JOURNAL OF BACTERIOLOGY, Sept. 2011, p. 4405–4416 Vol. 193, No. 17
0021-9193/11/$12.00 doi:10.1128/JB.05045-11

An ABC-Type Cobalt Transport System Is Essential for Growth of *Sinorhizobium meliloti* at Trace Metal Concentrations▽†

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Received 8 April 2011/Accepted 17 June 2011

We report expression and mutant phenotypes for a gene cluster in *Sinorhizobium meliloti*, designated *cbtJKL*, that has been shown to encode an ABC-type cobalt transport system. Transcription of *cbtJKL* initiated 384 nucleotides upstream from the *cbtJ* translation start codon, and the resulting 5′ region contained a putative B_{12} riboswitch. Expression of the *cbtJKL* genes appeared to be controlled by (cobalt-loaded) cobalamin interacting at the B_{12} riboswitch, since (i) a putative B_{12} riboswitch was located within this large upstream region, (ii) *cbtJ* transcription was repressed upon addition of cobalt or vitamin B_{12}, and (iii) deletions in the B_{12} riboswitch resulted in constitutive *cbtJKL* transcription. Insertion mutants in *cbtJKL* failed to grow in LB medium, and growth was restored through the addition of cobalt but not other metals. This growth phenotype appeared to be due to the chelation of cobalt present in LB, and *cbtJKL* mutants also failed to grow in minimal medium containing the chelating agent EDTA unless the medium was supplemented with additional or excess cobalt. In uptake experiments, $^{57}Co^{2+}$ accumulation was high in wild-type cells expressing the *cbtJKL* genes, whereas wild-type cells in which *cbtJKL* expression was repressed showed reduced accumulation. In *cbtJKL* mutant cells, $^{57}Co^{2+}$ accumulation was reduced relative to that of the wild type, and presumably, this residual cobalt transport occurred via an alternate ion uptake system(s) that is not specific to cobalt. In symbiosis, the alternate system(s) appeared to mediate cobalt transport into bacteroid cells, as low *cbtJKL* expression was detected in bacteroids and *cbtJKL* mutants formed N_2-fixing nodules on alfalfa.

Cobalt is an essential trace element for many living organisms, as it plays a key biological role as the centrally coordinated ion in cyclic tetrapyrroles known as corrin rings (19, 40). Corrinoids, including the coenzyme vitamin B_{12} (adenosylcobalamin [AdoCbl]) and its cobalamin (Cbl) derivatives, are coenzymes in a number of central metabolic reactions. Cobalt can also be associated directly with cobalt-dependent enzymes (noncorrin enzymes) (32). To acquire sufficient cobalt for metabolism, bacteria have high-affinity uptake systems to scavenge Co^{2+} from the environment, where it is often available only in trace amounts (16, 56, 57, 69). When external metal concentrations are very high, Co^{2+} accumulation may become toxic, and excess Co^{2+} can be removed from cells by efflux systems (43, 63).

The NikMNQO and CbiMNQO uptake systems preferentially transport Ni and Co, respectively, and are common among bacteria and archaea (see references 55 to 58 and 74). These systems are members of a class of modular transporters which have substrate-specific components that are integral membrane proteins (CbiMN), energy coupling factor (ECF) transporters that consist of an ATPase typical of the ATP binding cassette (ABC) superfamily (CbiO), and a characteristic transmembrane protein (CbiQ) (57). These systems lack a periplasmic/extracellular substrate binding protein (11).

There are also a number of secondary, non-ABC-type Ni^{2+}/Co^{2+} transporters (16) whose metal ligand preference correlates in many cases with the genomic localization of the transport genes, whether they are adjacent to clusters for Ni- or Co-containing enzymes or to those for enzymes involved in Cbl biosynthesis (27, 55). For example, the *Rhodococcus rhodochrous nhlF* gene, encoding cobalt permease, lies beside the gene encoding a nitrile hydratase that contains a noncorrin Co^{2+} (13, 33). Other divalent cation transport systems, such as ZupT (25) and Mg^{2+} transport systems (CorA) (64), can also mediate Co^{2+} uptake, but these are not likely to be physiologically relevant because of their poor affinity for Co^{2+}.

Various bacteria are known to take up cobalamins from the environment, and the system of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* consists of an outer membrane TonB-dependent transporter, BtuB (2), the ABC-type transport proteins BtuC and BtuD, and the periplasmic binding protein BtuF (5). Genetically, *btuF* is not linked to the *btuCED* operon (6). BtuC is the transmembrane component and BtuD the ATP-binding component. The function of BtuE is uncertain and not required for vitamin B_{12} transport (54). Although the *btuF* and *btuCD* genes are often annotated in bacteria, sequence similarities among ABC-type siderophore/heme/vitamin B_{12} family transport systems (34) make it difficult to identify the substrate(s) transported by these systems (74).

Riboswitches are conserved RNA elements in the 5′-untranslated region (5′-UTR) of prokaryotic mRNA molecules that modulate transcription attenuation or translation attenuation through the binding of specific effectors, such as vitamin B_{12}, lysine, glycine, adenine, guanine, or glucosamine-6-phosphate (71). The regulated genes are usually involved in the biosynthesis or transport of the particular effector metabolite.

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 1 July 2011.

For example, riboswitch sequences are often found 5′ of the *cbiMNQO* genes (74). In *E. coli* and *S. enterica*, a B_{12} riboswitch represses both transcription and translation of *btuB* (23, 50, 51), and conserved elements of the B_{12} riboswitch are found 5′ of the cobalamin biosynthesis (*cob*) operon (42). Within the B_{12} riboswitch region, there is a conserved motif called the B_{12} box that is essential for AdoCbl-dependent regulation (44, 45, 52).

Sinorhizobium meliloti is a Gram-negative alphaproteobacterium that forms N_2-fixing root nodules on its plant host, alfalfa. Co^{2+} is required for the growth of *S. meliloti* and other rhizobia (36, 70) and is required for efficient nitrogen fixation in the *Sinorhizobium*-alfalfa symbiosis (15). *S. meliloti* synthesizes vitamin B_{12}, and the Cbl-dependent enzymes methylmalonyl-coenzyme A (CoA) mutase, methionine synthase, and ribonucleotide reductase have been identified (9, 10, 14, 30, 62), as well as putative Cbl biosynthetic genes (4). The *S. meliloti* BluB protein was recently shown to catalyze a missing step in vitamin B_{12} synthesis by cannibalizing flavin to form 5,6-dimethylbenzimidazole, the lower ligand of vitamin B_{12} (7, 67). Recently, Co^{2+} was observed to be required for survival of an *S. meliloti* mutant that lacks the phosphotransferase system enzyme Hpr (48). BioM (CbiO homolog) and BioN (CbiQ homolog) in *S. meliloti* have been reported to import biotin (18), and these along with BioY appear to represent an ECF-type biotin transporter. A putative ABC-type cobalamin transporter (BtuF [Smb20056], BtuC [Smb20057], and BtuD [Smb20058]) was identified on the basis of sequence similarity to the siderophore/heme/vitamin B_{12} family and the presence of a B_{12} riboswitch in the 5′-UTR (69). Here we report that the ABC-type system encoded by the *smb20056*, *smb20057*, and *smb20058* genes transports Co^{2+} but not cobalamin. We demonstrate that this transport system is required for growth of free-living cells at trace element concentrations of cobalt and that it is not required for symbiotic N_2 fixation in *S. meliloti*. We designate the transport system genes *cbtJ* (*smb20056*), *cbtK* (*smb20057*), and *cbtL* (*smb20058*), and we present expression data suggesting that *cbtJKL* expression is repressed by Co^{2+} and cobalamin via a 5′ B_{12} riboswitch.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* cultures were grown at 37°C in Luria broth (LB), and *S. meliloti* cells were grown at 30°C in LB containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$. The defined M9-succinate medium contained 1× M9 salts (Difco) supplemented with 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 μg ml^{-1} biotin, and 43 nM $CoCl_2$ (10 ng $CoCl_2 \cdot 6H_2O$/ml) (60), with 15 mM succinate as the sole carbon source. MOPS (morpholinepropanesulfonic acid)-buffered minimal medium was used as previously described (73). One milliliter of a 1,000× trace element solution was added per liter of minimal medium. The trace element solution (1,000×) consisted of the following amounts of compounds per liter of H_2O: 1.0 g H_3BO_3, 1.0 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $CuSO_4 \cdot 5H_2O$, 0.5 g $MnCl_2 \cdot 4H_2O$, 1.0 g $NaMoO_4 \cdot 2H_2O$, 10.0 g EDTA, and 2.0 g NaFe-EDTA. We note that the 43 nM $CoCl_2$ that is routinely added to our M9 and MOPS minimal media (73) is in excess of that required for growth of *cbtJKL* mutants, and this accounts for the growth of *cbtJKL* mutant transconjugants on M9-succinate medium that is shown in Fig. 1A. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis of the minimal medium employed in our experiments, with no added $CoCl_2$, detected 2 nM cobalt.

Antibiotics were used at the following concentrations: streptomycin, 200 μg ml^{-1}; gentamicin, 60 μg ml^{-1} (10 μg ml^{-1} for *E. coli*); and tetracycline, 10 μg ml^{-1} (15 μg ml^{-1} for *E. coli*).

To identify substances required for growth of *S. meliloti* mutants in LB, cells were initially grown for 36 h in M9-succinate medium, washed three times with 0.85% NaCl, and then inoculated into LB (optical density at 600 nm [OD_{600}], ~0.01). Following incubation for 16 h, the LB-grown cells were used to inoculate fresh LB (OD_{600}, ~0.01) supplemented alternatively with each of the ingredients used in M9 medium. The subcultures were grown for 16 h, and growth rates were monitored by measuring the OD_{600}. To investigate whether Co^{2+} was required for growth in minimal medium, *S. meliloti* strains were grown in LB with 5 μM $CoCl_2$, washed three times with 0.85% NaCl, and then subcultured in MOPS medium (1) containing 2 mM inorganic phosphate (MOPS-P2) with $CoCl_2$ (0 to 20 nM). Subsequently, these MOPS-P2 cultures were subcultured again into MOPS-P2 medium with 0 to 20 nM $CoCl_2$, and growth (OD_{600}) was monitored.

DNA and RNA manipulations and microarrays. DNA isolation, transformation, restriction, and ligation were performed by standard procedures (61). Oligonucleotide synthesis (Table 2) and DNA sequencing were performed at MobixLab (McMaster University, Hamilton, Ontario, Canada). To identify the *cbtJ* transcriptional start site through primer extension reactions, total RNA was isolated from RmP110 grown in LB or M9-succinate medium as described previously (73). Primers ML18489 and ML18490 were end labeled with [γ-^{32}P]ATP, and following the primer extension reaction, the product was loaded onto a 6% acrylamide–7 M urea sequencing gel and electrophoresed alongside a sequencing ladder generated by using the same primer with plasmid pTH2240 as the DNA template.

