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A New Pathway for Salvaging the Coenzyme B$_{12}$ Precursor Cobinamide in Archaea Requires Cobinamide-Phosphate Synthase (CbiB) Enzyme Activity

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The ability of archaea to salvage cobinamide has been under question because archael genomes lack orthologs to the bacterial nucleoside triphosphate:5'–deoxyxobinamide kinase enzyme (cobU in Salmonella enterica). The latter activity is required for cobinamide salvaging in bacteria. This paper reports evidence that archaea salvage cobinamide from the environment by using a pathway different from the one used by bacteria. These studies demanded the functional characterization of two genes whose putative function had been annotated based solely on their homology to the bacterial genes encoding adenosylcobinamide acid and adenosylcobinamide-phosphate synthases (cbiP and cbiB, respectively) of S. enterica. A cbiP mutant strain of the archaean Halobacterium sp. strain NRC-1 was auxotrophic for adenosylcobinamide acid, a known intermediate of the de novo cobamide biosynthesis pathway, but efficiently salvaged cobinamide from the environment, suggesting the existence of a salvaging pathway in this archaea. A cbiB mutant strain of Halobacterium was auxotrophic for adenosylcobinamide-GDP, a known de novo intermediate, and did not salvage cobinamide. The results of the nutritional analyses of the cbiP and cbiB mutants suggested that the entry point for cobinamide salvage is adenosylcobinamide acid. The data are consistent with a salvaging pathway for cobinamide in which an amidohydrolase enzyme cleaves off the aminopropanol moiety of adenosylcobinamide to yield adenosylcobinamide acid, which is converted by the adenosylcobinamide-phosphate synthase enzyme to adenosylcobinamide-phosphate, a known intermediate of the de novo biosynthetic pathway. The existence of an adenosylcobinamide amidohydrolase enzyme would explain the lack of an adenosylcobinamide kinase in archaea.

To date, de novo coenzyme B$_{12}$ (Fig. 1) biosynthesis has only been reported to occur in prokaryotes (2, 13, 28, 30, 31, 38). This major biosynthetic pathway has mostly been studied in aerobic systems, with the majority of the work being focused on the anaerobic biosynthesis of the corrin ring in Salmonella enterica (11, 27), Propionibacterium freudenreichii subsp. shermanii (29), and Bacillus megaterium (6, 23, 24) and on aerobic biosynthesis of the corrin ring in Pseudomonas denitrificans (4). This large body of work has given considerable insight into the details of cobamide biosynthesis and has set the basis for comparisons with other organisms (26, 38).

At present, our knowledge of how archaea synthesize cobamides is very limited (7, 36, 39). It is clear that some archaea synthesize and require cobamides to live. For example, methanogenic archaea require cobamides for methanogenesis from H$_2$ and CO$_2$, acetate, or methanol (10). The extremely halophilic archaeon Halobacterium sp. NRC-1 has been shown to produce and require cobamides under certain growth conditions, but it is unclear why they are needed (39). Some archaea may possess cobamide-dependent ribonucleotide reductases that are required for DNA synthesis, as suggested by genome sequence analysis. In fact, cobamide-dependent ribonucleotide reductases have been isolated from Thermoplasma acidophilum and Pyrococcus furiosus (25, 34). The availability of several archaeal genome sequences has allowed researchers to predict which organisms may have complete de novo cobamide pathways and which may have only enough genetic information for precursor salvaging.

Analysis of the available archaean genome sequences revealed the absence of an archaean ortholog to the bacterial ATP:adenosylcobinamide (AdoCbi) kinase/GTP:adenosylcobinamide-phosphate (AdoCbi-P) guanylyltransferase (CobU in S. enterica). The transferase activity was shown to be required for de novo biosynthesis of cobamides and for the salvaging of unphosphorylated Cbi (19). The kinase activity, on the other hand, is only required for the salvaging of Cbi (8, 36) (Fig. 1). Recently, it was shown that the conserved archaean cobY gene is the nonorthologous replacement of the S. enterica cobU gene. The CobY protein has the nucleoside triphosphate (NTP):AdoCbi-P nucleotidyltransferase activity required for de novo synthesis of cobamides but lacks the NTP:AdoCbi kinase activity necessary to salvage Cbi via the pathway used by bacteria (5, 36, 39).

The lack of an NTP:AdoCbi kinase ortholog in archaea raises three important questions. (i) Are archaea able to salvage Cbi? (ii) If they can, does an alternative, nonorthologous replacement of the bacterial NTP:AdoCbi kinase exist in these prokaryotes? (iii) If a nonorthologous replacement of the bacterial NTP:AdoCbi kinase does not exist in archaea, does an alternative, uncharacterized Cbi-salvaging pathway exist? Previous studies of Methanobacterium thermoautotrophicum strongly suggested that this archaean can salvage Cbi (32). However, to the best of our knowledge, there are no reported studies of the pathway used by this or any other archaean to salvage Cbi.

