of S-adenosylmethionine (SAM) and methylmalonyl-CoA mutase converting methylmalonyl-CoA to succinyl-CoA are dependent on VB12 for their metabolic activities (5), making VB12 essentially required for the biosynthesis of nucleic acids, amino acids, and fatty acids. Thus, VB12 deficiency is critically associated with micronuclei formation and chromosomal abnormalities (6–8) and may contribute to adverse pregnancies along with neurologic morbidity and death in neonates (9–14).

In humans, VB12 can be absorbed from food of animal origin or may be provided by some gut microbes through fermentation of complex carbohydrates (14). However, only a few bacterial and archaeal species have the genetic complexity for VB12 biosynthesis via aerobic and/or anaerobic pathways depending on the nature of microorganisms (3, 15). For example, *Pseudomonas* species produce VB12 only under aerobic condition (3, 16), whereas Propionibacteria use both aerobic and anaerobic routes for its biosynthesis. It has been shown that VB12 biosynthetic pathways involve nearly 30 different enzymes, including *hemB*, *HemC*, *HemD*, *Cbi*, and *Cob* genes (17). CobA serves as a rate-limiting enzyme that converts uroporphyrinogen II to precorrin-2, which is eventually incorporated with DMB to form cobalamin (17). Interestingly, the *cobA* gene was initially annotated as *cysG* in some bacteria, including *Propionibacterium* spp. (18, 19); however, the specificity of this gene in VB12 biosynthesis remains elusive.

Propionibacteria are Gram-positive, facultative anaerobic, and nonmotile microorganisms with a high GC content that can be taxonomically classified into cutaneous (e.g., *Propionibacterium acnes*) and dairy (e.g., *Propionibacterium freudenreichii*) species (20, 21). Although *P*. *freudenreichii*, along with *Pseudomonas* species, is a major producer of VB12 and is widely used for industrial fermentation (2, 17), the regulatory elements that restrict VB12 biosynthesis in these bacteria, are not fully understood. It has been reported that VB12 biosynthesis is tightly regulated by noncoding RNAs (ncRNA), known as riboswitches (22–24), which are embedded within the 5′ UTR of VB12-synthesizing operons. However, the elucidation of the identity and mechanisms by which these riboswitches regulate gene expression and VB12 biosynthesis within *P*. *UF1*, which was recently isolated from gut microbiota of preterm infants fed human breast milk (25), still require further rigorous investigation.

*P*. *UF1 shares 90% sequence identity with *P. freudenreichii* (25). We have recently reported that *P*. *UF1* not only regulates the innate and T cell response to intestinal infection (25–27), but also controls the maturation of neonatal protective T cell immunity to resist pathogen infection (28). Here, to further elucidate the regulatory mechanisms exerted by this bacterium, we demonstrate that *P*. *UF1* abundantly produces VB12, which in turn regulates expression of the *cobA* operon through a riboswitch, *cbiM*Cb1, within this beneficial bacterium that may affect the host milieu and contribute to gut homeostasis.

Results

CobA as the Key Enzyme for Bacterial VB12 Biosynthesis. Biochemical studies demonstrate that CobA is essential for the conversion of...
uroporphyrinogen III to precorrin-2 (Fig. 1A), which is the branch point between the biosynthesis of VB12 and siroheme (18). However, whether CobA plays a critical role in the biosynthesis of VB12 within P. UF1 is currently unknown. Thus, the cobA gene was deleted from the bacterial chromosome by homologous recombination with a single crossover event, resulting in ΔcobA P. UF1 (Fig. 1B and SI Appendix, Table S1). For complementation of the cobA-deficient bacterial strain, the cobA gene, along with its native promoter, was integrated into the chromosome of ΔcobA P. UF1 (Fig. 1B and SI Appendix, Table S1). PCR and Western blot analysis demonstrated cobA expression in P. UF1 and C-ΔcobA P. UF1 but not in ΔcobA P. UF1 (Fig. 1C and D). HPLC analysis demonstrated that cobA deficiency led to complete abrogation of intracellular VB12 within ΔcobA P. UF1, referring to the VB12 standard, and the complementation of cobA mutation restored VB12 biosynthesis in C-ΔcobA P. UF1 (Fig. 1E). Furthermore, deletion of cobA significantly decreased VB12 production over time when cultured in either MRS medium or Poznan medium, a minimal medium containing no VB12 (SI Appendix, Fig. S1). These data highlight the pivotal role of CobA in critically directing VB12 biosynthesis within P. UF1.

