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ABC Transporter for Corrinoids in *Halobacterium* sp. Strain NRC-1†

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We report evidence for the existence of a putative ABC transporter for corrinoid utilization in the extremely halophilic archaeon *Halobacterium* sp. strain NRC-1. Results from genetic and nutritional analyses of *Halobacterium* showed that mutants with lesions in open reading frames (ORFs) Vng1370G, Vng1371Gm, and Vng1369G required a 10^5-fold higher concentration of cobalamin for growth than the wild-type or parent strain. The data support the conclusion that these ORFs encode orthologs of the bacterial cobalamin ABC transporter permease (*btuC*; Vng1370G), ATPase (*btuD*; Vng1371Gm), and substrate-binding protein (*btuF*; Vng1369G) components. Mutations in the Vng1370G, Vng1371Gm, and Vng1369G genes were epistatic, consistent with the hypothesis that their products work together to accomplish the same function. Extracts of *btuF* mutant strains grown in the presence of cobalamin did not contain any cobalamin molecules detectable by a sensitive bioassay, whereas *btuCD* mutant strain extracts did. The data are consistent with the hypothesis that the BtuF protein is exported to the extracellular side of the cell membrane, where it can bind cobalamin in the absence of BtuC and BtuD. Our data also provide evidence for the regulation of corrinoid transport and biosynthesis. *Halobacterium* synthesized cobalamin in a chemically defined medium lacking corrinoid precursors. To the best of our knowledge, this is the first genetic analysis of an archaeal corrinoid transport system.

Corrinoids belong to the family of cyclic tetrapyrroles that includes hemes, chlorophylls, and coenzyme F_{430} (16, 48). A complete corrinoid (also called cobamide) has upper and lower ligands that play important biochemical roles (16). The upper ligand forms a labile, covalent bond with the cobalt ion of the corrin ring (Co-C), while the lower ligand interacts with the cobalt ion via a coordination bond. The best-known cobamide is cobalamin (Cbl), which in its biologically active form has a 5′-deoxyadenosyl group as an upper ligand, hence the name adenosylcobalamin or coenzyme B_{12}. Cobamides are distinguished from one another by the nature of the lower ligand nucleotide base (39), which is 5,6-dimethylbenzimidazole (DMB) in the case of Cbl.

Because of the complex structure of corrinoids, biosynthesis of the complete Cbl molecule requires at least 24 genes (48). Only prokaryotes synthesize corrinoids, although many eukaryotes, including humans, require corrinoids for their metabolism (39, 40, 48). Active transport of corrinoids is a process found in both prokaryotes and eukaryotes. Because the levels of corrinoids in the environment are low, transport of corrinoids requires specific systems with high affinity. In prokaryotes, most of the work on corrinoid transport has been performed with the gram-negative bacteria *Escherichia coli* and *Salmonella enterica* (9, 13, 37, 40, 47). Corrinoid transport is a special problem to these bacteria because the molecule must pass through both an outer and an inner membrane and the periplasm (13). Transport across the outer membrane requires both the BtuB and TonB proteins (18, 36). Active transport across the inner membrane is achieved via an ATP-binding cassette (ABC) transport system encoded by the *btuC*, *btuD*, and *btuF* genes, which encode the membrane permease, ATPase, and periplasmic-binding protein components, respectively (4, 9, 12, 47). ABC transporters are widely distributed in all domains of life and drive the translocation of substrates across membranes by the hydrolysis of ATP.

No corrinoid transport systems have been described for archaea, although some archaea synthesize and require corrinoids for survival. For example, methanogenic archaea require cobamides for methanogenesis from H_2 and CO_2, acetate, or methanol (14). Active cobamide-dependent (class II) ribonucleotide reductases have been purified from both *Thermoplasma acidophilum* (45) and *Pyrococcus furiosus* (38), suggesting cobamides are used by these organisms. We recently showed that the extremely halophilic archaeon *Halobacterium* sp. strain NRC-1 requires corrinoids under certain growth conditions, although the reasons for their corrinoid requirement remain unknown (50). We also showed that *Halobacterium* salvages Cbl and several of its precursors when present in the medium at subnanomolar concentrations, suggesting that this archaeon possesses a high-affinity corrinoid transport system (51).

Based on genome sequence analyses, ABC transporters appear to be as ubiquitous in archaea as in bacteria (2). Therefore, we hypothesized that, like bacteria, archaea use an ABC transporter for the utilization of corrinoids. Substrate uptake systems of this type have been studied in *Sulfolobus solfataricus*, *P. furiosus*, and *Thermococcus litoralis* and have been shown to be composed of a permease, an ATPase, and an extracellular substrate-binding protein that is anchored to the cell membrane (1, 3, 15, 20, 22, 52). Presumably because archaea only have a single membrane and no periplasm, no orthologs of outer membrane transporters have been found.

Using *Halobacterium* sp. strain NRC-1 as a model system, we report genetic evidence of an ABC-type corrinoid transporter in archaea. The Vng1370G, Vng1370Gm, and Vng1369G

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† We dedicate this work to the memory of Robert Kadner, a pioneer in the field of corrinoid transport, a good friend and colleague.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Marker(s)[a]	Relevant genotype	Relevant characteristics and use	Reference or source[b]
Halobacterium strains				
MPK414		Δ*ura3*	Able to synthesize Cbl de novo	50
JE6738		Δ*ura3* Δ*cbiP*	Unable to synthesize cobamides de novo	51
JE7084		Δ*ura3* Δ*cbiP* Δ*btuCD*	In-frame deletion of *btuCD*; unable to synthesize cobamides de novo	
JE7108		Δ*ura3* Δ*btuCD*	In-frame deletion of *btuCD*	
JE7494		Δ*ura3* Δ*cbiP* Δ*btuCD ura3*::*cbiP*$^+$	Complementation of de novo pathway	
JE7495		Δ*ura3* Δ*cbiP* Δ*btuCD ura3*::*btuCD*$^+$	Complementation of *btuCD*	
JE7681		Δ*ura3* Δ*btuF*	In-frame deletion of *btuF*	
JE7682		Δ*ura3* Δ*cbiP* Δ*btuF*	In-frame deletion of *btuF*; unable to synthesize cobamides de novo	
JE7683		Δ*ura3* Δ*cbiP* Δ*btuF* Δ*btuCD*	In-frame deletions of *btuF* and *btuCD*; unable to synthesize cobamides de novo	
JE7684		Δ*ura3* Δ*cbiP* Δ*btuCD ura3*::*btuC*$^+$	Complementation of *btuC*	
JE7685		Δ*ura3* Δ*cbiP* Δ*btuCD ura3*::*btuD*$^+$	Complementation of *btuD*	
JE7796		Δ*ura3* Δ*cbiP* Δ*btuF ura3*::*cbiP*$^+$	Complementation of de novo pathway in *btuF* mutant	
JE7797		Δ*ura3* Δ*cbiP* Δ*btuF ura3*::*btuF*$^+$	Complementation of *btuF*	
S. enterica strains				
JE873		*metE205 ara-9* Δ*cob272* (*cobUST*)	Cobamide bioassay	Laboratory collection
JE2243		*metE205 ara-9 btuB101*::MudJ[c]	Cobamide bioassay	Laboratory collection
Plasmids				
pMPK428	5-FOA^s Mev^r	*ura3*$^+$	Generation of in-frame deletions of targeted genes	32
pMPK424	5-FOA^s Mev^r	*ura3*$^+$	Recombination at *ura3* locus	31
pCBIP7	5-FOA^s Mev^r	Δ*cbiP*	Generates *cbiP* deletion	51
pVNG1370-2	5-FOA^s Mev^r	*ura3*$^+$ Δ*btuCD*	Generates *btuCD* deletion	
pVNG1370-4	5-FOA^s Mev^r	*ura3*$^+$ *btuCD*$^+$	Recombination of *btuCD* into *ura3* locus	
pHsBTUC5	5-FOA^s Mev^r	*ura3*$^+$ *btuC*$^+$	Recombination of *btuC* into *ura3* locus	
pHsBTUD1	5-FOA^s Mev^r	*ura3*$^+$ *btuD*$^+$	Recombination of *btuD* into *ura3* locus	
pHsBTUF5	5-FOA^s Mev^r	*ura3*$^+$ Δ*btuF*	Generates *btuF* deletion	
pHsBTUF6	5-FOA^s Mev^r	*ura3*$^+$ *btuF*$^+$	Recombination of *btuF* into *ura3* locus	