Microarray chips were purchased from NimbleGen Systems Inc., Madison, WI. Cells from aerated log-phase cultures (250 ml) (OD_{600}, 0.4 to 0.8) were harvested by centrifugation, RNAs were extracted, and cDNAs were end labeled with biotin. Cells were grown in five different media in triplicate to give a total of 15 RNA samples. The media were LB, M9 plus 15 mM glucose, M9 plus 15 mM succinate, M9 plus 5 mM protocatechuate (PCA), and M9 plus 5 mM PCA plus 15 mM glucose. Hybridization as well as probe intensity analysis was performed by NimbleGen, following company procedures. The custom-made arrays contained 385,298 24-mer oligonucleotide probes targeting sequences within annotated start and end positions of 6,269 annotated *S. meliloti* features (mostly protein coding sequences). The raw data, consisting of probe intensities, were quantile normalized across all experimental replicates. The median intensity of all probes within the annotated region was used as an uncorrected measure of gene expression for each experiment. Background expression was estimated for each experiment by simulating a gene through 10,000 random samples drawn from the normalized intensities of a null probe set of randomly generated sequences. Data for the *cbtJKL* locus are presented in Table S1 in the supplemental material and in Fig. 1.

Construction of plasmid integration mutants and reporter gene fusion strains. To generate promoter fusions to the *gfp-lacZ* reporter genes, a 535-bp DNA fragment upstream of the *cbtJ* open reading frame (ORF) was PCR amplified using primers P20056F and P20056R and then cloned into the BglII-XhoI sites in pTH1703 to obtain pTH1968 (Fig. 1A). Single-crossover homologous recombination of pTH1968 into the *S. meliloti* genome resulted in the fusion of the *cbtJ* promoter region to *gfp-lacZ* and preserved a functional copy of the promoter and all genes at this locus in strain RmP831 (Fig. 1B). To construct *gfp-lacZ* fusions to *cbtJ* and *cbtL*, internal fragments of *cbtJ* (543 bp) and *cbtL* (571 bp) were PCR amplified using the 20056intF and 20056intR primers and the 20058intF and 20058intR primers, respectively, and cloned into the BglII-XhoI sites in pTH1703 to obtain transcriptional fusion plasmids pTH1969 and pTH2030, respectively (Fig. 1A). Cointegration of pTH1969 into the *S. meliloti* genome generated a fusion of the 5′ end of *cbtJ* to the *gfp-lacZ* genes and impaired the expression of all downstream genes in this locus in RmP833 (Fig. 1B). However, integration of pTH2030 into *S. meliloti* generated a 5′-*cbtL*::*gfp-lacZ* fusion that maintained functional copies of *cbtJ* and *cbtK* but impaired the expression of *cbtL*, *smb20059*, and *smb20060* in strain RmP889 (Fig. 1B). To demonstrate the function of the *smb20059* and *smb20060* genes in Co^{2+} uptake, a 486-bp fragment from the 3′ end of *cbtL* and a 1,201-bp fragment from the 3′ end of *smb20059* to the 5′ end of *smb20060* were PCR amplified using the 20058endF and 20058endR primers and the B20059F and ML19261 primers, respectively. These were cloned into the BglII-XhoI sites in pTH1703 to obtain pTH1970 and pTH2270, respectively (Fig. 1A). Integration of plasmid pTH1970 into the *S. meliloti* genome resulted in strain RmP835, in which the *cbtJ*, *cbtK*, and *cbtL* genes were functional, whereas the expression of *smb20059* and *smb20060* was disrupted (Fig. 1B). Integration of pTH2270 into *S. meliloti* resulted in a dysfunctional *smb20060* gene in RmP1485 (*smb20060*::*gfp*$^+$-*lacZ*) (Fig. 1B).

To abolish Cbl biosynthesis in *S. meliloti*, a *cobT* mutant was constructed. *S. meliloti* CobT (SMc00701) shares 91% amino acid sequence identity to the *Pseudomonas denitrificans* CobT protein, a subunit of the cobalt chelatase complex CobNST, which is required for insertion of cobalt into hydrogenobyrinic

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic or genotype[a]	Source or reference
Strains		
E. coli strains		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)	Lab collection
MT616	Conjugation helper strain carrying pRK600	20
S. meliloti strains		
RmP110	Rm1021 with changed wild-type *pstC*; Sm^r	74
RmP831	RmP110::pTH1968 *cbtJp*::*gfp*$^+$-*lacZ* Sm^r Gm^r (CbtJ$^+$ CbtK$^+$ CbtL$^+$)	This study
RmP833	RmP110::pTH1969 *cbtJ*::*gfp*$^+$-*lacZ* Sm^r Gm^r (CbtJ$^-$ CbtK$^-$ CbtL$^-$)	This study
RmP835	RmP110::pTH1970; 3′ end of *cbtK*::*gfp*$^+$-*lacZ*; Sm^r Gm^r (CbtJ$^+$ CbtK$^+$ CbtL$^+$)	This study
RmP889	RmP110::pTH2030 *cbtL*::*gfp*$^+$-*lacZ* Sm^r Gm^r (CbtJ$^+$ CbtK$^+$ CbtL$^-$)	This study
RmFL3108	RmP110::pFL3108 *cbtK*::*gfp*$^+$-*lacZ* Sm^r Gm^r (CbtJ$^+$ CbtK$^-$ CbtL$^-$)	This study
RmP1477	RmP110::*cobT*::pTH2293 Sm^r Gm^r	This study
RmP2361	RmP110 ΔB152 (pSymB Δ61,240–74,302), i.e., Δ*cbtJKL*	B. Peduska and T. M. Finan
RmP2364	RmP2361(pTH2653)	Peduska and Finan
Plasmids		
pFL3108	pTH1522 carrying 3′ end of *cbtK*::*gfp*$^+$-*lacZ* and 5′ end of *cobB*; Gm^r	8
pFL3283	pTH1522 carrying *cobT*::*gfp*$^+$-*lacZ*; Gm^r	8
pTH1703	Transcriptional reporter plasmid; Gm^r	8
pTH1919	pBBRMCS-3 with *tetR-tetA* (NsiI-BglII) from RK2	This study
pTH1968	pTH1703 carrying *cbtJp* (P20056F-P20056R); Gm^r	This study
pTH1969	pTH1703 carrying *cbtJ*::*gfp*$^+$-*lacZ* (20056intF-20056intR); Gm^r	This study
pTH1970	pTH1703 carrying 3′ end of *cbtL*::*gfp*$^+$-*lacZ* (20058endF-20058endR); Gm^r	This study
pTH2030	pTH1703 carrying *cbtK*::*gfp*$^+$-*lacZ* (20058intF-20058intR); Gm^r	This study
pTH2213	pTH1919 derivative with ΔBglII; Tc^r	This study
pTH2221	pTH2213 carrying Ω terminator (ML18596-ML18597); Tc^r	This study
pTH2224	pTH2221 carrying *gfp*$^+$-*lacZ*; Tc^r	This study
pTH2237	pTH2224 carrying *cbtJp*::*gfp*$^+$-*lacZ* (ML18589-ML18594); Tc^r	This study
pTH2238	pTH2224 carrying *cbtJp*-5′-UTR 5′-UTR::*gfp*$^+$-*lacZ* (ML18595-ML18589)	This study
pTH2239	pTH2224 carrying *cbtJp*::*gfp*$^+$-*lacZ* (MLML18590-ML18594); Tc^r	This study
pTH2240	pTH2224 carrying 5′-UTR and 5′ region of *cbtJ* (ML18590-ML18594); Tc^r	This study
pTH2256	pTH2224 carrying *cbtJp*-B12 riboswitch::*gfp*$^+$-*lacZ* (ML18561-ML18589, ML18560-ML18594, and ML18589-ML18594)	This study
pTH2270	pTH1703 carrying *smb20060*::*gfp*$^+$-*lacZ* (B20059F-ML19261); Gm^r	This study
pTH2293	pFL3283 with Δ*gfp*$^+$-*lacZ*; Gm^r	This study
pTH2303	pTH2224 carrying *cobPp* (ML20074-ML20075); Tc^r	This study
pTH2653	pLAFR1 carrying positions 56,613 to 81,543 of pSymB (i.e., includes the *cbtJKL* genes); Tc^r	Peduska and Finan

[a] For plasmids, the primer pairs used to amplify genes are indicated in parentheses. Gm, gentamicin; Sm, streptomycin; Tc, tetracycline; Cm, chloramphenicol.

acid a,c-diamide in vitamin B_{12} biosynthesis (12). A pTH1522 library fusion plasmid, pFL3283 (8), was restricted with SpeI-XhoI to remove the *gfp-lacZ* reporter genes, filled in by use of Klenow polymerase, and then self-ligated to obtain pTH2293, which carries the 3′ region of *cobS* (198 bp), the 5′ region of *cobT* (390 bp), and an intergenic region (50 bp) of the *cobST* genes. Strain RmP1477 (*cobT*) was generated following integration of pTH2293 into the *S. meliloti* chromosome and was selected on LB medium containing 10 μM AdoCbl. RmP1477 was a Cbl auxotroph and grew only on LB or M9 medium supplemented with AdoCbl.

To construct the replicating reporter plasmid pTH2224, the pTH1703 plasmid (8) was digested with PstI, filled in by use of Klenow polymerase, and then digested with XhoI to obtain a 4,037-bp fragment carrying the *gfp-lacZ* genes. The BglII site in pTH1919 (Table 1) was deleted by BglII digestion and fill-in by use of Klenow DNA polymerase to obtain pTH2213. A 136-bp Ω terminator from pTH1703 was PCR amplified using primers ML18596 and ML18597 and cloned into the SacI-XbaI sites in pTH2213 to obtain pTH2221. The pTH2221 plasmid was cut with KpnI, filled in by use of Klenow polymerase, cut with XhoI, and then ligated with the *gfp-lacZ* fragment (XhoI-ΔPstI) to yield pTH2224.

To measure transcription from the *cbtJ* promoter in RmP110 (wild type) and RmP1447 (*cobT* mutant), a 983-bp fragment upstream from *cbtJ* was PCR amplified using primers ML18589 and ML18594, cut with SpeI-BamHI, and cloned into the XbaI-BglII sites in pTH2224 to obtain the transcriptional fusion plasmid pTH2237 (see Fig. 6). In order to reveal the role of the B_{12} regulatory element in *cbtJ* expression, the putative B_{12} riboswitch (see Fig. 4C and 6A) upstream of the *cbtJ* ORF was deleted by two-step PCR. A 627-bp fragment upstream of the B_{12} riboswitch and another 221-bp region between the riboswitch and the *cbtJ* ORF were PCR amplified using primer pairs ML18589-ML19561 and ML19560-ML18594, respectively. The two products were purified and annealed as the template for a second PCR using primers ML18589 and ML18594. The second PCR product, carrying an 806-bp region lacking the B_{12} riboswitch sequence, was cloned into the SpeI-BamHI sites in pTH2224 to obtain the transcriptional *gfp-lacZ* fusion plasmid pTH2256 (see Fig. 6A). To further analyze the regulatory sequence, a 595-bp region upstream of the transcription start site and a 147-bp region within the 5′-UTR were PCR amplified using primer pairs ML18589-ML18595 and ML18590-ML18594, respectively, and then cloned into the SpeI-BamHI sites in pTH2224 to obtain pTH2238 and pTH2239, respectively (see Fig. 6A). A DNA fragment carrying the 147-bp region upstream of the *cbtJ* ORF and the 5′ *cbtJ* coding region (333 bp) was PCR amplified using primers ML18590 and ML18594 and then inserted into pTH2224 to obtain pTH2240, which was used for DNA sequencing reactions.