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In this paper, we provide genetic evidence for the ability of the extremely halophilic archaeon *Halobacterium* sp. strain NRC-1 to efficiently salvage exogenous Cbi via an alternative pathway to the one used by bacteria. These studies demanded the functional characterization of two genes whose putative function had been annotated exclusively on the basis of their homology to the bacterial adenosylcobyric acid (AdoCby) and AdoCbi-P synthases (*cbiP* and *cbiB*, respectively) present in *S. enterica* (Fig. 1).

**Materials and Methods**

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

**Chemicals, culture media, and growth conditions.** All chemicals used in this work were commercially available, high-purity compounds. When corrinoids were added to the medium, they were used at concentrations of 100 pM for *Halobacterium* studies and 15 nM for *S. enterica* studies. All corrinoids were added in their cyano form. Cbi dicyanide was purchased from Sigma (St. Louis, Mo.). Cobyric acid dicyanide [(CN)₂Cby] was a gift from Paul Renz (Universität-Hohenheim, Stuttgart, Germany), and mevinolin was purchased from Zymo Research (Orange, Calif.). 5-fluoroorotic acid (5-FOA) was purchased from Zymo Research (Orange, Calif.), and mevinolin was purchased from LKT Laboratories, Inc. (St. Paul, Minn.).

**Halobacterium studies.** Strains were grown in liquid peptone (Oxoid, Hampshire, England) medium (18) lacking trace metals. *Halobacterium* cultures were grown to stationary phase at 37°C with shaking for 5 days. Cells used as inocula were harvested by centrifugation (10,000 × g for 2 min) with a Microfuge 18 centrifuge (Beckman-Coulter, Fullerton, Calif.) and washed once in a chemically
defined medium (14). Cells were diluted 100-fold and used to inoculate the defined medium containing the appropriate corrinoid supplements. Cultures were grown at 37°C with shaking. Growth was monitored every 24 h by measuring the absorbance of the culture at 650 nm with a Spectronic 20D spectrophotometer. Growth was monitored every 24 h by measuring the absorbance of the culture at 650 nm with a Spectronic 20D spectrophotometer.

### TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Marker(s)</th>
<th>Relevant genotype</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Halobacterium strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPK414</td>
<td>Δura3</td>
<td>Strain with de novo cobamide biosynthetic capability</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>JE6738</td>
<td>Δura3 ΔcbiP</td>
<td>Strain with in-frame deletion of cbip</td>
<td>Laboratory collection</td>
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<td>JE6791</td>
<td>Δura3 ΔcbiB</td>
<td>Strain with in-frame deletion of cbib</td>
<td>Laboratory collection</td>
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<td>JE6930</td>
<td>Δura3 ΔcbiB ura3::cbiP+</td>
<td>Strain used to test for complementation of cbib</td>
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<tr>
<td>JE7001</td>
<td>Δura3 ΔcbiP ura3::cbiP+</td>
<td>Strain used to test for complementation of cbip</td>
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<td><strong>S. enterica strains</strong></td>
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<td>metE</td>
<td>S. enterica wild type for this study</td>
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<tr>
<td>pMPK428</td>
<td>5-FOA*, Mev*</td>
<td>Δura3*</td>
<td>Plasmid used to generate in-frame deletions of targeted genes</td>
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</tr>
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<td>pMPK424</td>
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<td>Δura3*</td>
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<td>pCBIP2</td>
<td>5-FOA*, Mev*</td>
<td>Δura3* ΔcbiP</td>
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<td>pCBIP7</td>
<td>5-FOA*, Mev*</td>
<td>Δura3* cbiP+</td>
<td>Plasmid used to recombine cbip into Δura3 locus</td>
<td></td>
</tr>
<tr>
<td>pVng1578-2</td>
<td>5-FOA*, Mev*</td>
<td>Δura3*ΔcbiB</td>
<td>Plasmid transformed into MPK414 to delete cbib</td>
<td></td>
</tr>
<tr>
<td>pVng1578-3</td>
<td>5-FOA*, Mev*</td>
<td>Δura3* cbiB+</td>
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<tr>
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<td>Ap*</td>
<td>Cloning vector used for complementation studies in S. enterica</td>
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<td>Ap*</td>
<td>cbiP+</td>
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</tr>
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<td>cbiB+</td>
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<tr>
<td>pMcCBIB1</td>
<td>Ap*</td>
<td>cbiB+</td>
<td>Plasmid used to provide M. mazei cbiB in trans</td>
<td></td>
</tr>
</tbody>
</table>

*a Abbreviations: Mev*, resistance to mevinolin; 5-FOA*, sensitivity to 5-fluoroorotic acid; Ap*, resistance to ampicillin.

b Unless otherwise stated, strains and plasmids were constructed during the course of this study.