[a] Abbreviations: Mev^r, resistance to mevinolin; 5-FOA^s, sensitivity to 5-FOA.
[b] Unless otherwise stated, strains and plasmids were constructed during the course of this study.
[c] Abbreviation of Mu dI1734 (10).

genes were predicted to encode the archaeal orthologs of the bacterial BtuC, BtuD, and BtuF proteins, respectively. These functions were required to salvage low nanomolar levels of corrinoids from the environment of this archaeon. We also report evidence for the regulation of this transport system and demonstrate that *Halobacterium* synthesizes Cbl (with the lower ligand DMB) de novo.

MATERIALS AND METHODS

Strains and plasmids. The genotypes of the *Halobacterium* and *S. enterica* strains and plasmids used in this work are described in Table 1.

Chemicals. Unless otherwise stated, all chemicals used in this work were commercially available, high-purity compounds. All corrinoids were added in their cyano-liganded form. Cobinamide dicyanide (Cbi) and cyanocobalamin were purchased from Sigma (St. Louis, MO). Cbi-GDP dicyanide (Cbi-GDP) was synthesized as previously described (46). Cobyric acid dicyanide (Cby) was a gift from Paul Renz (Universität Hohenheim, Stuttgart, Germany), 5-fluoroorotic acid (5-FOA) was purchased from Zymo Research (Orange, CA), and mevinolin was purchased from LKT Laboratories, Inc. (St. Paul, MN).

***Halobacterium* growth studies.** Strains were grown in liquid rich peptone (RP) medium (Oxoid, Hampshire, England) (28) lacking trace metals. *Halobacterium* cultures were grown for 4 days to stationary phase at 37°C with shaking. Cells were added to 5 ml chemically defined (CD) medium (17) at a dilution of 1:100, and cultures were grown at 37°C with shaking. Briefly, the CD medium contains the amino acids A, R, C, E, G, I, L, K, M, F, P, S, T, Y, and V; 11 mM glycerol; salts; and trace metals. Growth was monitored every 24 h for 6 days by measuring the absorbance of the culture at 650 nm with a Spectronic 20D spectrophotometer (Milton Roy, Rochester, NY). To determine cell viability (calculated as CFU), cells were plated onto solid RP medium (6.6% [wt/vol] agar). In all cases, media were supplemented with uracil (450 μM).

***Halobacterium* plasmid constructions.** Plasmids were propagated in *E. coli* strain DH5α, except where noted otherwise. Unless stated otherwise, *Halobacterium* strain MPK414 (Δ*ura3*) genomic DNA was used as the template for PCR and was prepared as previously described (50). The high-fidelity enzyme *Pfu* (Stratagene) was used for PCR amplification. All DNA fragments were digested with the appropriate restriction enzymes (indicated by the underlined portion in the name of the primers) and then gel purified using a QIA quick gel extraction kit (QIAGEN). Plasmid pMPK424 was prepared from the *E. coli dam* mutant strain GM2163 (New England Biolabs). All primers were purchased from Integrated DNA Technologies. Underlined portions of the primer sequences (see

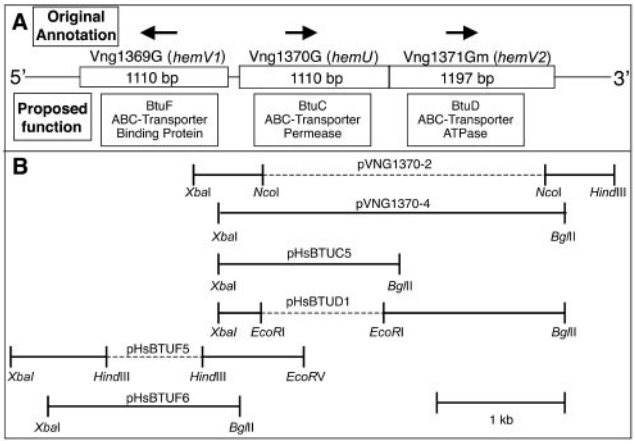

FIG. 1. Putative gene cluster in *Halobacterium* sp. strain NRC-1 indicating putative *btuF* (Vng1369G), *btuC* (Vng1370G), and *btuD* (Vng1371Gm) genes and plasmid constructions. (A) The reported ORF designation and original gene annotation are shown above each gene. Proposed functions are shown below each gene. The reported length (base pairs) of each gene is indicated. Arrows indicate directions of transcription. (B) Brackets connected by solid lines indicate the regions of DNA that were included in the indicated plasmids (pVNG1370-2, pVNG1370-4, pHsBTUC5, pHsBTUD1, pHsBTUF5, and pHsBTUF6). Dashed lines indicate regions that were not included in the plasmids. DNA restriction enzyme sites used for cloning are indicated below the brackets.

below) indicate introduced restriction sites. All plasmids were subsequently sequenced for verification. A diagram of the *Halobacterium* sp. strain NRC-1 DNA included in the most relevant plasmids is included in Fig. 1.

Plasmid pVNG1370-2. The primer sets VNG1370DelXbaI5′ (TCTAGATCT AGACGTCGGCAGCGATGTTGTGG)-VNG1370DelNcoI3′ (CCATGGCCA TGGGCGCAGCGTGATCGGTTCC) and VNG1370DelNcoI5′ (CCATGGCC ATGGGCTGTCGTGTCCGCAGTCG)-VNG1370DelHindIII3′ (AAGCTTA AGCTTACGAGCGTGATGGTCTGTCC) were used to PCR amplify 890-bp and 760-bp fragments, respectively. The former fragment was cloned into the XbaI/NcoI restriction sites of plasmid pMPK428 (32). The second fragment was then cloned into the NcoI/HindIII restriction sites of the resulting plasmid to create plasmid pVNG1370-2, which contains an in-frame deletion of *btuC* and *btuD* replacing bases 112 to 1110 of *btuC* and bases 1 to 1143 of *btuD* with a 6-bp NcoI restriction site. The gene product of this construct should be a nonfunctional peptide with amino acid residues 1 to 37 of BtuC fused to amino acid resides 382 to 398 of BtuD.