To generate an *S. meliloti cobP* promoter fusion, an 871-bp intergenic region between *cobP* and *smc04306* was PCR amplified using primers ML20074 and ML20075 and inserted upstream of *gfp-lacZ* in pTH2224 to obtain the transcriptional fusion plasmid pTH2303 (see Fig. 6B). β-Galactosidase (LacZ) activity and green fluorescent protein (GFP) were measured as previously described (8).

Co^{2+} uptake assays. *S. meliloti* RmP110 (wild type) was grown in LB or LB supplemented with 5 μM $CoCl_2$. Strain RmP833 (*cbtJ*::*gfp*$^+$-*lacZ*) was grown in LB supplemented with 5 μM $CoCl_2$ for 16 h, washed three times with 0.85% NaCl, and then subcultured in LB or LB supplemented with 5 μM $CoCl_2$. All cultures for Co^{2+} uptake assays were grown to an OD_{600} of approximately 1.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]
P20056F	ATCTCTCGAGGACGAACGTAATAGTATAAC
P20056R	TCATAGATCTTCCCCGAGTGTGAGGCCGCT
20056intF	GACACTCGAGAATTGCGGACGGCAGATCAC
20056intR	GGCCAGATCTGCCTCTTCTCGGCTTTTCAG
20058intF	GACTCTCGAGGCGAGCGGGGTTTCATGGTC
20058intR	GATCAGATCTCCGATGTCGAGATGGTTGGT
20058endF	TCGCCTCGAGCGCCGGAGGACGATGCGATC
20058endR	GTCCAGATCTCAGGCTCCGACGGCGGCGATTG
B20059F	GGAAGTCGACGGTCCGTTTCATACTGGCCG
ML19261	TTCAAGATCTCGTCCAGCCGCTTGTTGATC
ML18489	ATGAGCGGTCGTTTCAGAAAGCTGTTCATG
ML18490	ACAGTTGCGGGGGCAGCCACGGTTTTGGTC
ML18589	GAGCACTAGTACAGGCATTCCCCCATACAT
ML18590	CCGAACTAGTCTGTTGACGAACGTAATAGT
ML18593	CTTCGGATCCGCTAGCCTTGGCAAGTACGCTT TCGAAGCT
ML18594	GTTTGGATCCGCTAGCCATGAATAGTCCCCGA GTGT
ML18595	GGGTGGATCCGCTAGCGGTCCCAGTTAATGTG GAAC
ML18596	TAACGAGCTCCGGTGGATGACCTTTTGAAT
ML18597	CCGGTCTAGAGGTGATTGATTGAGCAAGCT
ML19560	CCGTCACGACGTTGGATCGATTTGGTCCCAGT TAATGTGGAACGC
ML19561	GTTCATTAACTGGGACCAAATCGATCCAACGT CACGGGC
ML20074	GCGCGAATTCCTGATCCGGACCGCTT
ML20075	CGAGGGTACCGCATGCCATTACCGCTTGCCAT AGCG

[a] Restriction sites that were engineered into the primers are underlined.

Cells were harvested by centrifugation at 4°C at 4,000 × *g* for 20 min, washed three times with transport buffer (50 mM MOPS, pH 7.4, 10 mM $MgCl_2$, and 15 mM succinate), and then resuspended in the buffer to an OD_{600} of about 2 for LB-grown RmP110 and to an OD_{600} of approximately 10 for other strains. Sixty microliters of the cell suspension was added to 510 μl of transport buffer, and following incubation at 30°C for 5 min, assays were initiated through the addition of 30 μl of 10 μM $CoCl_2$ labeled with $^{57}Co^{2+}$ (specific activity, 265 mCi/mmol) to a final concentration of 0.5 μM. Aliquots (0.1 ml) were removed from the assay mixture at different times, immediately passed through nitrocellulose membranes (pore size, 0.45 μm) (HAWP 02500; Millipore, Bedford, MA) that had been presoaked in the same buffer, and immediately washed with the transport buffer (total of 8 ml). The filters were dried and counted using a model 1480 automatic gamma counter (PerkinElmer). All transport assays were performed in triplicate. For chase experiments, a 100-fold excess of unlabeled $CoCl_2$ was added to the $^{57}Co^{2+}$ uptake assay solution at 10 min.

Plant growth and gene expression in nodules. Alfalfa growth in a nitrogen-deficient growth medium was set up as previously described (1). Plant shoots and nodules were obtained 4 weeks after inoculation. The plant shoots were dried in an oven, and the dry weights were used as an index of symbiotic N_2 fixation. Preparation of bacteroids from the nodules was carried out as described previously (73), and β-galactosidase activity assays were performed in microtiter plates as described previously (8). β-Galactosidase specific activities were expressed as follows: $(A_{420} \times 1{,}000)\ (\text{time})^{-1}\ (\text{amount of bacteroid extract})^{-1}\ (\text{concentration of protein})^{-1}$, where time is in minutes, amount of bacteroid extract is in milliliters, and concentration of protein is in mg ml^{-1}.

Biochemicals and radiochemicals. Cyanocobalamin (CNCbl or vitamin B_{12}), AdoCbl, and $CoCl_2 \cdot 6H_2O$ were purchased from Sigma-Aldrich Canada Ltd. $^{57}CoCl_2$ (specific activity, 381 Ci/mmol) was obtained from Amersham Biosciences, GE Healthcare. ICP-MS analysis of the culture media was performed by ActLabs, Ancanster, Ontario, Canada.

Microarray data accession number. The microarray data are available in the CIBEX database under accession number CBX157.

RESULTS

***cbtJKL* (*smb20056*, *smb20057*, and *smb20058*) genes are required for growth in LB medium.** The *cbtJKL* genes, located on the pSymB megaplasmid of *S. meliloti*, were originally annotated *smb20056*, *smb20057*, and *smb20058*, respectively (21, 24). These encode a periplasmic substrate-binding protein (CbtJ), a permease (CbtK), and an ATP-binding protein (CbtL) of an ABC-type transport system. As discussed below, these genes were reannotated *btuF*, *btuC*, and *btuD* in the current *S. meliloti* genome database (4). Based on our data and unpublished work (J. Cheng et al., submitted for publication), we have designated these genes the cobalt transporter genes *cbtJ*, *cbtK*, and *cbtL*, respectively, and we use these names throughout this work. A fourth gene, *smb20059*, annotated a putative *S*-adenosylmethionine (SAM)-dependent methyltransferase gene, is located directly downstream of *cbtL*. The last 2 nucleotides (nt) of *smb20059* overlap with the insertion sequence element IS*Rm5* (Fig. 1A).

Our interest in the *cbtJKL* gene cluster arose from the observation that *S. meliloti* plasmid integration recombinants which are genotypically *cbtJ*$^+$, *cbtK*, *cbtL*, and *smb20059* negative could not be recovered on LB medium (8) (SmFl3108 in Fig. 1). Microarray experiments using mRNA from wild-type cells showed that the *cbtJKL* and *smb20059* genes were highly expressed in cells grown in LB, whereas only background expression was observed in minimal medium (M9) with glucose, succinate, or protocatechuate as a carbon source (see Fig. S1 in the supplemental material). We therefore investigated whether *S. meliloti cbtJKL* mutants could be recovered on M9-succinate medium and found this to be the case. Recombinants RmP831, RmP835 (*smb20059* negative), and RmP1485, which are all *cbtJ*$^+$ *cbtK*$^+$ *cbtL*$^+$, were able to grow on both LB and M9-succinate media. However, mutants RmP833, RmFL3108, and RmP889, which are disrupted in one or more genes of the *cbtJKL* operon, could not grow on LB, although they could grow on M9-succinate (Fig. 1A). Both the microarray data and the overlapping structure of the *cbtJKL* genes strongly suggest that the *cbtJKL-smb20059* genes are transcribed as an operon. Accordingly, since the integration mutations have polar effects on downstream genes, we have formally established that *cbtL* is required for growth on LB and that *smb20059* is not, as strain RmP835 (*smb20059* mutant) grew on LB.

Co^{2+} is required for growth of *cbtJKL* mutants under free-living conditions. The results suggested that growth of *cbtJKL* mutant cells in LB required a component present in minimal medium. To identify that component(s), the ingredients of M9 medium, including the trace elements, were added individually to LB, and growth of the *cbtJKL* mutants was monitored by measuring the OD_{600}. The results from these experiments showed that *cbtJKL* mutants (RmP833, RmFL3108, and RmP889) grew poorly in LB unless the medium was supplemented with $CoCl_2$ (Fig. 2A and data not shown). Growth of the *cbtJKL* mutants was strongly dependent on the concentration of $CoCl_2$ added to the LB medium. At 0.5 μM $CoCl_2$, the mutants grew to an optical density that was approximately 25% that of the wild-type strain RmP110, and growth was essentially rescued with 5 μM $CoCl_2$ (Fig. 2A). Strain RmP2361, in which a 50-kb region including the *cbtJKL* genes was deleted, grew only in LB medium supplemented with cobalt. Cosmid clones carrying the wild-type *cbtJKL* genes allowed this mutant to grow in LB without added cobalt. Because cobalt transport is often linked to the transport of nickel and other metal ions (16), we investigated whether the apparent requirement of the *cbtJKL* mutants for Co^{2+} could be met by addition of Zn, Ni, or Fe ions. When LB was supplemented with 2 μM $ZnSO_4$, 2 μM $NiSO_4$, or 1 mM $FeCl_3$, none of the metal ions could