**Plasmid constructions.** Plasmids were propagated in the Escherichia coli strain DH5α except where noted. In all cases, Halobacterium sp. strain NRC-1 genomic DNA for PCR was prepared as previously described (39). Methanococcus mazei strain Goei1 DNA for PCR was a gift from Gerhard Gotschalk (Göttingen, Germany). All primers were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). Underlined portions of the primer sequences (see below) indicate introduced restriction sites.

**Halobacterium plasmids.** A diagram of the Halobacterium sp. strain NRC-1 DNA included in the most relevant plasmids is included in Fig. 2B.

1. **Plasmid pCBIPI.** The 5’ primer cbIP/HindIII #2 (GCACGTGGTCTAGATGAAAG) and the 3’ reverse primer cbIP/EcoRV3’ (CTGGAGTGGAATCCGGTAGCAAC) were used to amplify an 804-bp PCR fragment from strain MPK414 genomic DNA. Amplified DNA was cut with HindIII/EcoRV restriction enzymes (unless otherwise noted, the underlined portion of the sequence is the restriction enzyme site), purified with a QiAquick gel extraction kit (Qiagen, Valencia, Calif.), and cloned into the HindIII/SmaI restriction site of plasmid pMPK428, which contains the wild-type allele of the Halobacterium sp. Δura3 gene and a mevinolin resistance determinant (22). The resulting plasmid is referred to as pCBIP1.

2. **Plasmid pCBIP2.** Plasmid pCBIP2 (ΔcbiP ura3*) carries an in-frame deletion of the Halobacterium sp. strain NRC-1 cbip gene and was constructed as follows. The 5’ primer cbIP/HindIII 21 (CACGACGAGTAAGCTTTCGGC) and reverse 3’ primer cbIP/HindIII 39 (GCACGTGGTCTAGATGAAAG) were used to amplify an 804-bp fragment from MPK414 genomic DNA.
The fragment was cut with XbaI/HindIII restriction enzymes, gel purified, and cloned into the XbaI/HindIII restriction site of plasmid pCBIP2 to create plasmid pCBIP2. The latter contained an in-frame deletion of \( \text{cbiP} \) that replaced bases 303 to 1376 with a 6-bp HindIII restriction site, thus deleting 358 of the 512 amino acids. Plasmid pCBIP2 also carries the mevinolin resistance determinant and a wild-type allele of the \( \text{ura3} \) gene.

(ii) Plasmid pCBIP4. The 5' primer cbiComp EcoRI5' (TCTAGAATT CGAGCGGCGTCCGAGGACG) and reverse primer cbiCompBglII' (A GATCTTAGATCTAAAGAGCCGGCCCGTTGCAAAACAGCGTGACCG TAG) were used to amplify a 1,739-bp PCR product from strain MPK414 genomic DNA. The fragment was cloned into pGEM-T with the Promega pGEM-T cloning kit (Madison, Wis.) to yield the plasmid pCBIP4.

(iv) Plasmid pCBIP5. The fragment carried on plasmid pCBIP4 was excised as a 1,721-bp fragment with an EcoRI/BglII restriction site of plasmid pMPK428, which contains the wild-type \( \text{cbiB} \) allele of \( \text{S. enterica} \) \( \text{cbiB} \) gene and was constructed as follows. The 5' primer \( \text{Vng1578} \) EcoRI5' (GAATTCTCTAGATGA CGACCGCGCCGTCGAC) and reverse primer \( \text{Vng1578} \) BglII3' (GAA ACTGTGGTTGCATACG) were used to amplify an 841-bp fragment from MPK414 genomic DNA. The fragment was cut with XbaI/NcoI restriction enzymes, gel purified, and cloned into the XbaI/NcoI restriction site of plasmid pVNG1578-1 to create plasmid pVNG1578-2. The latter contained an in-frame deletion of \( \text{cbiB} \) that replaced bases 133 to 897 with a 6-bp NcoI restriction site, thus deleting 255 of the 308 amino acids. Plasmid pVNG1578-2 also carries the mevinolin resistance determinant and a wild-type allele of the \( \text{ura3} \) gene.