Controlling cobA Operon Expression by VB12. In bacteria such as Escherichia coli and Salmonella Typhimurium, VB12 interacts with the 5' UTR of the VB12 biosynthesis operon to repress translation of the corresponding genes, including the cob and btuB operons (29, 30). Thus, a demonstration of the central role of cobA in controlling VB12 biosynthesis within P. UF1 prompted us to assess the feedback regulation of cobA operon by VB12. We cloned the 5' UTR of the cobA operon (PcobA) into the first gene of the operon, cbiM, with His-tag or the flavin mononucleotide-binding fluorescent protein (FbFP) gene (31) to construct the CbiM–wild-type (WT) and FbFP–WT reporter bacterial strains, respectively (SI Appendix, Table S1). Here, when adding cobalt and DMB (2 substrates required for VB12 biosynthesis) or VB12 alone into FbFP–WT and CbiM–WT bacterial cultures, the expression levels of FbFP and cbiM were significantly decreased (Fig. 2A and B).

To further explore a correlation between VB12 concentration and the expression of these reporter genes, we first examined the effect of VB12 on FbFP expression. As shown in Fig. 2C, VB12 down-regulated the FbFP expression in a VB12-dose-dependent manner. The half-maximal inhibitory concentration (IC50) of VB12 was 75.2 ng/mL, and the expression of FbFP was completely abated at 500 ng/mL (Fig. 2C). In addition, Western blot analyses consistently demonstrated the dose-dependent inhibition of cbiM expression by VB12 using His-tag antibody (Fig. 2E). Furthermore, the same VB12-mediated gene suppression was also observed in P. UF1 using mouse serum antibodies generated against CobA (SI Appendix, Fig. S24). These data demonstrate that both endogenous and exogenous VB12 modulated the expression of cobA operon through its 5' UTR.

The cobA operon harbors cbiMNQO genes encoding proteins critical for cobalt transport and preocorrin-2 biosynthesis (Fig. 2D). To investigate whether VB12 regulates the entire cobA operon, we labeled the C termini of respective cbiN, cbiO, and cobA genes with His-tag to assess their expression by Western blot analyses. Our data demonstrate that the expression of cbiN and cbiO was dampened by adding VB12 to CbiN-ΔcobA P. UF1 and CbiOB-ΔcobA P. UF1 cultures (Fig. 2F and SI Appendix, Table S1), as observed for cbiM expression (Fig. 2E). Note that cobA expression displayed the dose-dependent inhibition but to a lesser extent in the CobA-ΔcobA P. UF1 strain (Fig. 2F and SI Appendix, Table S1). These data suggest that expression of the entire cobA operon is tightly controlled by VB12.

![Fig. 1.](https://example.com/figure1.png)

Fig. 1. cobA is essential for VB12 biosynthesis within P. UF1 (A) Proposed biosynthetic pathway for VB12 produced by P. UF1 in which cobA is responsible for converting uroporphyrinogen III to precorrin-2. (B) Genetic organization of P. UF1, ΔcobA P. UF1, and C-ΔcobA P. UF1 strains. cmR, chloramphenicol-resistant gene. hygB, hygromycin B. (C) PCR identification of P. UF1, ΔcobA P. UF1, and C-ΔcobA P. UF1 strains using primers P1/P2 and P3/P4 as shown in B. (D) Western blot (WB) analysis of cobA expression in P. UF1, ΔcobA P. UF1, and C-ΔcobA P. UF1 strains using mouse serum antibodies against CobA. The large surface layer protein (LspA) served as a reference control. (E) HPLC chromatograms of VB12 extracted from P. UF1, ΔcobA P. UF1, and C-ΔcobA P. UF1 strains. The bar graph shows the intracellular levels of VB12 in the indicated strains. Data are representative of 3 independent experiments. Error bars indicate SEM. **P < 0.01; ****P < 0.0001, 2-tailed unpaired t test.
VB12 possesses analogs such as cyanocobalamin (manufactured form), methylcobalamin (active form), hydroxocobalamin (storage form), and adenosylcobalamin (active form) (14). To elucidate a possible differential regulation by the VB12 analogs, CbiMΔcobA P. UF1 and FbFPΔcobA P. UF1 cultures (SI Appendix, Table S1) were treated with various concentrations of the VB12 analogs to analyze the cbiM and FbFP expression in these bacterial strains. The expression of cbiM and FbFP was similarly down-regulated by all the analogs (SI Appendix, Fig. S2 B and C). These data demonstrate that VB12 and its analogs control expression of the cobA operon through its 5′ UTR within P. UF1.