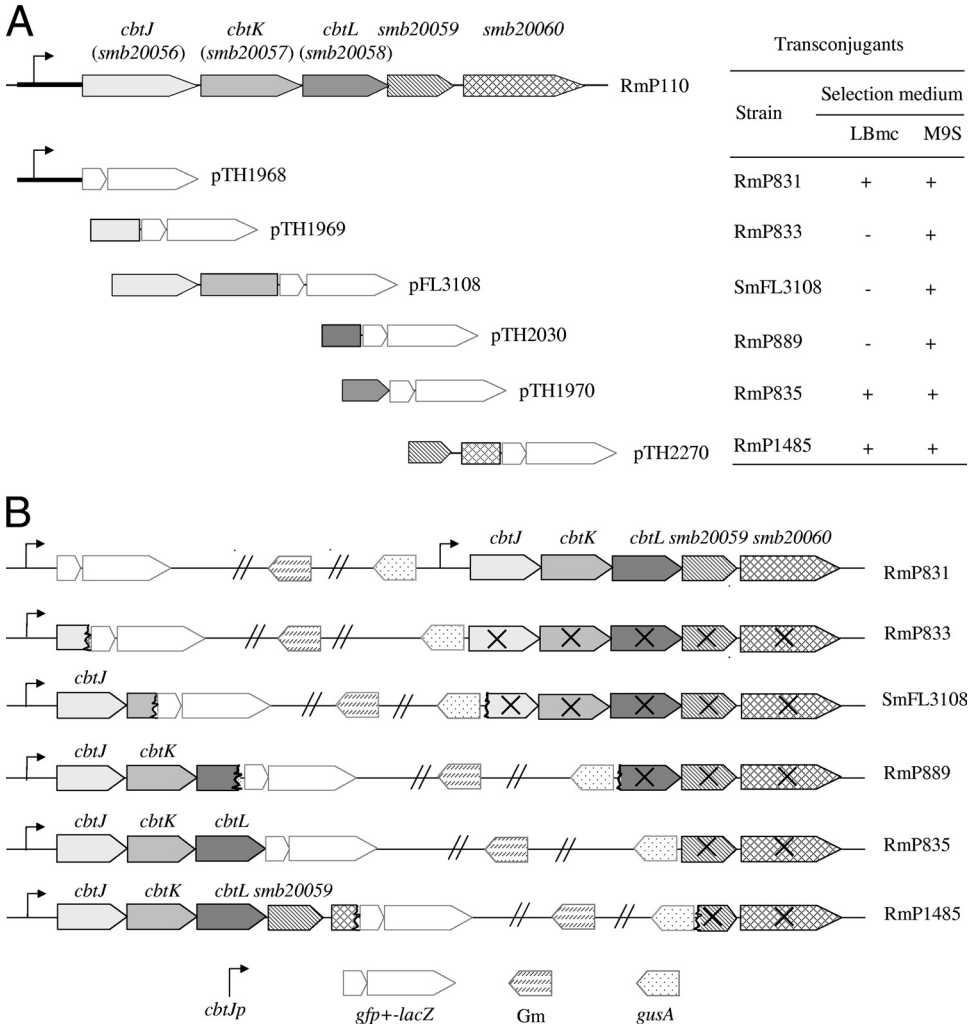


FIG. 1. Schematic of the *cbtJ-smb20060* region on the pSymB megaplasmid of *S. meliloti*. (A) DNA fragments were PCR amplified and cloned upstream of the promoterless *gfp*$^+$*-lacZ* genes in pTH1703 to obtain pTH1968, pTH1969, pTH2030, pTH1970, and pTH2270. Plasmid pFL3108 was a pTH1522 derivative carrying a genomic DNA fragment (8). Upon integration of the reporter fusion clones into the *S. meliloti* genome, transconjugant colonies grew (+) or did not grow (−) when selected on LB or M9-succinate containing streptomycin and gentamicin (Gm). Strains RmP831, RmP833, SmFL3108, RmP889, RmP835, and RmP1485 are representative transconjugant colonies selected for each integrated plasmid. (B) Diagram showing the genome organization of strains RmP831, RmP833, SmFL3108, RmP889, RmP835, and RmP1485 following recombination of the reporter plasmids. X's indicate the genes whose transcription was disrupted in each strain.

rescue the growth of *S. meliloti* RmP833, RmFL3108, or RmP889 (Fig. 2B). These results indicate that the growth defect of the *cbtJKL* mutants is Co^{2+} specific.

The M9 and MOPS-buffered minimal media we routinely employ for growth of *S. meliloti* contain 43 nM $CoCl_2$ (70). Both the wild-type RmP110 strain and the *cbtJKL* mutant strains grew in these minimal media. ICP-MS analysis of the LB medium employed in our experiments detected 100 nM cobalt, a level similar to those previously reported (47). A 100 nM cobalt concentration is >50-fold higher than the 1.7 nM concentration reported to be sufficient to support the growth of *S. meliloti* in minimal media (31) and over twice the 43 nM $CoCl_2$ level we employ in minimal media. It was thus surprising that growth of the *cbtJKL* mutants in LB required supplementation with cobalt. However, since the yeast extract and tryptone present in LB are strong chelators of metal ions (44), we concluded that there was insufficient bioavailable or free cobalt in LB to support the growth of *cbtJKL* mutants. Consistent with this suggestion, when a 1 μM concentration of the chelating agent EDTA was added to M9-succinate minimal medium containing 43 nM $CoCl_2$, the *cbtJKL* deletion mutant RmP2364 failed to grow, whereas both the wild-type RmP110 strain and the complemented mutant, RmP2364 (Δ*cbtJKL* strain plus *cbtJKL*), grew well. Moreover, in the presence of 1 μM EDTA, increasing the $CoCl_2$ concentration to 420 nM allowed the *cbtJKL* deletion mutant to grow (see Fig. S2 in the supplemental material).

An *smb20056*::Tn*5* mutant of Rm1021 was reported in a collection of mutants of *S. meliloti* whose growth in LB was sensitive to a high concentration of salt (350 mM NaCl) (41). Accordingly, we examined the *cbtJ* mutant RmP833 (*smb20056*::*gfp*$^+$*-lacZ*) for growth in LB containing 5 μM

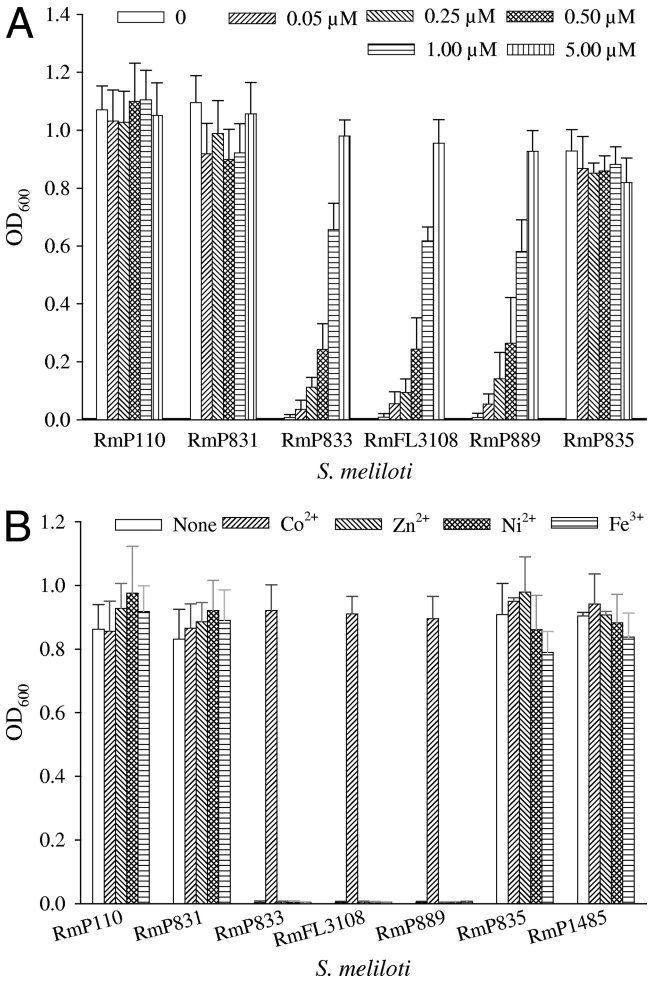


FIG. 2. Growth of *S. meliloti* wild-type RmP110 and plasmid integration mutants in LB or LB with Co^{2+}, Zn^{2+}, Ni^{2+}, or Fe^{3+} added. *S. meliloti* strains were cultured in M9-succinate medium, washed with 0.85% NaCl, and then subcultured into LB. After growing for 16 h, the precultures were inoculated into fresh LB or LB supplemented with $CoCl_2$ (0 to 5 μM) (A) or $CoCl_2$ (5 μM), $ZnSO_4$ (2 μM), $NiSO_4$ (2 μM), or $FeCl_3$ (1 mM) (B). The OD_{600} was measured after 16 h of incubation.

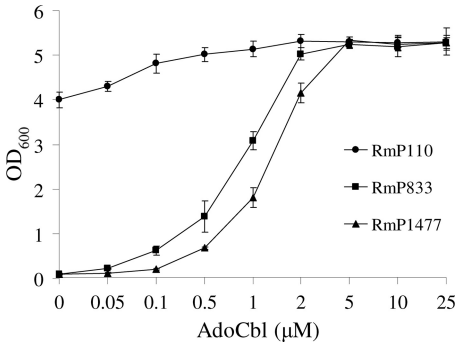


FIG. 3. Growth of *S. meliloti* RmP110 (wild type), RmP833 (*cbtJKL*), and RmP1477 (*cobT*) in LB medium supplemented with AdoCbl or left unsupplemented. The strains were initially grown in LB containing 5 μM AdoCbl, washed with 0.85% NaCl, and then inoculated into LB medium supplemented with different concentrations of AdoCbl. The OD_{600} was recorded after incubation for 24 h. Assays were performed in triplicate, and values represent the means ± standard deviations (SD).

$CoCl_2$ and found that it grew like the wild-type RmP110 strain, whether or not the medium contained 350 mM NaCl. Thus, under our conditions, the *cbtJKL* mutants were not sensitive to high salt concentrations. It is possible that the growth differences may reflect differing sources of the LB components.

Growth of a vitamin B_{12} auxotroph and *cbtJKL* mutants in the presence of AdoCbl. Given the presence of cobalt in vitamin B_{12} and the similarity of CbtJ to the cobalamin-binding protein BtuF, we investigated whether the addition of AdoCbl would allow *cbtJKL* mutants to grow in LB medium. To interpret these experiments, we determined the concentration of cobalamin that was necessary for growth of an *S. meliloti* Cbl biosynthesis mutant. The data showed that the Cbl biosynthesis mutant RmP1477 (*cobT*) and the *cbtJKL* mutant RmP833 had similar growth profiles in LB containing AdoCbl, although the *cbtJKL* mutant grew more rapidly than the *cobT* mutant at lower concentrations of AdoCbl (Fig. 3). Thus, while the data suggest that AdoCbl can substitute for cobalt in the *cbtJKL* mutant, the *cbtJKL* mutant cells appeared to be unimpaired in the ability to take up AdoCbl. Hence, the CbtJKL system does not appear to transport Cbl.

The *cbtJ* 5′ region contains a promoter and a vitamin B_{12} riboswitch. To map the transcriptional start site(s) upstream of *cbtJ*, we performed primer extension with a primer (ML18489) that overlaps the *cbtJ* ATG start codon and a primer (ML18490) 340 nt upstream of the start codon (Fig. 4 and data not shown). RNA isolated from LB-grown wild-type cells revealed a transcript that initiated 384 nt upstream of *cbtJ* in both extension reactions (Fig. 4). The deduced −35 and −10 hexanucleotide promoter sequence, 5′-CTTGAC-N_{17}-ATTAA C-3′, showed similarity to the recently derived *S. meliloti* promoter consensus sequence 5′-CTTGAC-N_{17}-CTATAT-3′, particularly within the more conserved −35 region (38).