Plasmid VNG1370-4. The primer set VNG1370-Comp-XbaI-5′ (TCTAGTTC TAGATGTGATCGCGGTGTTGCTGG)–VNG1371-Comp-BglII-3′ (AGATC AAGATCTTGGCTGCCGTGCGACCCATG) was used to PCR amplify a 2,620-bp fragment that was cloned into the XbaI/BglII restriction site of plasmid pT7-7 (44). This cloned insert was excised with an XbaI/BglII restriction enzyme digest from a plasmid prepared from strain GM2163. The DNA fragment was then cloned into the XbaI/BglII sites of plasmid pMPK424 (31). The resulting plasmid, pVNG1370-4, contains the putative operon containing Vng1370G and Vng1371Gm. In addition to the two open reading frames (ORFs), 225 bp of genetic material 5′ of Vng1370G and 70 bp 3′ of Vng1371Gm were included to preserve any transcriptional regulation.

Plasmid pHsBTUC5. The primer set VNG1370-Comp-XbaI-5′–Hs-BTUC-Comp-BglII-3′ (ACATCAAGATCTAAAAGCCGCGCCGGTTGCCAACTCC ACGTCGAGG) was used to PCR amplify a 1,370-bp fragment that was cloned into the XbaI/BglII sites of pMPK424 (31). The resulting plasmid contains the entire *btuC* ORF, 225 bp 5′ of *btuC* to preserve transcriptional regulation, and a 16-bp sequence derived from the *bop* transcriptional terminator (11) (included in the 3′ reverse primer) to ensure termination of the *btuC* mRNA transcript.

Plasmid pHsBTUD1. The primer sets VNG1370-Comp-XbaI-5′–Hs-BtuD-Comp-EcoRI-3′ (TGTTCTGAATTCAACGGTGCGCAGCGTGATC) and Hs-BtuD-Comp-EcoRI-5′ (TGTTCAGAATTCATCATCACCGCCCTGATCG)–Hs-BtuD-Comp-BglII-3′ (ACATCAAGATCTAAAAGCCGCGCCGGTTGTC CACGTAATACGTTCC) were used to PCR amplify 340-bp and 1,310-bp DNA fragments, respectively. Both DNA products were cut with EcoRI restriction enzyme and ligated together with T4 ligase (MBI Fermentas, Amherst, NY). Using the ligated DNA as the template, the primer set VNG1370-Comp-XbaI-5′–Hs-BtuD-Comp-BglII-3′ was used to PCR amplify a 1,650-bp DNA fragment, which was cloned into the XbaI/BglII sites of plasmid pMPK424 (31). The resulting plasmid contains a wild-type allele of *btuD*, as well as the 225 bp 5′ of *btuC* to include the transcription start site. To include this sequence, as well as 70 bp 5′ of the *btuD* ORF (to include the ribosome-binding site), part of *btuC* was included but as an in-frame deletion. A 6-bp EcoRI restriction site replaced bases 118 to 1041 of *btuC*. This construct should not encode a functional BtuC peptide but should ensure the production of a *btuD* mRNA transcript. As described for plasmid pHsBTUC5, a transcriptional terminator sequence was included 3′ of the *btuD* ORF.

Plasmid pHsBTUF5. The primer sets VNG1369-Del-XbaI-5′ (GATATCTCT AGATGCCCATCAGCCAGTACATC)–VNG1369-Del-HindIII-3′ (AGATCT AAGCTTGAGTGTGATCGCGGTGTTGC) and VNG1369-Del-HindIII-5′ (TCTAGAAAGCTTAACACCACCATCAACACGACG)–VNG1369-Del-EcoRV-3′ (TCTAGAGATATCACTTGGACGACGACGAACAG) were used to PCR amplify 920-bp and 840-bp DNA fragments, respectively. The former fragment was first cloned into the XbaI/HindIII sites of pMPK428 (32). The second fragment was cloned into the constructed plasmid to create plasmid psBTUF5. The resulting plasmid contains an in-frame deletion of *btuF*, which replaces bases 169 to 948 with a 6-bp HindIII site, thus removing 260 of the 369 amino acid residues of the resulting peptide.

Plasmid pHsBTUF6. The primer set HsBTUF-Comp-XbaI-5′ (TGAAGATC TAGAGTGCGCAGCGTGATCGGTTC)–HsBTUF-Comp-BglII-3′ (ACTACT AGATCTAAAAGCCGCGCCGGTTGAGGAATGAAACGGTGTCG) was used to PCR amplify a 130-bp DNA fragment that was cloned into the XbaI/BglII sites of the pMPK424 (31). The resulting plasmid, pHsBTUF6, contains the entire *btuF* ORF, 170 bp 5′ of the start site to preserve transcriptional regulation, and the same 16-bp terminator sequence included in plasmid pHsBTUC5.

***Halobacterium* strain constructions. (i) In-frame deletion mutants.** In-frame deletions of the *btuCD* and *btuF* loci were generated using previously described methodology (30). Briefly, deletion strains were constructed by transforming the desired *Halobacterium* sp. strain NRC-1 derivative of MPK414 (*ura3*) with a pMPK428-derived plasmid containing a deletion of the desired gene as described previously (25). Flanking sequences around the deletion of over 700 bp allowed efficient recombination of the fragment into the chromosome. Mevinolin-resistant mutants were selected as previously described (25) and replated on medium containing 5-FOA to select for loss of the plasmid (30). Colonies resistant to 5-FOA were screened by PCR to identify desired recombinants. DNA sequencing was used to confirm the presence of an in-frame deletion. Plasmid pVNG1370-2 (Δ*btuCD*) was transformed into MPK414 and JE6738 (Δ*cbiP*) to generate strains JE7108 (Δ*btuCD*) and JE7084 (Δ*cbiP* Δ*btuCD*), respectively. Plasmid pHsBTUF5 (Δ*btuF*) was transformed into MPK414, JE6738 (Δ*cbiP*), and 7084 (Δ*cbiP* Δ*btuCD*) to generate strains JE7861 (Δ*btuF*), JE7862 (Δ*cbiP* Δ*btuF*), and JE7683 (Δ*cbiP* Δ*btuCD* Δ*btuF*), respectively.