Identifying a VB12 Riboswitch within 5′ UTR of the cobA Operon.

Riboswitches are noncoding RNA (ncRNA) regulatory elements that specifically bind small-molecular ligands such as VB12 to modulate gene expression (24). To identify potential regulatory elements, the 5′ UTR (309 bp) of the cobA operon was used to perform a comparative analysis. Conserved secondary structure and sequence homology analyses demonstrated the presence of a potential VB12 riboswitch (Rfam accession, RF00174; 140 bp), designated as cbiMCbl riboswitch, which contains 3 major stem loops (SLs): SL1, SL2, and SL3 (Fig. 3A).

Mechanistically, SL domains interact with ligands and the targeting RNA to regulate gene expression within various microorganisms (32). Thus, to determine the regulatory function of these SLs, we tested the effect of each SL domain on the expression of cbiM and FbFP by P. UF1. Here the FbFP reporter assays demonstrated that the SL1-deleted riboswitch (∆SL1) lost the VB12 dose-dependent regulation, and the IC50 of VB12 was 12,962 ng/mL, which was 256-fold higher than that of the WT riboswitch (Fig. 3 B and C). Consistently, the repression of cbiM expression was observed for ∆SL1 compared with the WT riboswitch in P. UF1 (Fig. 3D). Furthermore, to precisely elucidate the sites or regions required for VB12-mediated gene regulation within P. UF1, site-directed mutations were introduced into SL1 of the cbiMCbl riboswitch. While the WT riboswitch exhibited ∼10-fold repression of FbFP expression at 250 ng/mL VB12, site mutation of 12 or 13 in SL1 only retained marginal reduction of protein expression even at 2,500 ng/mL VB12 (Fig. 3E and SI Appendix, Fig. S3C). In contrast, site mutations of 5 to 6, 7 to 8, or 9 to 10 did not impact the regulatory activity of this riboswitch (Fig. 3E and SI Appendix, Fig. S3C). Furthermore, deletion of SL3 resulted in a total loss of gene expression (SI Appendix, Fig. S2 A and B), whereas deletion of the SL3 loop region, sites 12′ to 15′, abolished the dose-dependent down-regulation of the downstream gene.
expression in P. UF1 (Fig. 3F and SI Appendix, Fig. S3D). In contrast, site mutation of the loop region did not down-regulate the downstream gene expression by VB12 (Fig. 3F and SI Appendix, Fig. S3D), indicating that these sites may cooperatively maintain the regulatory activity of the SL3 domain in this bacterium. These findings emphasize that VB12-mediated regulation is highly dependent on the structure of the cbIMCbI riboswitch within P. UF1.

Regulation of cbIM Expression by Ribosome-Binding Site–Mediated Base Pairing. VB12-element exhibits a conserved RNA regulatory sequence in various VB12 riboswitches of numerous microorganisms (15, 33). To investigate whether the VB12 element exists within cbIMCbI riboswitch, the known VB12 riboswitches and cbIMCbI riboswitch were compared by sequence alignment analysis using LocARNA, whereby a conserved VB12 element and various secondary structures (e.g., Pkn) were identified within the cbIMCbI riboswitch (Fig. 4A). cbIMCbI riboswitch contained a conserved core region, 5’-GCCACUG-3’, which partially overlapped with the SL2 domain (Fig. 4A). Importantly, 2 groups of Watson–Crick base pairs, between Pkn and Pkn’ and between antisequester and ribosome-binding site (RBS) sequester, were found within the cbIMCbI riboswitch (Fig. 4A).