A 574-bp DNA sequence upstream of the *cbtJ* ORF was analyzed against the Rfam RNA database (http://rfam.sanger.ac.uk/), and a 201-nt region (nt +1 to +201 in Fig. 4) was identified as similar to a conserved RNA structure known as a B_{12} riboswitch (42). A highly conserved sequence called the B_{12} box (70) was located at nucleotides +182 to +192. Characterized B_{12} boxes lie upstream of *btuB* in *E. coli* (37) and *S.* Typhimurium (50) and upstream of the *cbiA* gene (the first gene in the *cob* operon) in *S.* Typhimurium (53). In a survey of conserved RNA structural features associated with genes involved in vitamin B_{12} metabolism and transport in bacteria, Vitreschak et al. (69) identified the same B_{12} riboswitch and B_{12} box upstream of *S. meliloti cbtJ* (*smb20056*). In that publication and a recent updated annotation of the *S. meliloti* genome (4), the *cbtJ* (*smb20056*), *cbtK* (*smb20057*), and *cbtL* (*smb20058*) genes were reannotated *btuFCD*—encoding a vitamin B_{12} transport system. Our data suggest that this system transports cobalt, not vitamin B_{12}, hence the designation *cbtJKL*.

Repression of *cbtJKL* expression by cobalamin and involvement of Co^{2+} in the B_{12} riboswitch. In view of the B_{12} riboswitch-like element identified above and the growth properties of the *cbtJKL* mutants, the effects of exogenous AdoCbl and

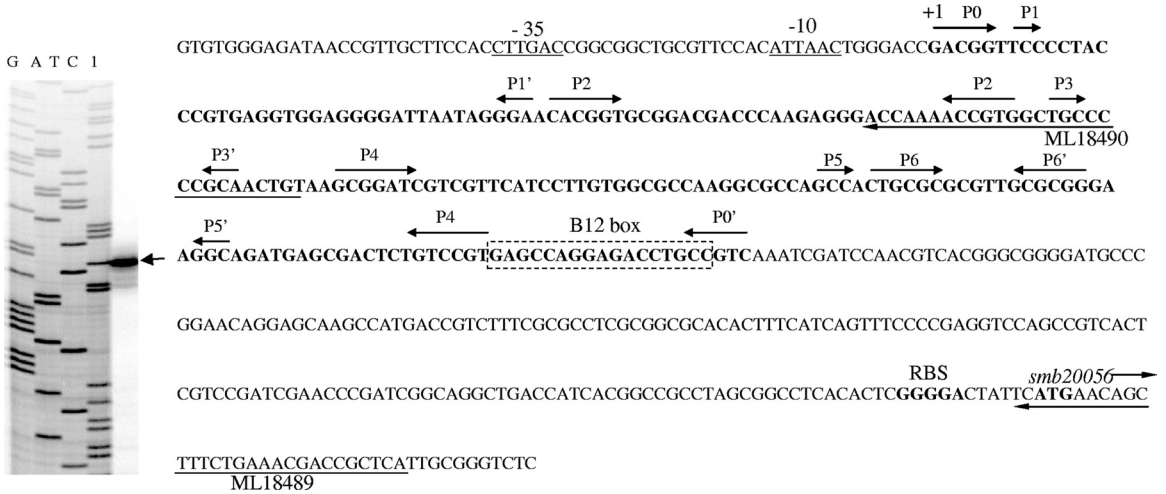


FIG. 4. Characterization of the region upstream of *cbtJ*. The sequences of the *cbtJ* −35 and −10 promoter recognition sequences and the transcription start site (+1) are indicated. The primers used for mapping the transcription start site are indicated by arrows under the sequences. The sequence of the 201-nt B_{12} riboswitch is shown in bold. The B_{12} box is framed by a dashed-line box. The stem-loop sequences P0/P0′ to P6/P6′ are indicated by arrows above the respective sequences, based on the conserved structure of the B_{12} riboswitch (67). The ribosome binding site (RBS) and start codon of *cbtJ* are shown in bold. The autoradiograph image shows a sequencing gel and the ^{32}P-labeled ML18490 extension products obtained with RNA from RmP110 grown in LB (lane 1). Data obtained with primer ML18489 are not shown but were consistent with those obtained with ML18490. The arrow indicates the primer extension product.

$CoCl_2$ on *cbtJKL* expression were determined. Transcription was examined in the transcriptional *gfp-lacZ* gene fusion strains RmP831 and RmP833, which are phenotypically wild type and mutant, respectively, for the *cbtJKL* genes (Fig. 1). In M9-succinate minimal medium, higher levels of *cbtJ* transcription were found in the *cbtJKL* mutant than in the wild-type background (Fig. 5). Addition of $CoCl_2$ or AdoCbl (or cyanocobalamin [data not shown]) to the medium attenuated *cbtJ* transcription. We note that *cbtJ* transcription was much more sensitive to the addition of cobalt than to the addition of AdoCbl. Accordingly, whereas 10 μM AdoCbl reduced *cbtJ* transcription by approximately 70%, only 10 nM $CoCl_2$ was required for a similar reduction in *cbtJ* transcription (Fig. 5A and B).

To define *cis*-acting sequences involved in the modulation of *cbtJ* transcription by Co^{2+} or Cbl, the full-length promoter region and fragments lacking portions of this region were examined for the ability to drive *gfp* transcription in a replicating reporter plasmid (pTH2224) (Fig. 6A). In the wild-type strain RmP110, the *cbtJ* promoter in pTH2237 and pTH2239 initiated transcription to levels that were 40-fold higher than that in cells containing the empty vector pTH2224 (1,400 units) (Fig. 6A). Transcription was repressed upon the addition of 10 nM $CoCl_2$ or 10 μM AdoCbl, and this repression was not observed in constructs in which the putative B_{12} riboswitch region (nt +1 to +201 or nt +1 to +387) was deleted in pTH2256 or pTH2238 (Fig. 6A). These data suggested that there are no other regulatory regions (such as binding sites for a transcriptional regulator) upstream of the B_{12} riboswitch.

As a control for the above experiments, we investigated whether Co^{2+} could also modulate the expression of Cbl biosynthesis (*cob*) genes in *S. meliloti*. A predicted B_{12} riboswitch and a B_{12} box were previously located at nt −153 to −137 upstream of the *cobP* (*smc04305*) start codon (69) (Fig. 4).

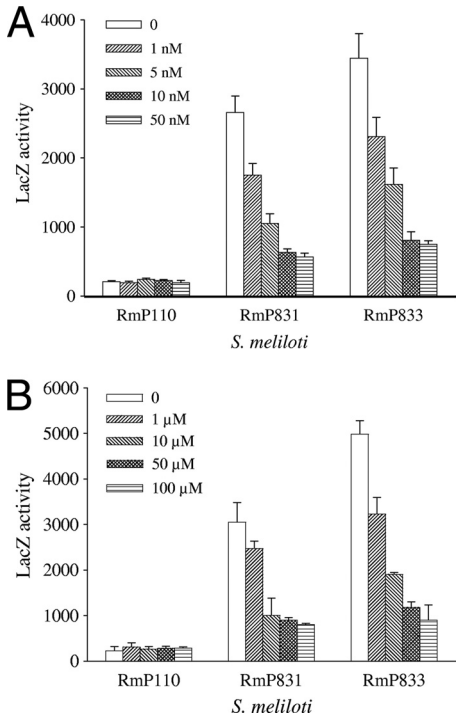
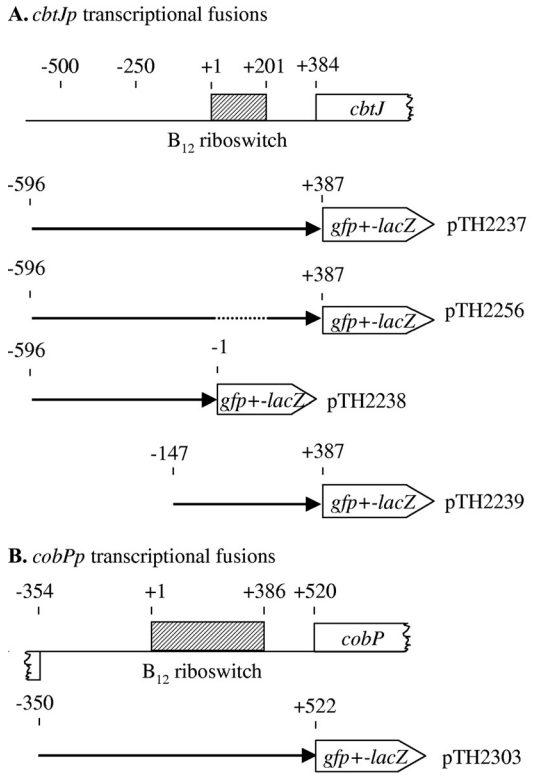


FIG. 5. Nanomolar concentrations of $CoCl_2$ and micromolar concentrations of AdoCbl affect *cbtJ* expression in *S. meliloti*. Cells were grown in M9-succinate medium, washed with 0.85% NaCl, and then inoculated into M9-succinate with 0 to 50 nM $CoCl_2$ (A) or M9-succinate with 0 to 100 μM AdoCbl and no $CoCl_2$ (B). After growth for 36 h, LacZ (β-galactosidase) activity was determined. The fusion strains were RmP110 (wild type), RmP831 (*cbtJp*::*gfp-lacZ* fusion), and RmP833 (*cbtJKL* mutant with *cbtJ*::*gfp-lacZ* fusion).



C. Expression of *cbtJ* and *cobP* transcriptional fusions

	Relative GFP fluorescence					
	RmP110				RmP1477	
	None	$CoCl_2$	AdoCbl	$CoCl_2$ + AdoCbl	AdoCbl	$CoCl_2$ + AdoCbl
pTH2237	40539	8447	19758	6026	41476	41470
pTH2256	50153	47660	45696	51519	62472	62563
pTH2238	38152	38075	32422	30148	ND	ND
pTH2239	47072	12792	11841	5639	ND	ND
pTH2303	19909	4540	9490	4460	17472	17213

FIG. 6. Regulation of *cbtJ* and *cobP* promoter (*cbtJp* and *cobPp*) activities by $CoCl_2$ and AdoCbl. (A and B) Transcriptional fusions in *S. meliloti* RmP110 (wild type) and RmP1477 (*cobT*) grown in M9-succinate without exogenous $CoCl_2$ (none) or with 10 nM $CoCl_2$, 10 μM AdoCbl, or 10 nM $CoCl_2$ and 10 μM AdoCbl. (A) The DNA fragments upstream of *cbtJ* were cloned as transcriptional *gfp-lacZ* fusions in the promoterless, broad-host-range replicating plasmid pTH2224. The location of the transcription start site is marked +1, and the dashed line indicates the deleted B_{12} riboswitch. (B) The region upstream of *cobP* was inserted upstream of the *gfp-lacZ* genes and 5′ of the putative B_{12} riboswitch, designated +1. (C) GFP expression (fluorescence emission [OD_{600}]) given as averages for three independent assays, with SD of <10%. The relative GFP fluorescence was <1,400 units for RmP110 and RmP1477 carrying the empty plasmid pTH2224. ND, not determined.