Construction of complementation strains. Complementation studies were performed with a single copy of the appropriate wild-type gene(s) in question placed at the *ura3* locus. The same *ura3*-based gene replacement method for the isolation of deleted genes was used. PCR and DNA sequencing were used to confirm the presence of the correct gene at the *ura3* locus. Plasmid pCBIP7 (*cbiP*$^+$) was transformed into JE7084 (Δ*cbiP* Δ*btuCD*) and JE7682 (Δ*cbiP* Δ*btuF*) to generate strains JE7494 (Δ*cbiP* Δ*btuCD ura3*::*cbiP*$^+$) and JE7796 (Δ*cbiP* Δ*btuF ura3*::*cbiP*$^+$), respectively. Plasmids pVNG1370-4 (*btuCD*$^+$), pHsBTUC5 (*btuC*$^+$), and pHsBTUD1 (*btuD*$^+$) were transformed into JE7084 (Δ*cbiP* Δ*btuCD*) to generate JE7495 (Δ*cbiP* Δ*btuCD ura3*::*btuCD*$^+$), JE7684 (Δ*cbiP* Δ*btuCD ura3*::*btuC*$^+$), and JE7685 (Δ*cbiP* Δ*btuCD ura3*::*btuD*$^+$), respectively. Plasmid pHsBTUF6 (*btuF*$^+$) was transformed into JE7682 (Δ*cbiP* Δ*btuF*) to generate strain JE7797 (Δ*cbiP* Δ*btuF ura3*::*btuF*$^+$).

***Halobacterium* corrinoid extraction assays.** Ten milliliters of dense *Halobacterium* culture was used to inoculate 1 liter of liquid RP or CD medium supplemented with various concentrations of Cbl. The cultures were grown to full density (4 days in RP medium and 6 days in CD medium) at 37°C with shaking at 180 rpm. Serial dilutions of the cells were plated on solid medium to calculate total CFU. Cells were harvested at 4,300 × *g* for 10 min in a Beckman-Coulter J21 centrifuge, washed by gently resuspending them in 200 ml of medium salts (4.3 M NaCl, 81 mM $MgSO_4$, 27 mM KCl, 14 mM sodium citrate), and pelleted again. This was repeated twice, and the cell pellet was resuspended in 25 ml of methanol and incubated for 2 h at 65°C with gentle shaking. The suspension was cleared by centrifugation at 40,000 × *g* for 2 h in a Beckman-Coulter J25-I centrifuge. The supernatant was then dried under vacuum by using a Savant concentrator, and the sample was resuspended in 1 ml of buffer (100 mM phosphate buffer [pH 6.5], 10 mM KCN) and incubated under light for 10 min to

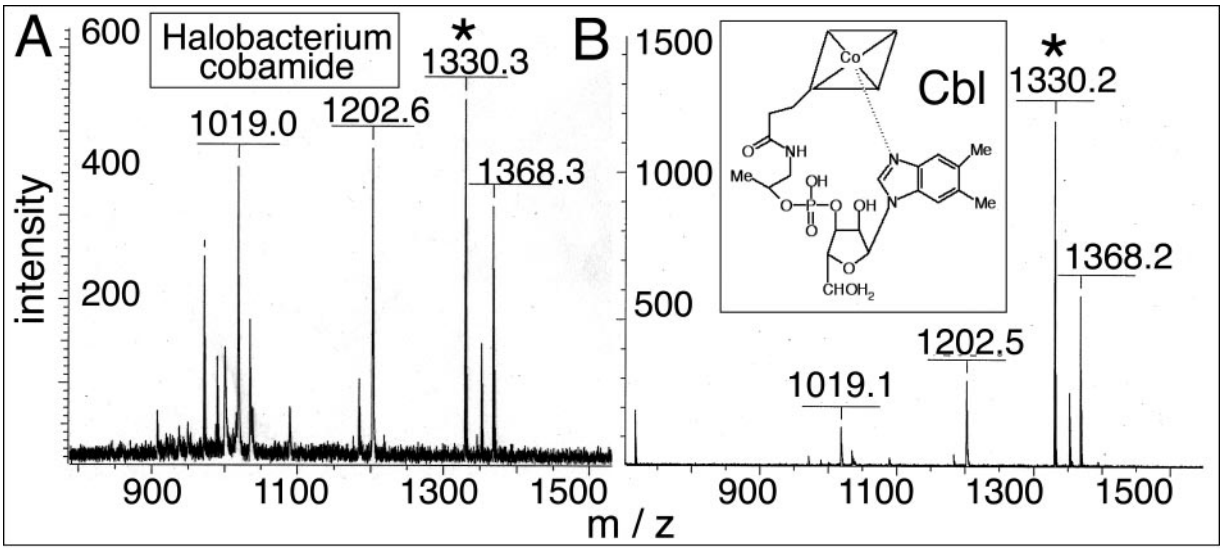

FIG. 2. Mass spectrometry analysis of the de novo synthesized cobamide extracted from *Halobacterium*. Shown is the MALDI-TOF mass spectrometry analysis of the HPLC-purified cobamide extracted from *Halobacterium* cells producing corrinoids de novo (A) and authentic Cbl (B). The signals with *m*/*z* values of 1,330.3 and 1,330.2 (indicated by asterisks) were consistent with the molecular mass of Cbl (without the upper cyano ligand), where $z = +1$. No significant signals were detected above an *m*/*z* value of 1,500 in either case. Me, methyl.

derivatize any corrinoids to their cyano form. Total cell protein was determined by the Bio-Rad (Hercules, CA) Bradford protein assay. Samples were prepared by resuspending pelleted cells in 5 M NaOH.

Detection of corrinoids. The presence of Cbl or other corrinoids was assessed by means of a bioassay. For this purpose, *S. enterica* strains JE873 (*metE cobUST*) and JE2243 (*metE btuB*) were used as indicator strains in an overlay on minimal no-carbon E medium (5) supplemented with glycerol and $MgSO_4$. Two microliters of *Halobacterium* corrinoid extract or 2 pmol of authentic Cbl was spotted onto the agar overlay. The inoculated plates were incubated aerobically at 37°C for 24 h. The last step of cobamide biosynthesis in strain JE873 is blocked, making growth dependent on complete cobamides. Cell growth around the application site on overlays containing strain JE873 would indicate the presence of Cbl or another cobamide in the extract. Strain JE2243 was used as a negative control because it cannot transport Cbl (due to a lesion in the gene encoding the outer membrane corrinoid transporter BtuB) and will not respond to its presence in the extracts.

High-performance liquid chromatography (HPLC) analysis of corrinoids. *Halobacterium* corrinoid extracts were filtered using Corning Spin-X centrifuge filters. Corrinoids were separated by using a Beckman-Coulter HPLC system equipped with a Luna (Phenomenex) 5-μm C_{18} column (150 by 4.6 mm) developed with a modification of the system reported elsewhere (6) at a flow rate of 1 ml/min. The column was equilibrated with a buffer system containing 98% A and 2% B. For quantification of Cbl in the extracts, 2 min after injection, the column was developed for 10 min with a linear gradient until the final composition reached 100% B. For the purification of corrinoids for mass spectrometry analysis, the column was developed for 55 min to 100% B 5 min after injection. The solvents used were as follows: A, 100 mM phosphate buffer (pH 6.5)–10 mM KCN; B, 100 mM phosphate buffer (pH 8.0)–10 mM KCN-acetonitrile (1:1). Corrinoids were detected using a Beckman-Coulter photodiode array detector. Authentic Cbl was used as the standard.

Mass spectrometry. The HPLC-purified corrinoid in *Halobacterium* extracts was prepared for mass spectrometry analysis as previously described (49). This sample, as well as authentic Cbl, was submitted for analysis to the mass spectrometry facility at the University of Wisconsin—Madison Biotechnology Center. The mass spectrum was obtained using a Bruker Daltronics (Billerica, MA) BILFLEX III matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer.

