Corrinoids belong to the family of cyclic tetrapyrroles that includes hemes, chlorophylls, and coenzyme F₄₃₀ (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 B₁₂. 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 H₂ and CO₂, acetate, or methanol (14). Active cobamide-dependent (class II) ribonucleotide dehydratases 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, Vng1371Gm, and Vng1369G
TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Marker(s)  | Relevant genotype | Relevant characteristics and use | Reference or source |
|-------------------|------------|-------------------|----------------------------------|---------------------|
| **Halobacterium strains** |            |                   |                                  |                     |
| MPK414            | ∆ara3      |                   | Able to synthesize Cbl de novo   | 50                  |
| JE6738            | ∆ara3, ∆chiP |                | Unable to synthesize cobamides de novo | 51                  |
| JE7084            | ∆ara3, ∆chiP, ∆btuCD |    | In-frame deletion of btuCD; unable to synthesize cobamides de novo |  |
| JE7108            | ∆ara3, ∆btuCD |                | In-frame deletion of btuCD pathway |  |
| JE7494            | ∆ara3, ∆chiP, ∆btuCD, ura3::chiP | | Complementation of de novo pathway |  |
| JE7495            | ∆ara3, ∆chiP, ∆btuCD, ura3::btuCD | | Complementation of btuCD |  |
| JE7681            | ∆ara3, ∆btuF |                | In-frame deletion of btuF |  |
| JE7682            | ∆ara3, ∆chiP, ∆btuF | | In-frame deletion of btuF; unable to synthesize cobamides de novo |  |
| JE7683            | ∆ara3, ∆chiP, ∆btuF, ∆btuCD | | In-frame deletions of btuF and btuCD; unable to synthesize cobamides de novo |  |
| JE7684            | ∆ara3, ∆chiP, ∆btuCD, ura3::btuCD | | Complementation of btuC |  |
| JE7685            | ∆ara3, ∆chiP, ∆btuCD, ura3::btuD | | Complementation of btuD |  |
| JE7796            | ∆ara3, ∆chiP, ∆btuF, ura3::chiP | | Complementation of de novo pathway in btuF mutant |  |
| JE7797            | ∆ara3, ∆chiP, ∆btuF, ura3::btuF | | Complementation of btuF |  |
| **S. enterica strains** |            |                   |                                  |                     |
| JE873             | metE205 ara-9, Δcob272 (cobUST) | | Cobamide bioassay | Laboratory collection |
| JE2243            | metE205 ara-9, btuB101::MudF | | Cobamide bioassay | Laboratory collection |
| **Plasmids**      |            |                   |                                  |                     |
| pMPK428           | 5-FOA, Mev' | ura3'             | Generation of in-frame deletions of targeted genes | 32                  |
| pMPK424           | 5-FOA, Mev' | ura3'             | Recombination at ura3 locus      | 31                  |
| pCBIP7            | 5-FOA, Mev' | ∆chiP             | Generates chiP deletion          | 51                  |
| pVNG1370-2        | 5-FOA, Mev' | ura3', ∆btuCD    | Generates btuCD deletion         |                     |
| pVNG1370-4        | 5-FOA, Mev' | ura3', btuCD'    | Recombination of btuCD into ura3 locus |  |
| pHsBTUC5          | 5-FOA, Mev' | ura3', btuC'     | Recombination of btuC into ura3 locus |  |
| pHsBTUD1          | 5-FOA, Mev' | ura3', btuD'     | Recombination of btuD into ura3 locus |  |
| pHsBTUF5          | 5-FOA, Mev' | ura3', ∆btuF    | Generates btuF deletion          |                     |
| pHsBTUF6          | 5-FOA, Mev' | ura3', ∆btuF'   | Recombination of btuF into ura3 locus |  |

*a Abbreviations: Mev', resistance to mevinolin; 5-FOA', 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).

**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 (∆ana5) 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
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 btuC 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 excised 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 bta mRNA transcript. As described for plasmid pHSBTUC5, a transcriptional terminator sequence was included 3’ of the btuD ORF.