This region was fused to *gfp* in plasmid pTH2303, and assays were performed as described for the *cbtJ* promoter constructs (Fig. 6B). The levels of GFP detected under the various growth conditions demonstrated that the *cobP* promoter was negatively regulated by AdoCbl and $CoCl_2$, in a similar fashion to that observed for the *cbtJ* promoter (Fig. 6A). We infer that this regulation occurs via the B_{12} riboswitch and that Co^{2+}-mediated repression of both the *cobP* and *cbtJ* promoters occurs via newly synthesized Co^{2+}-containing Cbl.

To gain further insight into the differential regulatory effects of AdoCbl and $CoCl_2$ on the *cbtJ* and *cobP* promoters, transcription from these promoters was examined in the *cobT* mutant RmP1477, in which Cbl biosynthesis was eliminated (Fig. 6A and B). In the *cobT* mutant, transcription from the *cbtJ* and *cobP* promoters (pTH2237 and pTH2303, respectively) was not attenuated upon addition of AdoCbl or $CoCl_2$ plus AdoCbl. These results suggest that AdoCbl transport into *S. meliloti* is inefficient, such that even at an extracellular concentration of 10 μM AdoCbl, the internal concentration of AdoCbl is insufficient to repress expression via the B_{12} riboswitch. In contrast to the case for the wild-type background, the addition of 10 nM cobalt (as in the treatment with $CoCl_2$ plus AdoCbl) in the *cobT* mutant background had no effect on *cbtJ* and *cobP* transcription. Thus, both the *cbtJ* and *cobP* promoters behaved similarly with respect to cobalt-dependent regulation. These data suggest that cobalt-mediated regulation occurs via newly synthesized Co^{2+}-containing Cbl interacting at the B_{12} riboswitch.

Reduced Co^{2+} accumulation correlates with reduced *cbtJKL* transcription. To directly investigate cobalt transport, *S. meliloti* wild-type (RmP110) and *cbtJKL* mutant (RmP833) cells were examined for the ability to transport and accumulate $^{57}Co^{2+}$. RmP110 cells grown in LB showed the fastest time-dependent Co^{2+} accumulation, and this activity decreased by about 80% in RmP110 cells grown in LB supplemented with 5 μM $CoCl_2$ (Fig. 7A). RmP833 cells cultured in LB with 5 μM $CoCl_2$ showed 40 to 60% of the Co^{2+} uptake activity of LB-grown RmP110 cells, whereas similar low levels of Co^{2+} transport were observed in RmP833 cultured in LB and RmP110 grown in LB with 5 μM $CoCl_2$. These data suggest that in the *cbtJKL* mutant (RmP833) cells, an alternate system(s) that can transport Co^{2+} was induced by exogenous Co^{2+}.

To examine the characteristics of $^{57}Co^{2+}$ uptake, we investigated whether the addition of a 100-fold excess of nonradioactive cobalt would release the $^{57}Co^{2+}$ accumulated by cells over a 10-min incubation period (Fig. 7B). No release of $^{57}Co^{2+}$ from the cells was observed, indicating the unidirectional accumulation of $^{57}Co^{2+}$ by *S. meliloti*. Moreover, the lack of $^{57}Co^{2+}$ release from the cells also suggests that nonspecific binding of $^{57}Co^{2+}$ to the cells did not occur, as we

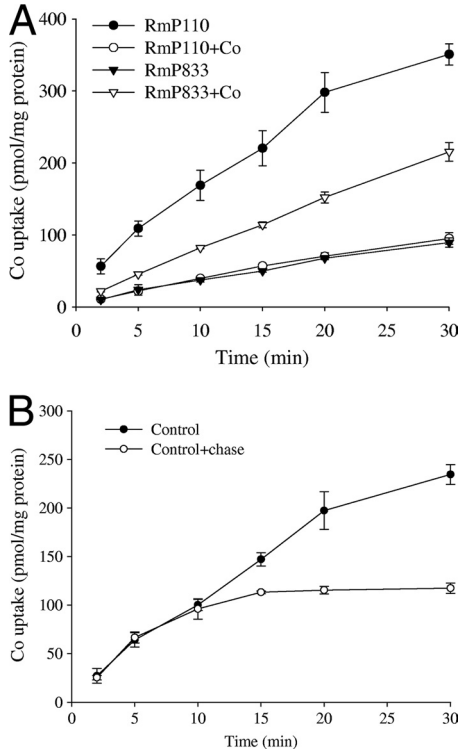


FIG. 7. Cobalt ($^{57}Co^{2+}$) accumulation by the wild type (RmP110) and a *cbtJKL* mutant (RmP833) of *S. meliloti*. Cells were grown in LB or LB supplemented with 5 μM $CoCl_2$, washed, and then resuspended in transport buffer (50 mM MOPS, pH 7.5, 10 mM $MgSO_4$, and 15 mM succinate). Transport assays were initiated by the addition of $CoCl_2$ ($^{57}Co^{2+}$; specific activity, 265 mCi/mmol) to cell suspensions at a final concentration of 0.5 μM. (A) $^{57}Co^{2+}$ accumulation by RmP110 and RmP833 cells grown in LB or LB with 5 μM $CoCl_2$. (B) $^{57}Co^{2+}$ accumulation by RmP110 cells grown in LB or with the addition of 50 μM unlabeled $CoCl_2$ at 10 min. Each data point represents the average for three independent measurements. The error bars represent SD. Green fluorescence readings (mean emission at 510 nm/OD_{600} ± SD) for the cells used in this experiment were 92 ± 11 (RmP110), 80 ± 8 (RmP110 plus $CoCl_2$), 4,927 ± 282 (RmP833), and 1,400 ± 43 (RmP833 plus $CoCl_2$).

would expect the excess nonradioactive cobalt to displace such nonspecifically bound $^{57}Co^{2+}$.

Low *cbtJKL* expression in alfalfa nodules. To investigate *cbtJ* expression in root nodules and whether the *cbtJKL* genes are required for nodule formation and symbiotic N_2 fixation, various *S. meliloti* strains were inoculated onto alfalfa seedlings. After growth for 4 weeks under nitrogen-deficient conditions in Leonard jars, plant dry shoot weights were used as an index of N_2 fixation. The dry shoot weights of plants (averages ± standard errors) inoculated with RmP831, RmP833, and RmP835 (35.1 ± 3.9, 33.1 ± 0.8, and 28.9 ± 4.8 mg/plant, respectively) were similar to that of wild-type RmP110 (32.8 ± 4.6 mg/plant). These results suggest that disruption of the *cbtJKL* operon had no effect on symbiotic N_2 fixation.

To investigate *cbtJKL* expression in *S. meliloti* bacteroids, we measured β-galactosidase activity from a *cbtJ*::*gfp-lacZ* gene fusion and an *nifH*::*gfp-lacZ* gene fusion in bacteroids from 4-week-old nodules. As expected, the *nifH* gene, encoding nitrogenase reductase, was highly expressed (mean LacZ specific activity ± standard error, 12,070 ± 1,172), at a level about 110-fold over the RmP110 background level (107 ± 16). The LacZ activities from the *cbtJ-cbtL*::*gfp-lacZ* fusion genes in RmP831, RmP833, and RmP835 bacteroids were expressed at levels about 2.5-fold above the background level (LacZ specific activity, 280 ± 11, 262 ± 21, and 255 ± 22, respectively). These results suggest that the *cbtJKL* genes have little role in symbiotic N_2 fixation and are expressed only at low levels. It is likely that an alternate transporter(s) is responsible for Co^{2+} uptake into bacteroids, as Co^{2+} plays an essential role in *Rhizobium-Medicago* symbiosis (15, 31).

DISCUSSION

Defining pathways for the transport of cobalt into bacteria is of general importance because trace elements, including cobalt, are important modulators of biological processes. Cobalt availability has been shown to limit methane production by methylotrophic methanogens (22) and mercury methylation by the sulfate-reducing bacterium *Desulfococcus multivorans* (17), and it has been suggested to influence the composition of phytoplankton in the ocean (59). The abundant marine cyanobacterium *Prochlorococcus* requires cobalt for growth, and both it and *Synechococcus* appear to synthesize and excrete cobalt-binding ligands which enhance cobalt assimilation (60).

Here we present evidence that the *cbtJKL* (*smb20056* to *smb20058*) genes encode a cobalt transport system. The CbtJ, CbtK, and CbtL proteins appear to be typical of a solute-binding protein-dependent ABC-type transporter, and this represents one of the first examples of an ABC system for the transport of cobalt. The conclusion that the transported ligand is cobalt is based primarily on the finding that *cbtJKL* mutants do not grow in LB unless this medium is supplemented with cobalt. This growth requirement appears to be specific for cobalt, as other metals, such as Ni, Zn, and Fe, failed to promote growth (Fig. 2A and B). Moreover, *cbtJKL* expression was downregulated in response to the addition of exogenous cobalt (Fig. 5A), and $^{57}Co^{2+}$ accumulation was correlated with *cbtJKL* expression (Fig. 7). In another report, we also demonstrate that the solute binding protein CbtJ can bind to cobalt (Cheng et al., submitted).

Other cobalt transport systems have been described (16, 33, 56, 57, 74), and frequently these systems contain B_{12} riboswitches, as is the case for the *cbtJKL* genes described here. Recently, an ABC transporter (FecDE and CeuE) in *Helicobacter mustelae* was reported to contribute to nickel and cobalt acquisition. The authors concluded that this transporter was not specific for nickel, as a *fecD* mutant showed reduced cellular cobalt levels and increased cobalt resistance (65).

Another uptake system(s) present in *S. meliloti* can also transport cobalt, as the growth of *cbtJKL* mutants in LB supplemented with cobalt requires its uptake via another system(s). A growth phenotype similar to that of the *cbtJKL* mutants was reported for a *Ralstonia eutropha hoxN* mutant defective in a high-affinity, nickel-specific permease. The ability of this *hoxN* mutant to grow with H_2 as an energy source was restored by increasing the concentration of nickel in the medium (13). While the identity of the alternate *S. meliloti* transporter(s) remains to be determined, one candidate is the Mg^{2+} transporter CorA (28, 64). It is unclear whether its

affinity for Co would be physiologically sufficient, although the cellular cobalt requirements are presumably very low. Interestingly, in the *cbtJKL* background, the addition of cobalt to the medium appeared to induce the alternate cobalt uptake system(s) (Fig. 7). *S. meliloti* does not have homologs of the CbiMNQO or NiCoT cobalt transporters (56, 74). However, a gene (*smb20556*) designated *cbtC*, which has an upstream B_{12} riboswitch region, has been suggested to encode a putative cobalt transport protein (55). While we have not directly examined *smb20556* for a role in cobalt uptake, in other work we observed that deletion of the *smb20556* gene region had no effect on growth in LB or minimal medium (data not shown).