RESULTS AND DISCUSSION

***Halobacterium* synthesizes Cbl de novo.** To identify the cobamide synthesized by *Halobacterium*, we extracted corrinoids from strain JE7681 (Δ*btuF cbiP*$^+$) grown in CD medium. Strain JE7681 synthesized corrinoids de novo but did not salvage exogenous corrinoids due to the lack of BtuF function (see below). Corrinoids were resolved using HPLC as previously described (see Materials and Methods). A peak with the diagnostic UV-visible spectrum of corrinoids eluted 35.1 min after injection, the same retention time as authentic Cbl (data not shown). The material under this peak was used in bioassays.

S. enterica strain JE873 (*metE* Δ*cobUST*) was the indicator strain used in bioassays to detect the presence of cobamides in *Halobacterium* corrinoid extracts (26). *S. enterica* strain JE2243 (*metE btuB*) lacking the outer membrane corrinoid transporter BtuB protein (19) was used as a negative control. A fraction containing the putative *Halobacterium* cobamide supported the growth of strain JE873, but not JE2243, consistent with the presence of a cobamide (data not shown). The MALDI-TOF mass spectrum of the *Halobacterium* cobamide contained a molecular ion signal with an *m*/*z* of 1,330.3 that was consistent with Cbl lacking an upper ligand. Authentic Cbl was used as a control, and its spectrum was strikingly similar to that of the *Halobacterium* cobamide (Fig. 2A). Other peaks were observed, but these were also present in the mass spectrometry profile of authentic Cbl (Fig. 2B). On the basis of these results, we concluded that *Halobacterium* sp. strain NRC-1 synthesizes a cobamide with DMB as the lower α ligand (i.e., Cbl).

Methanothermobacter marburgensis strain Marburg is the only other archaeon shown to produce Cbl, but it only did so when DMB was supplied in the medium (43). Cobamides have been isolated with the lower ligand 5-methylbenzimidazole from *Archaeoglobus fulgidus* and *T. acidophilum* (23), adenine from *Methanosarcina barkeri* (35), and 5-hydroxybenzimidazole from *M. marburgensis* strain Marburg (24). It appears that the differences between the cobamides made by different species of prokaryotes correlate not with the biological functions of the

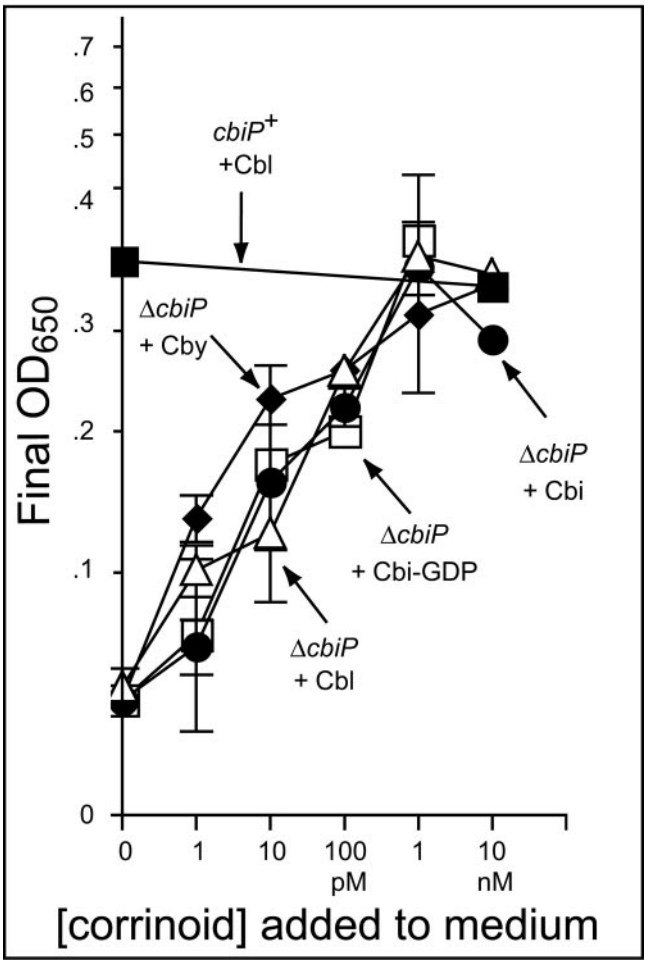

FIG. 3. Corrinoid transport efficiency of *Halobacterium* strains. Corrinoid-dependent growth of *Halobacterium* sp. strain NRC-1 in CD liquid medium with various concentrations of corrinoids at 37°C is reported as the absorbance at 650 nm (OD_{650}) after 6 days of growth. Separate panels include the effects of a Δ*btuCD* mutation (A), complementation of Δ*btuCD* mutant strains (B), a Δ*btuF* mutation (C), and complementation of Δ*btuF* mutant strains (D). After 6 days, cells reached maximum density, and this value is indicative of the ability to grow in the given medium. The mean cell densities of duplicated experiments are reported ± the standard deviations. Strains are identified by their genotypes. The corrinoids added to the medium are indicated next to the genotypes. The strains used were MPK414 (*cbiP*$^+$) and JE6738 (Δ*cbiP*).

cobamide but with the metabolic conditions of the organism in its natural habitat (23).

***Halobacterium* has an efficient corrinoid transport system.** Strain JE6738 (Δ*cbiP*) was used to assess the ability of *Halobacterium* to assimilate low concentrations of various corrinoids. Strain JE6738 cannot synthesize corrinoids de novo due to the lack of CbiP (Cby synthase enzyme) and is dependent on exogenous cobamides or corrinoid precursors for growth (50). Strain JE6738 did not grow without added corrinoids, as opposed to strain JE6735 (*cbiP*$^+$), which did not require corrinoids to grow (Fig. 3). The growth response of strain JE6738 was assessed as a function of the concentration of incomplete cobamides (i.e., Cby, Cbi, Cbi-GDP) and Cbl. The growth responses of strain JE6738 to all corrinoids tested were very similar. At least 1 nM corrinoid was required for growth equivalent to a functional de novo pathway, while a 100 pM concentration of every corrinoid tested allowed intermediate growth. Concentrations of Cbl of up to 100 μM did not significantly increase growth any further and may have a slight inhibitory effect (Fig. 4A, inverted solid triangles). The concentration of corrinoid needed to support optimal growth of *Halobacterium* was very similar to the one needed for *S. enterica* and *E. coli* (4, 29). This result strongly suggested the existence of a transport system for corrinoids in *Halobacterium*.

Bioinformatic analysis of the B_{12} utilization (*btu*) genes of *Halobacterium*. In bacteria, the *btuC*, *btuD*, and *btuF* genes encode the corrinoid ABC transporter permease, ATPase, and corrinoid-binding periplasmic protein, respectively.

BtuC. ORF Vng1370G (gi: 1570394) shared 39% identity and 58% similarity with the BtuC protein of *E. coli* (7). We predict the Vng1370G gene is cotranscribed with the predicted *btuD* gene ortholog (Vng1371Gm [gi: 16554494]).

BtuD. The putative Vng1371Gm protein shared 29% identity and 45% similarity with the *E. coli* BtuD protein.