To elucidate whether these base pairs are important for VB12-mediated regulatory activity, site mutation and region deletion were introduced into these sequences, and their impacts on cbIM and FbFP expression were analyzed (Fig. 4A). Here single-site (e.g., AR2) or multisite mutations (e.g., AR3) in the antisense and RBS sequester distinctly weakened the regulatory activity of the cbIMCbI riboswitch, along with the increased levels of cbIM expression compared to the WT riboswitch (Fig. 4B). Furthermore, the regulatory activity of cbIMCbI riboswitch was abolished by mutated AR1 or R3 (Fig. 4B). In addition, the multisite mutation R1 led to complete loss of cbIM expression (Fig. 4B). Similarly, disruption of Pkn and Pkn’ base pairing differentially impacted the regulatory functions of cbIMCbI.
riboswitch. For example, the site mutations N1, N2, N3, N4, N5 and AN2 rendered the modified riboswitches unable todown-regulate cbIM expression (Fig. 4B). While mutations AN1 and AN3 decreased the regulatory activity of the riboswitches, deletion of Pkn’ (AN5) resulted in complete loss of regulation and cbIM expression (Fig. 4B). Furthermore, site mutation of Pkn (AN4) demonstrated comparable regulatory activity to the WT riboswitch (Fig. 4B). In addition, cbIM expression assays were largely consistent with FbFP reporter assays in these riboswitch-modified strains (Fig. 4B and SI Appendix, Fig. S4A). However, R3- and N4-modified strains demonstrated the VB12 dose-dependent down-regulation of cbIM expression but not of FbFP expression (Fig. 4B and SI Appendix, Fig. S4A). These data suggest that Pkn, Pkn’, antiseguester, and RBS

Fig. 4. cbIMCbl riboswitch regulates cbIM expression by RBS-mediated base pairing. (A) Multiple alignment of VB12 riboswitches. The colored background denotes P0 and P3 stems and their complementary sequences (P0’ and P3’). The conserved core region is shown in italic, bold, and black letters. RBSs are shown in bold and black letters. Red underscores denote the proposed antiseguester and RBS sequester. Purple underscores denote the candidate pseudoknots (Pkn) and complementary pseudoknots (Pkn’). EC, E. coli; ST, S. Typhimurium; SC, Streptomyces coelicolor. The summary table shows the sequences of respective mutations. (B) Western blot analysis of cbIM expression by a series of riboswitches mutated in Pkn, Pkn’, sequester, or antiseguester. (C) Western blot analysis of cbIM expression by riboswitches with paired double mutations in Pkn and Pkn’ or sequester and antiseguester. (D) Western blot analysis of cbIO and tetR expression by WT and the double-mutant riboswitch R:AR3.
sequester are the key regulatory domains of the cbiMCbl riboswitch in P. UF1.

Thus far, it is unclear whether the regulatory activity of the cbiMCbl riboswitch depends on Watson–Crick base pairing within the regulatory (Pkn and antisequester) and their complementary (Pkn′ and RBS sequester) domains. Thus, to elaborate on this notion, we introduced a second mutation into these single mutant bacterial strains to rescue their base pairing (Fig. 4A). Here, most of the double-mutant strains were still unable to regulate the regulatory (Pkn and antisequester) and their complementary (Pkn′ and RBS sequester) domains. Thus, to characterize the mechanistic complexities potentially regulating VB12 in the newly discovered P. UF1 bacterium, we first focused on the genomic region of bacterial CobA. CobA catalyzes the SAM-dependent bismethylation of uroporphyrinogen III synthase (40) to form precorrin-2, the primary precursor of VB12 (18). Thus, deleting cobiA from the bacterial chromosome abolished VB12 production in the ΔcobiA P. UF1 strain. VB12 was fully restored by complementing ΔcobiA P. UF1 with the WT cobiA gene, illuminating the critical role of cobiA in bacterial VB12 biosynthesis.

We also demonstrated that endogenous and exogenous VB12 tightly controlled the expression of cobiA operon via its 5′ UTR within P. UF1 bacterium; a feedback regulatory mechanism via VB12 that was also observed in E. coli and S. Typhimurium (29, 30). Importantly, we documented that VB12 completely inhibited the expression of downstream genes by the cbiMCbl riboswitch at 750 μM, which was significantly higher than env8HyCbl in E. coli (34). This differential regulatory activity may be ascribed to differential scales of VB12 biosynthesis in different bacteria such as P. UF1.