Plasmid pHsBTU6. The primer set HsBTUF-Comp-XbaI-5’ (TGAAGATCGGTGACGGTGACGCTGTTCTC)–HsBTUF-Comp-BglII-3’ (ACTAGTCATAGGCAGCAGCCGCTGACGCTGTTCTC) was used to PCR amplify a 130-bp DNA fragment that was cloned into the XbaI/BglII sites of the pHsBTUF5. The resulting plasmid, pHsBTUF6, should replace bases 169 to 948 with a 6-bp HindIII restriction site.

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 pMPK414-deriv plasmid containing a deletion of the desired gene and a functional terminator, 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 pHVNG1370-2 (btuCD) was transformed into MPK414 and JEs738 (ΔbtuB) to generate strains JE1078 (ΔbtuCD) and JE7084 (ΔbtuB ΔbtuCD), respectively. Plasmid pHsBTUF5 (ΔbtuF) was transformed into MPK414, JEs738 (ΔbtuB), and JE7804 (ΔbtuB ΔbtuCD) to generate strains JE7861 (ΔbtuBΔbtuF), JE7862 (ΔbtuB ΔbtuF ΔbtuCD), and JE7863 (ΔbtuB ΔbtuCD ΔbtuF), respectively.

Construction of complementation strains. Complementation studies were performed by introducing a single wild-type gene(s) into a desired strain 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 pCBF17 (cbpB) was transformed into JE7084 (ΔbtuB ΔbtuCD) and JE7682 (ΔbtuB ΔbtuF) to generate strains JE7494 (ΔbtuB ΔbtuCD ura3ΔcbpB+) and JE7796 (ΔbtuB ΔbtuF ura3ΔcbpB+), respectively. Plasmids pVNG1370-4 (btuCD+), pHsBTUC5 (btuC+), and pHsBTUF1 (btuF+) were transformed into JE7084 (ΔbtuB ΔbtuCD) to generate JE7495 (ΔbtuB ΔbtuCD ura3ΔbtaFΔbtaCΔbtaD), JE7684 (ΔbtuB ΔbtuCD ura3ΔbtaCΔbtaD), and JE7685 (ΔbtuB ΔbtuCD ura3ΔbtaFΔbtaCΔbtaD), respectively. Plasmid pHsBTUF6 (btuF+) was transformed into JE7682 (ΔbtuB ΔbtuCD) to generate strain JE7797 (ΔbtuB ΔbtuF ura3ΔbtaCΔbtaD).

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 C6. 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 the culture was used to inoculate 1 liter of liquid RP or CD medium supplemented with various concentrations of C6. 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 the
derivate 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 MgSO₄. 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 corrinoid 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 C₈ 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. 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 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 Daltonics (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 (ΔbtfF *chiP*) 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 ΔcobUST) was the indicator strain used in bioassays to detect the presence of cobamides in *Halobacterium* cobrinoid extracts (26). *S. enterica* strain JE2243 (metE btb) 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).
Separate panels include the effects of a \(\Delta cbip\) mutation (A), complementation of \(\Delta btuCD\) mutant strains (B), a \(\Delta btuF\) mutation (C), and complementation of \(\Delta btuF\) 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 (\(\Delta cbip\)).

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 \(37^\circ\)C is reported as the absorbance at 650 nm (OD 650) after 6 days of growth. Concentrations of Cbl of up to 100 \(\mu\)M 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. 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 \(\mu\)M 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 \(S.\ eneterica\) 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 \(btu\) 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 \(B_{12}\)-binding 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 (\(\Delta cbip\ \Delta btuCD\)) and JE7862 (\(\Delta cbip \Delta btuF\)) 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 JE7862 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 (\(\Delta cbip \Delta btuCD^+ \Delta btuF^+\)) (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 \(\mu\)M Cbl. Because cells are unlikely to encounter 100 \(\mu\)M 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 Cbi-GDP were tested and found to support growth equivalent to growth on Cbl.
Cbl-dependent growth of \textit{Halobacterium} mutants. Cbl-dependent growth of \textit{Halobacterium} mutants was corrected when wild-type alleles of Cbl supported wild-type growth (data not shown). Cbl-GDP did not support the growth of either strain, while 1 \textmu M Cbl supported wild-type growth (data not shown).