BtuF. ORF Vng1369G is encoded divergently from the putative *btuCD* operon and had 28% identity and 44% similarity to the *E. coli* BtuF protein. The *btuF* gene did not appear to be part of an operon. The *Halobacterium* BtuF protein is predicted to have an N-terminal signal peptide that directs it to the extracellular side of the cellular membrane via the Sec pathway (33, 34, 53). The signal (residues 1 to 21) is predicted to be cleaved after residue Ala24 (8). The BtuF protein has a C-terminal hydrophobic domain (amino acid residues 348 to 367) preceding a stretch of six hydroxylated amino acid residues, which may indicate anchoring to the extracellular side of the cell membrane (2).

A structure-based sequence alignment between *Halobacterium* BtuF and *E. coli* BtuF (the latter's crystal structure bound to B_{12} has been solved [21]) was used to identify possible B_{12}-biding residues. Of the 11 residues in *E. coli* BtuF that make direct contacts with the B_{12} molecule, *Halobacterium* BtuF has three that are identical (*E. coli* BtuF residues S8, P9, and A10) and two are similar substituted hydrophobic residues (Y28F and W174Y). Based on this comparison, it is unclear if *Halobacterium* BtuF plays the same role or binds B_{12} like *E. coli* BtuF.

***btuC* (Vng1370G), *btuD* (Vng1371Gm), and *btuF* (Vng1369G) of *Halobacterium* are required for corrinoid utilization.** Strains JE7084 (Δ*cbiP* Δ*btuCD*) and JE7862 (Δ*cbiP* Δ*btuF*) were used to determine if *Halobacterium btuC*, *btuD*, and *btuF* functions are required for corrinoid utilization. In these strains, the lesion in *cbiP* blocks de novo cobamide synthesis, thus rendering cell growth dependent on corrinoid transport (50). Strains JE7084 and JE7682 were grown in CD liquid medium with various concentrations of Cbl. These strains failed to grow when provided with 10 nM Cbl, a concentration that was sufficient for growth of JE6738 (Δ*cbiP btuCD*$^+$ *btuF*$^+$) (Fig. 4A [open triangles versus inverted closed triangles] and C [closed diamonds versus closed triangles]). No significant growth was observed until the medium was supplemented with 100 μM Cbl. Because cells are unlikely to encounter 100 μM corrinoid in nature, this observed growth was most likely due to nonspecific transport. These strains were also tested for the ability to assimilate incomplete cobamides. At 10 nM, Cby, Cbi, and

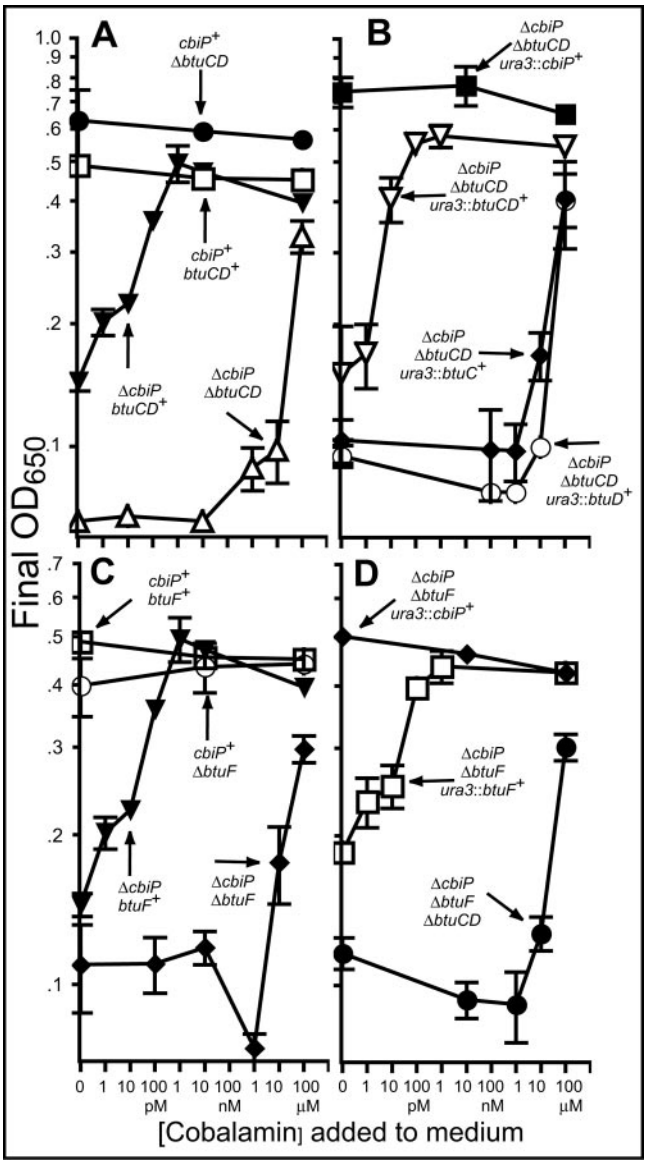

FIG. 4. Nutritional studies of *Halobacterium* sp. strain NRC-1 *btu* mutants. Cbl-dependent growth of *Halobacterium* sp. strain NRC-1 mutants in CD liquid medium with the indicated concentrations of Cbl at 37°C is reported as the absorbance at 650 nm (OD_{650}) after 6 days of growth. After 6 days, cells reach maximum density, and this value is indicative of the ability to grow in the given medium. The mean cell densities of duplicate experiments are reported ± the standard deviations. The strains are indicated by their genotypes. The strains used were MPK414 (*cbiP*$^+$ *btuCD*$^+$ *btuF*$^+$), JE6738 (Δ*cbiP btuCD*$^+$ *btuF*$^+$), JE7084 (Δ*cbiP* Δ*btuCD btuF*$^+$), JE 7108 (*cbiP*$^+$ Δ*btuCD btuF*$^+$), JE7494 (Δ*cbiP* Δ*btuCD btuF*$^+$ *ura3*::*cbiP*$^+$), JE7495 (Δ*cbiP* Δ*btuCD btuF*$^+$ *ura3*::*btuCD*$^+$), JE7684 (Δ*cbiP* Δ*btuCD btuF*$^+$ *ura3*::*btuC*$^+$), JE7685 (Δ*cbiP* Δ*btuCD btuF*$^+$ *ura3*::*btuD*$^+$), JE7681 (*cbiP*$^+$ Δ*btuF btuCD*$^+$), JE7682 (Δ*cbiP* Δ*btuF btuCD*$^+$), JE7683 (Δ*cbiP* Δ*btuCD* Δ*btuF*), JE7796 (Δ*cbiP* Δ*btuF ura3*::*cbiP*$^+$ *btuCD*$^+$), and JE7797 (Δ*cbiP* Δ*btuF btuCD*$^+$ *ura3*::*btuF*$^+$).

Cbi-GDP did not support the growth of either strain, while 1 μM Cbl supported wild-type growth (data not shown).