The obvious inability of the *cbtJKL* mutants to grow in LB medium contrasts with the growth phenotype observed in minimal medium, where a clear growth phenotype was observed only upon addition of a chelating agent. Strikingly, nanomolar quantities of cobalt restored growth in minimal medium, whereas micromolar quantities were required for growth of *cbtJKL* mutants in LB. This clear phenotypic difference appears to result from the chelation/binding of cobalt by the yeast extract and tryptone components present in LB. These bind tightly to metal ions, and this dramatically affects their availability to cells (29). For example, Ramamoorthy and Kushner (49) detected no free copper in a nutrient broth solution (0.3% beef extract and 0.5% peptone) containing 3 mM copper. While such bioavailability effects are generally considered with respect to the toxicity of metal ions, here the metal binding influenced the availability of cobalt as a nutrient. Thus, while ICP-MS analysis detected 100 nM Co^{2+} in LB, the actual concentration of free cobalt available to the cells must be much less than the 2 nM concentration required for growth of *cbtJKL* mutants in minimal media (36, 70).

Growth experiments suggest that the CbtJKL system does not transport cobalamins. Thus, while either cobalt or AdoCbl restored growth to *cbtJKL* mutants on LB, the concentrations of AdoCbl required for growth of the *cbtJKL* mutants were similar to the AdoCbl concentrations required to grow a cobalamin biosynthesis mutant (Fig. 3). We note that the *cbtJKL* genes were highly expressed in the *cobT* mutant (Fig. 6), but in both the *cobT* and *cbtJKL* mutants, it appears that cobalamin was transported by a low-affinity system. In contrast, *E. coli*, *S.* Typhimurium, and *Halobacterium* possess high-affinity cobalamin transport systems, and the addition of 1 nM (or less) cobalamin is sufficient to allow cobalamin auxotrophs to grow like the wild-type strains (6, 68, 72). The absence of a high-affinity cobalamin transport system in *S. meliloti* suggests that the soil environment in which *S. meliloti* lives lacks sufficient cobalamin or incomplete corrinoids to support the cost of carrying these accessory uptake genes. However, the related alphaproteobacterium *Rhodobacter sphaeroides* strain 2.4.1 possesses a BtuBFCD-like high-affinity cobalamin transporter (RSP_2402 to RSP_2405) (26). Interestingly, this strain also appears to possess *cbtJKL* homologues (RSP_3392 to RSP_3390) with a B_{12} riboswitch in the predicted 5′ region (Cheng et al., submitted).

Transcription of the *cbtJKL* genes increased upon cobalt depletion, and our data suggest that this regulation occurred via a B_{12} riboswitch located in the mRNA 5′ of *cbtJ*. The data are consistent with a model whereby cobalt-loaded vitamin B_{12} interacts at the B_{12} riboswitch and terminates *cbtJKL* transcription. Gallo et al. (24) have shown that the corrin ring plays a crucial role in the switching structure of the *btuB* riboswitch. Data obtained with transcriptional and translational reporter fusions (Fig. 6 and data not shown) suggest that the *cbtJ* riboswitch functions via termination of transcription. However, more detailed *in vitro* and *in vivo* analyses of this region are required to demonstrate a precise mechanism. The presence of free metal ions in the cytoplasm is toxic to the cell (47). In the case of noncorrin enzymes such as nitrile hydratase, the transported cobalt is bound to a chaperon prior to its insertion into the protein (75). Upon entry to the cytoplasm via the CbtJKL transporter, we assume that cobalt is rapidly incorporated into B_{12}.

In previous reports based on informatics, the *smb20056*, *smb20057*, and *smb20058* genes were designated *btuFCD* genes encoding a vitamin B_{12} transport system (4, 69). In another report, on the basis of mRNA transcript nucleotide sequencing, Mao et al. (39) suggested that a small, 180-nucleotide open reading frame (*vbismb0078*) is located in the 5′-UTR of *cbtJ*. As discussed above, our experimental data suggest that this region contains a B_{12} riboswitch. We note that the transcribed region upstream of *cbtJ* is 384 nucleotides in length and that the B_{12} riboswitch spans a 201-nt region (nt +1 to +201) (Fig. 4). The 384 nucleotides are considerably longer than the equivalent B_{12} riboswitch-containing regions upstream of the *btuB* genes in *E. coli* and *S.* Typhimurium (37, 50). It is therefore possible that additional regulatory elements may be present. In this respect, it is interesting that an AraC-type transcription regulator gene, *smb20055*, lies upstream of *cbtJ*; however, disruption of this gene had no effect on *cbtJ* expression (data not shown).

S. meliloti is known to require micronutrient concentrations of cobalt for growth (31, 36), and the ribonucleotide reductase and methionine synthase enzymes from *S. meliloti* use vitamin B_{12} as a coenzyme (9, 30). In the early 1960s, trace element concentrations of cobalt were shown to be required for symbiotic N_2 fixation (15). Cobalamin biosynthesis is required for symbiotic N_2 fixation (66), and as expected, the *cobT* mutant (RmP1477) formed Fix^- nodules on alfalfa (data not shown). Moreover, Taga and Walker (66) recently showed that the cobalamin-dependent ribonucleotide reductase encoded by *nrdJ* is required for symbiotic N_2 fixation, as a cobalamin-independent ribonucleotide reductase failed to restore symbiotic N_2 fixation to an *nrdJ* mutant of *S. meliloti*. Under the plant growth conditions employed in this study, the *cbtJKL* genes were expressed at low levels in N_2-fixing bacteroids, and thus the intracellular cobalt levels in bacteroids appeared to be sufficient to repress *cbtJKL* expression. Indeed, the Fix^+ phenotype of *cbtJKL* mutants demonstrates that sufficient cobalt for symbiotic N_2 fixation is taken up by an alternate transporter(s) in bacteroids. To demonstrate a cobalt requirement for symbiotic N_2 fixation, it was necessary for Delwiche et al. to explicitly remove cobalt from all components of the plant nutrient solution (15). It remains to be determined whether such plant growth conditions will reveal a symbiotic role for the *cbtJKL* system.

ACKNOWLEDGMENTS

We thank Rahat Zaheer for primer extension analyses and Catharine White and Allyson MacLean for comments and suggestions on

this work. We thank Christopher Wood for use of a model 1480 automatic gamma counter and anonymous reviewers for helpful comments.

This work was supported with funding from NSERC (Canada), from Genome Canada through the Ontario Genomics Institute, and from the Ontario Research and Development Challenge Fund to T.M.F.