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

\textit{btuC}, \textit{btuD}, and \textit{btuF} mutations are epistatic. If the products of the \textit{btuC}, \textit{btuD}, and \textit{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 (\textit{cbiP} \textit{Delta}btuCD \textit{Delta}btuF), JE7684 (\textit{cbiP} \textit{Delta}btuCD \textit{ura3}:\textit{btuF}'), and JE7685 (\textit{cbiP} \textit{Delta}btuCD \textit{ura3}:\textit{btuF}'') 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 \(\geq 100\) \textmu M 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 \textit{BtuF} but not \textit{BtuCD} functions prevents Cbl-cell association in \textit{Halobacterium}. To test if \textit{BtuC}, \textit{BtuD}, and \textit{BtuF} are required for the assimilation of exogenous Cbl, \textit{Halobacterium} \textit{Delta}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.

\textit{S. enterica} strain JE873 (\textit{metE} \textit{Delta}cobU\textit{ST}) was used to test for Cbl presence in \textit{Halobacterium} cells. None of the corrinoid extracts from \textit{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 (\textit{cbiP}'), JE7084 (\textit{Delta}cbiP \textit{Delta}btuCD \textit{Delta}btuF), JE7495 (\textit{Delta}cbiP \textit{Delta}btuCD \textit{ura3}:\textit{btuF}'), and JE7797 (\textit{cbiP} \textit{Delta}btuCD \textit{ura3}:\textit{btuF}'') but not in strain JE7682 (\textit{Delta}cbiP \textit{Delta}btuF) (Fig. 5). Collectively, these results suggested that the \textit{btuF} gene is required for the presence of Cbl in extracts but that the \textit{btuCD} genes are not. This phenotype of strain JE7682 was corrected by reintroduction of a wild-type copy of the \textit{btuF} gene into the chromosome (Fig. 5). None of the spotted extracts supported the growth of \textit{S. enterica} strain JE2243 (\textit{metE} \textit{btuB}), indicating that the growth of JE873 was due specifically to Cbl (results not shown).

Quantification of Cbl in \textit{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 \times 10^8 CFU/
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/H11001/H9004 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'-H11032 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 (ΔcbiP ΔbtuF) and JE7682 (ΔcbiP ΔbtuF) were tested. These strains were grown in RP medium supplemented with 100 nM Cbl. Compared to strain JE6738 (ΔcbiP), 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 *Methanothrophus thermotrophicus* strain ΔH (42) and *Methanopyrus kandleri* AV19 (43). We suggest a change in their nomenclature to reflect their involvement in vitamin B12 uptake. *Halobacterium* strain Marburg, a close relative of *Halobacterium* NRC-1, contains a putative ortholog to the *btuC* gene. It will be interesting to determine whether the presence of the *btuC* gene in *Halobacterium* strain Marburg, a close relative of *Halobacterium* NRC-1, is due to horizontal gene transfer or to a common ancestor of *Halobacterium* and *Halobacterium* NRC-1. The presence of a putative ortholog to the *btuC* gene in *Halobacterium* strain Marburg suggests that a nonorthologous transport system exists in the hyperhalophilic archaeon *Halobacterium* NRC-1: a major role for the twin-arginine translocation pathway? Microbiology 148:3335–3346.

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 ORF Vng0641G, Vng0653G, and Vng0673G), glutamate mutase (encoded by ORF 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(1)ribonoid 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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Kraüter, B., H. P. E. Kohler, and E. Stupperich. 1987. The corrinoid from *Methanopyrus kandleri* AV19 (43). 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, *Marburgena* gregii strain Marburg, a close relative of *M. thermotrophicus* 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 Fe3+–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.

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