The observed block of corrinoid transport in strains JE7084 and JE7682 was corrected when wild-type alleles of *Halobacterium btuC* and *btuD* or *btuF* were introduced into the chromosome. Strains JE7495 (Δ*cbiP* Δ*btuCD ura3*::*btuCD*$^+$) (Fig. 4B, inverted open triangles) and JE7797 (Δ*cbiP* Δ*btuF ura3*::*btuF*$^+$) (Fig. 4D, open squares) grew when 1 nM Cbl was added.

As expected, a lesion in the *btuCD* or *btuF* locus in a *cbiP*$^+$ strain did not interfere with growth under the conditions tested. Strains JE7108 (*cbiP*$^+$ Δ*btuCD*), JE7494 (Δ*cbiP* Δ*btuCD ura3*::*cbiP*$^+$), JE7681 (*cbiP*$^+$ Δ*btuF*), and JE7796 (Δ*cbiP* Δ*btuF ura3*::*cbiP*$^+$) grew without corrinoid supplementation (Fig. 4A [closed circles], B [closed squares], C [open squares], and D [closed diamonds], respectively).

***btuC*, *btuD*, and *btuF* mutations are epistatic.** If the products of the *btuC*, *btuD*, and *btuF* genes work together as a transport system, the phenotypes caused by any combination of the mutations would be the same, i.e., would be epistatic. To test this idea, strains JE7683 (Δ*cbiP* Δ*btuCD* Δ*btuF*), JE7684 (Δ*cbiP* Δ*btuCD ura3*::*btuC*$^+$), and JE7685 (Δ*cbiP* Δ*btuCD ura3*::*btuD*$^+$) were tested for the ability to utilize Cbl. When growing in CD liquid medium, all three strains displayed the same phenotype and did not grow unless ≥100 μM Cbl was added to the medium (Fig. 4B and D). These data suggested that the products of these three genes work together to transport Cbl.

Absence of BtuF but not BtuCD functions prevents Cbl-cell association in *Halobacterium*. To test if BtuC, BtuD, and BtuF are required for the assimilation of exogenous Cbl, *Halobacterium* Δ*cbiP* mutants were grown in liquid RP medium with and without 100 nM Cbl. RP medium allows cobamide-independent growth, and all of the strains in these studies grew at similar rates in this medium regardless of exogenous corrinoids (data not shown). Possible reasons for why corrinoid auxotrophs grew in RP medium are discussed below.

S. enterica strain JE873 (*metE* Δ*cobUST*) was used to test for Cbl presence in *Halobacterium* cells. None of the corrinoid extracts from *Halobacterium* cells grown without Cbl supported the growth of strain JE873 (Fig. 5), indicating that the de novo corrin ring biosynthetic pathway was not functional and that there was no contaminating Cbl in the RP medium. When 100 nM Cbl was added to the medium, Cbl was found in the extracts of strains JE6738 (Δ*cbiP*), JE7084 (Δ*cbiP* Δ*btuCD*), JE7495 (Δ*cbiP* Δ*btuCD ura3*::*btuCD*$^+$), and JE7797 (Δ*cbiP* Δ*btuF ura3*::*btuF*$^+$) but not in strain JE7682 (Δ*cbiP* Δ*btuF*) (Fig. 5). Collectively, these results suggested that the *btuF* gene is required for the presence of Cbl in extracts but that the *btuCD* genes are not. This phenotype of strain JE7682 was corrected by reintroduction of a wild-type copy of the *btuF* gene into the chromosome (Fig. 5). None of the spotted extracts supported the growth of *S. enterica* strain JE2243 (*metE btuB*), indicating that the growth of JE873 was due specifically to Cbl (results not shown).

Quantification of Cbl in *Halobacterium*. The Cbl associated with cell extract was quantified and expressed as picomoles of Cbl per gram of total cell protein. In RP medium, no Cbl was detected in strain JE6738 grown in medium supplemented with 100 pM Cbl (<20 pmol Cbl per g protein), but 2,680 pmol Cbl per g protein (1,176 molecules of Cbl per CFU) accumulated in the same strain when the medium contained 100 nM Cbl (Fig. 6A). When CD medium was supplemented with 100 pM Cbl, cultures reached 79% of the cell density (2.0×10^8 CFU/

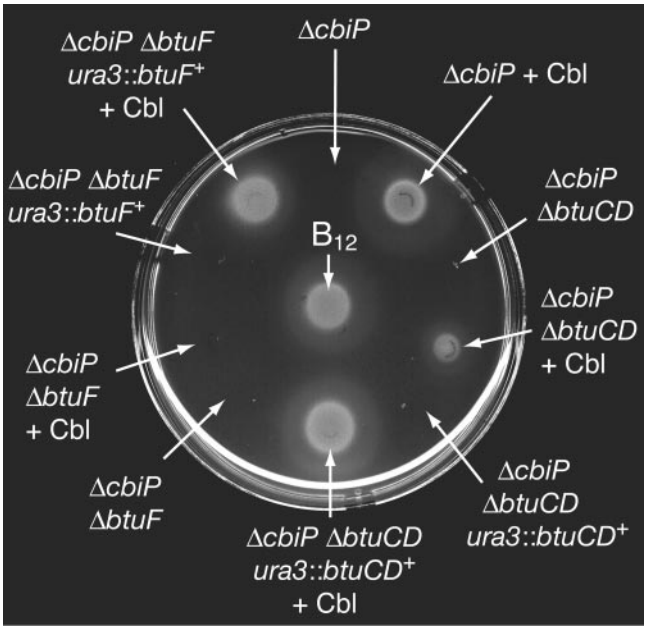

FIG. 5. Bioassay for the detection of Cbl extracted from *Halobacterium* cells. Shown is the response of *S. enterica* indicator strain JE873 (*metE205* Δ*cobUST*) to 2 μl of *Halobacterium* corrinoid extracts and 2 pmol of authentic Cbl. Growth around the area of application indicated the presence of Cbl in the extract. Arrows indicate the genotype of the strain from which the extract was obtained. All *Halobacterium* strains were grown in liquid RP medium with or without 100 nM Cbl (also indicated by arrows) prior to Cbl extraction.