VB12 analogs displayed similar regulatory activities in the riboswitch of P. UF1 but not in E. coli (32), possibly as a result of diverse sequences and secondary structures of VB12 riboswitches. Our structure-based analyses revealed that SL1 and SL3 were highly required for the regulatory function of the cbiMCbl riboswitch, which belong to receptor domains conserved within the VB12 riboswitches from E. coli and S. Typhimurium (32, 33, 41). Furthermore, we also demonstrated that Pkn in SL2 and the complementary Pkn′ were crucial regulatory domains within the cbiMCbl riboswitch, whose regulatory function was notably not dependent on Watson–Crick base pairing within the 2 domains. In contrast, the regulation of env8HyCbl depends on the Watson–Crick base pairing of the “kissing loop” between SL2 and the RBS region (32, 34), indicating that the cbiMCbl riboswitch within P. UF1 may use a different mechanism to control gene expression. Interestingly, we also identified a Watson–Crick base pairing between the RBS sequester and antisequester, whose base pairing was essentially required for regulatory activity of the riboswitch. In E. coli, it is well documented that the env8HyCbl riboswitch regulates gene expression at both transcriptional and translational levels (32, 34). In Listeria monocytogenes and S. Typhimurium, VB12 riboswitches are incapable of controlling their stable abundance, which may contribute to human health (3, 35–37). One of these metabolites is VB12, which crucially impacts the cross-talk between gut microbes and the host (3, 38). Although previously reported data demonstrate how pathogens, including Salmonella (29, 39), regulate the biosynthesis of this vitamin, the control of VB12 biosynthesis in bacteria with bifidogenic properties, particularly probiotic bacteria such as P. UF1, is currently obscure.

Thus, to characterize the mechanistic complexities potentially regulating VB12 in the newly discovered P. UF1 bacterium, we first focused on the genomic region of bacterial CobA. CobA catalyzes the SAM-dependent bismethylation of uroporphyrinogen III synthase (40) to form precorrin-2, the primary precursor of VB12 (18). Thus, deleting cobiA from the bacterial chromosome abolished VB12 production in the ΔcobiA P. UF1 strain. VB12 was fully restored by complementing ΔcobiA P. UF1 with the WT cobiA gene, illuminating the critical role of cobiA in bacterial VB12 biosynthesis.

We also demonstrated that endogenous and exogenous VB12 tightly controlled the expression of cobiA operon via its 5′ UTR within P. UF1 bacterium; a feedback regulatory mechanism via VB12 that was also observed in E. coli and S. Typhimurium (29, 30). Importantly, we documented that VB12 completely inhibited the expression of downstream genes by the cbiMCbl riboswitch at 750 μM, which was significantly higher than env8HyCbl in E. coli (34). This differential regulatory activity may be ascribed to differential scales of VB12 biosynthesis in different bacteria such as P. UF1.

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The transcription of the downstream genes (24, 41), indicating that various VB12 riboswitches may exhibit a distinct regulatory model for gene expression in bacteria with different natures and functions, mainly pathogenic or beneficial bacteria.

The probiotics are defined as live microbial feed supplements with beneficial properties, which potentially benefit the host when administered in adequate amounts (42). *Propionibacterium* species are currently of great interest for their beneficial effects as probiotics and are being applied to human dietary consumption, including Swiss cheese (43). We previously demonstrated that P. UF1 increases the frequency of protective T cells involved in fortifying the mucosal barrier function and regulating the intestinal inflammation against pathogenic infection (25). Accordingly, the impact on the microbiome and the host immune homeostasis can significantly enhance VB12 within P. UF1. It is conceivable that the genetic modification of this riboswitch biosynthesis, and that the genetic modification of this riboswitch could significantly enhance VB12 within P. UF1 (Fig. 6). This riboswitch may serve as an attractive modulator within the intestinal microflora, and that P. UF1 increases the frequency of protective T cells involved in fortifying the mucosal barrier function and regulating the intestinal inflammation against pathogenic infection (25).

In summary, there is currently great interest in sustaining the probiotics and are being applied to human dietary consumption, including Swiss cheese (43). We previously demonstrated that P. UF1 increases the frequency of protective T cells involved in fortifying the mucosal barrier function and regulating the intestinal inflammation against pathogenic infection (25).

**Experimental Procedures**

**Bacterial Strains and Growth.** *E. coli* NEB 5-alpha (New England Biolabs) used for plasmid construction and *E. coli* Rosetta (DE3; Sigma-Aldrich) for protein expression were grown in Luria–Bertani medium at 37 °C. The P. UF1 and its genetically modified strains were grown at 30 °C in MR5 medium (Difco) with 1% (wt/vol) sodium lactate (Thermo Fisher Scientific) or Poznan medium (46) in an anaerobic chamber (model AS-580; Anaerobe Systems). Antibiotics were added to the medium at the following final concentrations: 1 μg/mL chloramphenicol (Sigma-Aldrich) and 10 μg/mL ampicillin (for *E. coli* isogenic strains). For deleting the *cobA* gene from P. UF1, a 644-bp internal fragment of *cobA* was PCR amplified from the P. UF1 chromosome using primers cobAbamF and cobAbamR. The purified fragment was cloned into the pUCC plasmid (25), for plasmid construction and introduced into P. UF1 or ∆cobA P. UF1 by electroporation.