REFERENCES

1. **Bardin, S., S. Dan, M. Osteras, and T. M. Finan.** 1996. A phosphate transport system is required for symbiotic nitrogen fixation by *Rhizobium meliloti*. J. Bacteriol. **178:**4540–4547.
2. **Bassford, P. J., Jr., C. Bradbeer, R. J. Kadner, and C. A. Schnaitman.** 1976. Transport of vitamin B_{12} in *tonB* mutants of *Escherichia coli*. J. Bacteriol. **128:**242–247.
3. Reference deleted.
4. **Becker, A., et al.** 2009. A portal for rhizobial genomes: RhizoGATE integrates a *Sinorhizobium meliloti* genome annotation update with postgenome data. J. Biotechnol. **140:**45–50.
5. **Borths, E. L., K. P. Locher, A. T. Lee, and D. C. Rees.** 2002. The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter. Proc. Natl. Acad. Sci. U. S. A. **99:**16642–16647.
6. **Cadieux, N., et al.** 2002. Identification of the periplasmic cobalamin-binding protein BtuF of *Escherichia coli*. J. Bacteriol. **184:**706–717.
7. **Campbell, G. R. O., et al.** 2006. *Sinorhizobium meliloti bluB* is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B_{12}. Proc. Natl. Acad. Sci. U. S. A. **103:**4634–4639.
8. **Cowie, A., et al.** 2006. An integrated approach to functional genomics: construction of a novel reporter gene fusion library for *Sinorhizobium meliloti*. Appl. Environ. Microbiol. **72:**7156–7167.
9. **Cowles, J. R., H. J. Evans, and S. A. Russell.** 1969. B_{12} coenzyme-dependent ribonucleotide reductase in *Rhizobium* species and the effects of cobalt deficiency on the activity of the enzyme. J. Bacteriol. **97:**1460–1465.
10. **Cowles, J. R., and H. J. Evans.** 1968. Some properties of the ribonucleotide reductase from *Rhizobium meliloti*. Arch. Biochem. Biophys. **127:**770–778.
11. **Davidson, A. L., and J. Chen.** 2004. ATP-binding cassette transporters in bacteria. Annu. Rev. Biochem. **73:**241–268.
12. **Debussche, L., et al.** 1992. Assay, purification, and characterization of cobaltochelatase, a unique complex enzyme catalyzing cobalt insertion in hydrogenobyrinic acid a,c-diamide during coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*. J. Bacteriol. **174:**7445–7451.
13. **Degen, O., M. Kobayashi, S. Shimizu, and T. Eitinger.** 1999. Selective transport of divalent cations by transition metal permeases: the *Alcaligenes eutrophus* HoxN and the *Rhodococcus rhodochrous* NhlF. Arch. Microbiol. **171:** 139–145.
14. **De Hertogh, A. A., A. M. Patrica, and H. J. Evans.** 1964. Effect of cobalt on the oxidation of propionate by *Rhizobium meliloti*. J. Bacteriol. **87:**746–747.
15. **Delwiche, C. C., C. M. Johnson, and H. M. Reisenauer.** 1961. Influence of cobalt on nitrogen fixation by *Medicago*. Plant Physiol. **36:**73–78.
16. **Eitinger, T., J. Suhr, L. Moore, and J. A. Smith.** 2005. Secondary transporters for nickel and cobalt ions: theme and variations. Biometals **18:**399–405.
17. **Ekstrom, E. B., and F. M. Morel.** 2008. Cobalt limitation of growth and mercury methylation in sulfate-reducing bacteria. Environ. Sci. Technol. **42:**93–99.
18. **Entcheva, P., D. A. Phillips, and W. R. Streit.** 2002. Functional analysis of *Sinorhizobium meliloti* genes involved in biotin synthesis and transport. Appl. Environ. Microbiol. **68:**2843–2848.
19. **Escalante-Semerena, J. C.** 2007. Conversion of cobinamide into adenosylcobamide in bacteria and archaea. J. Bacteriol. **189:**4555–4560.
20. **Finan, T. M., B. Kunkel, G. F. Devos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167:**66–72.
21. **Finan, T. M., et al.** 2001. The complete sequence of the 1,683-kb pSymB megaplasmid from the N_2-fixing endosymbiont *Sinorhizobium meliloti*. Proc. Natl. Acad. Sci. U. S. A. **98:**9889–9894.
22. **Florencio, L., A. J. Field, and G. Lettinga.** 1994. The importance of cobalt for individual trophic groups in an anaerobic methanol-degrading consortium. Appl. Environ. Microbiol. **60:**227–234.
23. **Galibert, F., et al.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293:**668–672.
24. **Gallo, S., S. Mundwiler, R. Alberto, and R. K. Sigel.** 2011. The change of corrin-amides to carboxylates leads to altered structures of the B12-responding btuB riboswitch. Chem. Commun. (Cambridge) **47:**403–405.
25. **Grass, G., et al.** 2005. The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. J. Bacteriol. **187:**1604–1611.
26. **Gray, M. J., N. K. Tavares, and J. C. Escalante-Semerena.** 2008. The genome of *Rhodobacter sphaeroides* strain 2.4.1 encodes functional cobinamide salvaging systems of archaeal and bacterial origins. Mol. Microbiol. **70:**824–836.
27. **Hebbeln, P., and T. Eitinger.** 2004. Heterologous production and characterization of bacterial nickel/cobalt permeases. FEMS Microbiol. Lett. **230:**129–135.
28. **Hmiel, S. P., M. D. Snavely, C. G. Miller, and M. E. Maguire.** 1986. Magnesium transport in *Salmonella typhimurium*: characterization of magnesium influx and cloning of a transport gene. J. Bacteriol. **168:**1444–1450.
29. **Hughes, M., and R. K. Poole.** 1991. Metal speciation and microbial growth—the hard (and soft) facts. J. Gen. Microbiol. **137:**725–734.
30. **Inukai, S., K. Sato, and S. Shimizu.** 1977. Relationship of cobalt requirement to vitamin B_{12}-dependent methionine synthesis in *Rhizobium meliloti*. Agric. Biol. Chem. **41:**2229–2234.
31. **Kliewer, M., and H. J. Evans.** 1962. Effect of cobalt deficiency on the B_{12} coenzyme content of *Rhizobium meliloti*. Arch. Biochem. Biophys. **97:**427–429.
32. **Kobayashi, M., and S. Shimizu.** 1999. Cobalt proteins. Eur. J. Biochem. **261:**1–9.
33. **Komeda, H., M. Kobayashi, and S. Shimizu.** 1997. A novel transporter involved in cobalt uptake. Proc. Natl. Acad. Sci. U. S. A. **94:**36–41.
34. **Köster, W.** 2001. ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B_{12}. Res. Microbiol. **152:**291–301.
35. Reference deleted.
36. **Lowe, R. H., and H. J. Evans.** 1962. Cobalt requirement for the growth of rhizobia. J. Bacteriol. **83:**210–211.
37. **Lundrigan, M. D., W. Koster, and R. J. Kadner.** 1991. Transcribed sequences of the *Escherichia coli btuB* gene control its expression and regulation by vitamin B_{12}. Proc. Natl. Acad. Sci. U. S. A. **88:**1479–1483.
38. **MacLellan, S. R., A. M. MacLean, and T. M. Finan.** 2006. Promoter prediction in the rhizobia. Microbiology **152:**1751–1763.
39. **Mao, C., C. Evans, R. Jensen, and B. Sobral.** 2008. Identification of new genes in *Sinorhizobium meliloti* using the Genome Sequencer FLX system. BMC Microbiol. **8:**72.
40. **Martens, H., H. Barg, M. Warren, and D. Jahn.** 2002. Microbial production of vitamin B_{12}. Appl. Microbiol. Biotechnol. **58:**275–285.
41. **Miller-Williams, M., P. C. Loewen, and I. J. Oresnik.** 2006. Isolation of salt-sensitive mutants of *Sinorhizobium meliloti* strain Rm1021. Microbiology **152:**2049–2059.
42. **Nahvi, A., J. E. Barrick, and R. R. Breaker.** 2004. Coenzyme B_{12} riboswitches are widespread genetic control elements in prokaryotes. Nucleic Acids Res. **32:**143–150.
43. **Nies, D. H.** 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. **27:**313–339.
44. **Nou, X., and R. J. Kadner.** 1998. Coupled changes in translation and transcription during cobalamin-dependent regulation of *btuB* expression in *Escherichia coli*. J. Bacteriol. **180:**6719–6728.
45. **Nou, X., and R. J. Kadner.** 2000. Adenosylcobalamin inhibits ribosome binding to *btuB* RNA. Proc. Natl. Acad. Sci. U. S. A. **97:**7190–7195.
46. Reference deleted.
47. **Outten, C. E., and T. V. O'Halloran.** 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science **292:**2488–2492.
48. **Pinedo, C. A., R. M. Bringhurst, and D. J. Gage.** 2008. *Sinorhizobium meliloti* mutants lacking PTS enzymes HPr or EIIA are altered in diverse processes including carbon metabolism, cobalt requirements and succinoglycan production. J. Bacteriol. **190:**2947–2956.
49. **Ramamoorthy, S., and D. J. Kushner.** 1975. Binding of mercuric and other heavy metal ions by microbial growth media. Microb. Ecol. **2:**162–176.
50. **Ravnum, S., and D. I. Andersson.** 1997. Vitamin B_{12} repression of the *btuB* gene in *Salmonella typhimurium* is mediated via a translational control which requires leader and coding sequences. Mol. Microbiol. **23:**35–42.
51. **Ravnum, S., and D. I. Andersson.** 2001. An adenosyl-cobalamin (coenzyme-B_{12})-repressed translational enhancer in the *cob* mRNA of *Salmonella typhimurium*. Mol. Microbiol. **39:**1585–1594.
52. **Richter-Dahlfors, A. A., S. Ravnum, and D. I. Andersson.** 1994. Vitamin B_{12} repression of the *cob* operon in *Salmonella typhimurium*: translational control of the *cbiA* gene. Mol. Microbiol. **13:**541–553.
53. **Richter-Dahlfors, A. A., and D. I. Andersson.** 1992. Cobalamin (vitamin B_{12}) repression of the *cob* operon in *Salmonella typhimurium* requires sequences within the leader and the 1st translated open reading frame. Mol. Microbiol. **6:**743–749.
54. **Rioux, C., and R. Kadner.** 1989. Vitamin B_{12} transport in *Escherichia coli* K12 does not require the *btuE* gene of the *btuCED* operon. Mol. Gen. Genet. **217:**301–308.
55. **Rodionov, D. A., A. G. Vitreschak, A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of the vitamin B_{12} metabolism and regulation in prokaryotes. J. Biol. Chem. **278:**41148–41159.
56. **Rodionov, D. A., P. Hebbeln, M. S. Gelfand, and T. Eitinger.** 2006. Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: evidence for a novel group of ATP-binding cassette transporters. J. Bacteriol. **188:**317–327.
57. **Rodionov, D. A., et al.** 2009. A novel class of modular transporters for vitamins in prokaryotes. J. Bacteriol. **191:**42–51.
58. **Roth, J. R., J. G. Lawrence, M. Rubenfield, S. Kiefferhiggins, and G. M. Church.** 1993. Characterization of the cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium*. J. Bacteriol. **175:**3303–3316.
59. **Saito, M. A., J. W. Moffett, S. W. Chisholm, and J. Waterbury.** 2002. Cobalt limitation and uptake in *Prochlorococcus*. Limnol. Oceanogr. **47:**1629–1636.

60. **Saito, M. A., G. Rocap, and J. W. Moffett.** 2005. Production of cobalt binding ligands in a *Synechococcus* feature at the Costa Rica upwelling dome. Limnol. Oceanogr. **50:**279–290.
61. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
62. **Sato, K., S. Inukai, and S. Shimizu.** 1974. Vitamin B_{12}-dependent methionine synthesis in *Rhizobium meliloti*. Biochem. Biophys. Res. Commun. **60:** 723–728.
63. **Schmidt, T., and H. G. Schlegel.** 1994. Combined nickel-cobalt-cadmium resistance encoded by the *ncc* locus of *Alcaligenes xylosoxidans* 31A. J. Bacteriol. **176:**7045–7054.
64. **Smith, R. L., J. L. Banks, M. D. Snavely, and M. E. Maguire.** 1993. Sequence and topology of the CorA magnesium transport systems of *Salmonella typhimurium* and *Escherichia coli*. Identification of a new class of transport protein. J. Biol. Chem. **268:**14071–14080.
65. **Stoof, J., E. J. Kuipers, G. Klaver, and A. H. van Vliet.** 2010. An ABC transporter and a TonB ortholog contribute to *Helicobacter mustelae* nickel and cobalt acquisition. Infect. Immun. **78:**4261–4267.
66. **Taga, M. E., and G. C. Walker.** 2010. *Sinorhizobium meliloti* requires a cobalamin-dependent ribonucleotide reductase for symbiosis with its plant host. Mol. Plant Microbe Interact. **23:**1643–1654.
67. **Taga, M. E., N. A. Larsen, A. R. Howard-Jones, C. T. Walsh, and G. C. Walker.** 2007. BluB cannibalizes flavin to form the lower ligand of vitamin B_{12}. Nature **446:**449–453.
68. **Van Bibber, M., C. Bradbeer, N. Clark, and J. R. Roth.** 1999. A new class of cobalamin transport mutants (*btuF*) provides genetic evidence for a periplasmic binding protein in *Salmonella typhimurium*. J. Bacteriol. **181:**5539–5541.
69. **Vitreschak, A. G., D. A. Rodionov, A. A. Mironov, and M. S. Gelfand.** 2003. Regulation of the vitamin B12 metabolism and transport in bacteria by a conserved RNA structural element. RNA **9:**1084–1097.
70. **Watson, R. J., R. Heys, T. Martin, and M. Savard.** 2001. *Sinorhizobium meliloti* cells require biotin and either cobalt or methionine for growth. Appl. Environ. Microbiol. **67:**3767–3770.
71. **Winkler, W. C., and R. R. Breaker.** 2005. Regulation of bacterial gene expression by riboswitches. Annu. Rev. Microbiol. **59:**487–517.
72. **Woodson, J. D., A. A. Reynolds, and J. C. Escalante-Semerena.** 2005. ABC transporter for corrinoids in *Halobacterium* sp. strain NRC-1. J. Bacteriol. **187:**5901–5909.
73. **Yuan, Z. C., R. Zaheer, and T. M. Finan.** 2006. Regulation and properties of PstSCAB, a high-affinity, high-velocity phosphate transport system of *Sinorhizobium meliloti*. J. Bacteriol. **188:**1089–1102.
74. **Zhang, Y., D. A. Rodionov, M. S. Gelfand, and V. N. Gladyshev.** 2009. Comparative genomic analyses of nickel, cobalt and vitamin B_{12} utilization. BMC Genomics **10:**78.
75. **Zhou, Z., Y. Hashimoto, and M. Kobayashi.** 2009. Self-subunit swapping chaperone needed for the maturation of multimeric metalloenzyme nitrile hydratase by a subunit exchange mechanism also carries out the oxidation of the metal ligand cysteine residues and insertion of cobalt. J. Biol. Chem. **284:**14930–14938.