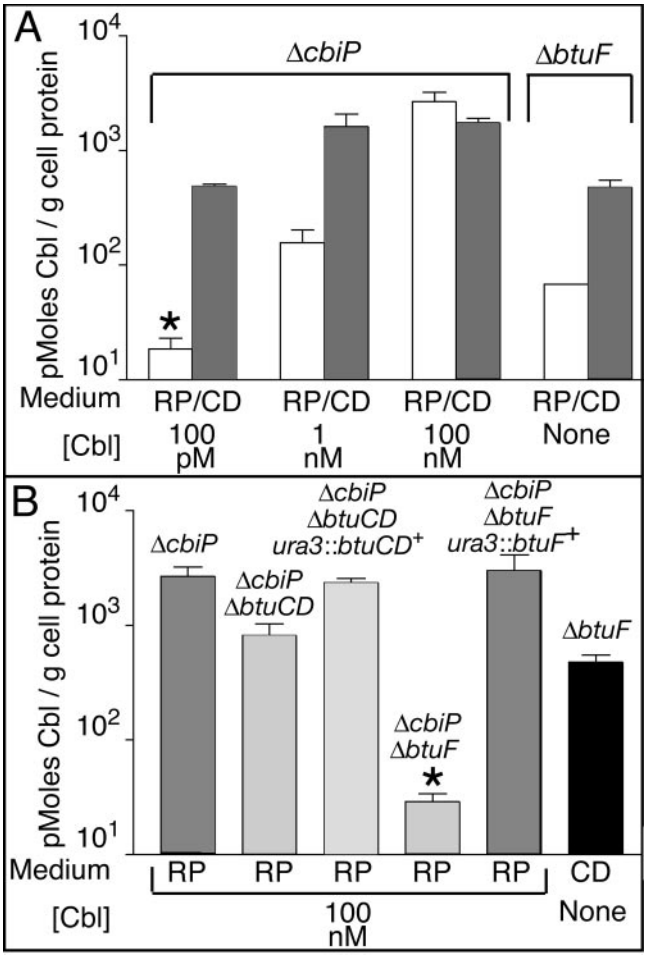

FIG. 6. Quantification of Cbl in *Halobacterium* cells. Shown is the amount of Cbl extracted from *Halobacterium* cells indicated as picomoles of Cbl per gram of total cell protein. The genotype of the strain from which Cbl was extracted is indicated above each column. Under each column, the type of liquid medium (RP, RP medium; CD, CD medium) and the concentration of Cbl added are indicated. Asterisks above a column indicate that the values are below the detection limit of the assay. The mean values of duplicated experiments are reported ± the standard deviations. The strains used were JE6738 (Δ*cbiP btuCD*$^+$ *btuF*$^+$), JE7084 (Δ*cbiP* Δ*btuCD btuF*$^+$), JE7495 (Δ*cbiP* Δ*btuCD btuF*$^+$ *ura3*::*btuCD*$^+$), JE7681 (*cbiP*$^+$ Δ*btuF btuCD*$^+$), JE7682 (Δ*cbiP* Δ*btuF btuCD*$^+$), and JE7797 (Δ*cbiP* Δ*btuF btuCD*$^+$ *ura3*::*btuF*$^+$).

ml) of a culture grown in RP medium and they accumulated 490 pmol Cbl per g protein (Fig. 6A). Cbl-cell association reached a maximum level when CD medium was supplemented with 1 nM Cbl (1,620 pmol Cbl per g protein), consistent with growth data that showed maximum growth at this concentration (Fig. 4, open triangles). The relationship between CFU and total cell protein did not vary significantly between growth media, suggesting that levels of Cbl can be compared. Strain JE7681 (*cbiP*$^+$ Δ*btuF*) was used to determine how much Cbl was synthesized de novo by *Halobacterium*. When grown in RP medium, strain JE7681 synthesized 70 pmol Cbl per g protein compared to 480 pmol Cbl per g protein when grown in CD medium (Fig. 6A). These data suggest regulation of the corrinoid transport system as a function of nutrient availability. At this point, no specific nutrient(s) to which the cells may respond has been identified. Additionally, no obvious transcriptional regulatory sequences in the DNA sequences 5′ of either the Cbl biosynthetic or transport genes have been identified (data not shown).

To determine how much a lesion in the *btuCD* or *btuF* locus would affect the assimilation of Cbl, strains JE7084 (Δ*cbiP* Δ*btuCD*) and JE7682 (Δ*cbiP* Δ*btuF*) were tested. These strains were grown in RP medium supplemented with 100 nM Cbl. Compared to strain JE6738 (Δ*cbiP*), the presence of Cbl in strain JE7084 was reduced 69% to 820 pmol Cbl per g protein, whereas no Cbl was detected in strain JE7682 extract (<30 pmol Cbl per g protein) (Fig. 6B). The phenotypes of strains JE7084 and JE7682 were corrected by reintroduction of the wild-type alleles of *btuCD* and *btuF*, respectively (Fig. 6B). During the quantification studies, Cbl was the only corrinoid detected by HPLC in strains lacking de novo capabilities, suggesting that the Cbl molecules did not have to be modified for usage.

It is possible that in the *btuCD* mutant strain, Cbl is associated with the cells but is inaccessible to metabolism. The Cbl molecules may still be associated with the BtuF protein, which is predicted to be anchored to the extracellular side of the membrane by its C-terminal hydrophobic domain. Without the BtuC and BtuD proteins in the membrane, BtuF may be binding Cbl molecules but not releasing them. In vitro binding studies with *Halobacterium* BtuF and Cbl, as well as localization studies, are needed to test if BtuF may be binding Cbl on the outer surface of the cell membrane.

Conclusions. We have identified a corrinoid transport system in the hyperhalophilic archaeon *Halobacterium* sp. strain NRC-1. Genes encoding this system were annotated as *hemU*, *hemV2*, and *hemV1* (27). We suggest a change in their nomenclature to *btuC*, *btuD*, and *btuF*, respectively, to reflect their role in corrinoid transport.

Most other available archaeal genome sequences are predicted to contain orthologs to the *btuC*, *btuD*, and *btuF* genes. Two notable exceptions lacking *btuC*, *btuD*, and *btuF* were *Methanothermobacter thermautotrophicus* strain ΔH (42) and *Methanopyrus kandleri* AV19 (41). Both of these archaea appear to contain genetic information for an entire cobamide de novo biosynthetic pathway. The latter may have evolved to rely on endogenously synthesized corrinoids. However, *M. marburgensis* strain Marburg, a close relative of *M. thermautotrophicus* strain ΔH, has been shown to assimilate exogenous corrinoids (43), suggesting that a nonorthologous transport system exists in this archaeon and thus may exist in other archaea.

Identification of *btuC*, *btuD*, and *btuF* orthologs in other archaea based on sequence analysis alone may be problematic. Corrinoid transport systems have amino acid sequences very similar to ABC-type Fe^{3+}, siderophore, and heme transport systems. Many archaea have several putative orthologs to these systems, and they are not always encoded in close proximity to each other or cobamide biosynthetic genes, making it difficult to identify or match the components of transport systems. Identification of these transport systems may have to rely on more classical genetic and biochemical approaches, like the ones used in the work reported here.

Putative Cbl-dependent enzymes in *Halobacterium*. It is unknown why *Halobacterium* requires corrinoids to grow in CD medium. The ability of RP medium to allow growth of *Halobacterium* corrinoid mutants suggests that these strains are auxotrophic for a nutrient present in this medium. Analysis of the genome sequence predicts that *Halobacterium* synthesizes at least three cobamide-dependent enzymes, methylmalonyl-coenzyme A mutase (encoded by ORFs Vng0481G, Vng0653G, and Vng0673G), glutamate mutase (encoded by ORFs Vng2286G and Vng2288G), and class II ribonucleotide reductase (encoded by ORF Vng1644G) (27). These enzymes would likely require adenosylcobalamin as the coenzyme; thus, Cbl would have to be adenosylated after transport by an ATP:co(I)rrinoid adenosyltransferase (CobA in *S. enterica*). *Halobacterium* contains a putative *cobA* ortholog (Vng1574G in *Halobacterium*), but its function has not been demonstrated experimentally. Nutritional analyses of mutants defective for these enzymes may determine if growth in CD medium requires Cbl.

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