To overexpress the *cobA* operon in ∆cobA P. UF1, the operon with its cognate promoter was also amplified from P. UF1 with primers cibm-sbf-F and cibm-sbf-R. After digestion with SbfI and HindIII, the purified PCR products were cloned into the pACYC184 vector. The thus constructed plasmid served as the baseline for the calculation of fluorescence intensity.

**Bacterial Cultures.** Bacterial cultures were centrifuged at 15,000 × *g* for 10 min at 4 °C and stored twice in PBS. The cells were disrupted by boiling for 15 min in 0.1 M phosphate buffer containing 0.01% potassium cyanide at pH 6.0. After centrifugation at 15,000 × *g* for 2 min, the supernatants were collected and passed through 0.22-μm filters (EMD Millipore). VB12 in the filtrates was quantified using the Agilent 1200 Infinity II LC system composed of an automated sampler (G4282B), a gradient pump (G4281B), and a variable wavelength detector (G4284B).

**VB12 Extraction and Analysis.** Bacterial cultures were centrifuged at 15,000 × *g* for 10 min at 4 °C and stored twice in PBS. The cells were disrupted by boiling for 15 min in 0.1 M phosphate buffer containing 0.01% potassium cyanide at pH 6.0. After centrifugation at 15,000 × *g* for 2 min, the supernatants were collected and passed through 0.22-μm filters (EMD Millipore). VB12 in the filtrates was quantified using the Agilent 1200 Infinity II LC system composed of an automated sampler (G4282B), a gradient pump (G4281B), and a variable wavelength detector (G4284B).

**FlFp Reporter Assays.** To measure FlFP fluorescence intensity, bacteria were resuspended in the following mobile phase (46) containing 0.15 M NaH2PO4 (pH 3.5) and methanol (75:25, vol/vol) in an Agilent C18 column (Eclipse Plus C18, 3.5 μm, 5 μl × 150 mm column) at 20 °C, with a flow rate of 1.0 mL/min. The total HPLC runtime for each sample was 15 min, and the injection volume was 20 μL. The detector wavelength was set at 362 nm. Quantitation was based on peak area and the standard curve of VB12.

**Preparation of Anti-CobA Serum Antibodies.** To generate CobA polyclonal antibodies, the pET21b-cobA expression plasmid was constructed by PCR amplification of the cobA gene using primers 21-cobA-bamF and 21-cobA-xhoR.
CobA expression was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG). Cell lysates were separated by 12% SDS-PAGE, and the CobA proteins were excised from the gel and used to immunize C57BL/6 mice, resulting in anti-CobA serum antibodies. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Florida (protocol 201708484). Mice were maintained in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care.

Western Blot Analysis. Bacteria were cultivated for 3 d and then washed 3 times, followed by lysozyme digestion (10 mg/ml) for 2 h at 37°C. Cell lysates were separated by 12% SDS-PAGE, transferred to PVDF membranes (Sigma-Aldrich), and blocked with Odyssey blocking buffer (Li-Cor Biosciences). Subsequently, membranes were incubated with anti-His-tag antibody (Thermo Fisher Scientific), anti-TeT antibody (Takara Bio), anti-CobA serum antibodies, or anti-LspA serum antibodies (available in our laboratory) for 2 h at room temperature in the blocking buffer with 0.1% Tween 20. After washing with TBST (20 mM Tris, 150 mM NaCl and 0.1% Tween 20, pH 7.4), membranes were incubated with IRDye 680RD goat anti-mouse secondary antibody (Li-Cor Biosciences) for 1 h at room temperature in the blocking buffer with 0.1% Tween 20. After washing with TB5, the proteins were detected using the Odyssey infrared imaging system (Li-Cor Biosciences). LspA served as an internal control.

qRT-PCR. Total RNA was extracted from various bacterial cultures using TRIzol reagent (Thermo Fisher Scientific), and the RNA mixtures were further purified using the Avertin Total RNA Mini Kit (Bio-Rad). The cDNA was synthesized from total RNA using the iScript Advanced cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96 real-time PCR system (Bio-Rad) using the primers listed in SI Appendix, Table S2. Results were normalized to those obtained from the grol2 gene.

Data Availability. All relevant data supporting the findings of this study are available in the paper and supplemental materials. The data that support the findings of this study are available from the corresponding author on reasonable request.

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Table S2. Results were normalized to those obtained from the grol2 gene.

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