(v) Plasmid pVNG1578-3. The plasmid pVNG1578-3 (\( \text{cbiB}^+ \text{ura3}^+ \)) carries a wild-type allele of the \( \text{Halobacterium} \) sp. strain NRC-1 \( \text{cbiB} \) gene and was constructed as follows. The 5' primer \( \text{Vng1578} \) BglII5' (GAATCTTACTAGATGACCGCGCCCGTCGAC) and reverse primer \( \text{Vng1578} \) EcoRI3' (GTAGTGGTTGCATACG) were used to amplify an 1,398-bp PCR product from strain JE6693 (a derivative of MPK414) with an in-frame deletion on Vng1577, deleting bases 103 to 408 (S. Escalante-Semerena, laboratory collection) genomic DNA. The fragment was cut with XbaI/BglII restriction enzymes, gel purified, and cloned into the XbaI/BglII restriction site of plasmid pMPK424 (21) (prepared from the mutant strain GM2163 dam) (New England Biolabs, Manchester, Mass.) to yield plasmid pVNG1578-3 (\( \text{cbiB}^+ \text{ura3}^+ \)). The latter contains the cloned fragment flanked by a sequence that would allow recombination at the \( \text{ura3} \) locus of \( \text{Halobacterium} \) sp. strain NRC-1. The resulting plasmid carried a wild-type copy of the \( \text{cbiB} \) gene, including 47 bases upstream of the putative start codon and 200 bases upstream of the putative operon.

To include these sequences, part of ORF Vng1577 was also cloned, but it carried an in-frame deletion spanning from residue 35 to residue 136 (of 152). Including these sequences should preserve the regulation of \( \text{cbiB} \) in its own operon without including other genes. Flanking the 3' end was a 16-bp sequence derived from the \( \text{bop} \) transcription terminator sequence (9) to ensure transcriptional termination of the \( \text{cbiB} \) mRNA transcript.

**S. enterica** plasmid pCBIP9. The plasmid pCBIP9 contained a wild-type allele of **S. enterica** cbiP under the control of the lac promoter and ribosome-binding site and was constructed as follows. The fragment carried on plasmid pCBIP5 (Escalante-Semerena, laboratory collection) included only the **S. enterica** cbiP ORF and was excised as a 1,520-bp fragment with an NdelXhol digest, gel purified, and cloned into the Ndel/Xhol restriction site of plasmid pT7-7 (33) to produce plasmid pCBIP9 (cbiP+).
M. mazei plasmids. (i) Plasmid pMmCBIP1. Plasmid pMmCBIP1 (cbiP<sup>+</sup>) contained a wild-type allele of M. mazei strain Goe1 cbiP (ORF Mma0093) under the control of the lac promoter and ribosome-binding site and was constructed as follows. The 5′-primer MmCH003-BamH1 (5′-AGCTATATATAAAAGCGCCGCGCG-3′) and the reverse primer MmCH003-SalI1 (5′-CCGCTTGTGTCGACCAGTCGACTTTTCAAGCTTCTGCG-3′) were used to amplify a 1.352-bp PCR product from M. mazei genomic DNA. The fragment was treated with polynucleotide kinase, cut with SacI, gel purified, and cloned into the NdeI/SalI site of pT7-7 (prepared by cutting plasmid pT7-7 with NdeI, blunt ending with the MBI Fermentas [Amherst, N.Y.] DNA polymerase I large [Klenow] fragment, and digesting with SalI to produce the plasmid pMmCBIP1 (cbiP<sup>+</sup>).

(ii) Plasmid pMmCBIB1. Plasmid pMmCBIB1 (cbiB<sup>+</sup>) contained a wild-type allele of M. mazei strain Goe1 cbiB (ORF Mma2059) under the control of the lac promoter and ribosome-binding site and was constructed as follows. The 5′-primer MmCHb005-5′NdeI (5′-AGCTATTATATTAGATCGATACGCGGACGACG-3′) and the reverse primer MmCHb005-5′SalI (5′-ATTGATCTGGAGTAAGTCGCGACTTTTCAAGCTTCTGCG-3′) were used to amplify a 1.025-bp PCR product from M. mazei genomic DNA. The fragment was cut with NdeI/SalI restriction enzymes, gel purified, and cloned into the NdeI/SalI restriction site of plasmid pT7-7 to produce plasmid pMmCBIB1 (cbiB<sup>+</sup>).

Halobacterium strain constructions. (i) Construction of a ΔcbiP mutant strain. An in-frame deletion of cbiP in the chromosome of strain MPK414 (ΔcbiP) was generated by using previously described methodology (20). Briefly, strain JE6738 (ΔcbiB ΔcbiP) was constructed by transforming strain MPK414 with plasmid pCBP2 as described previously (15). Flanking sequences of over 700 bases on each side of the deleted cbiP gene ensured efficient recombination of the fragment into the chromosome. Mevinolin-resistant transformants were selected as described previously (15) and replated on medium containing 5-FOA to select for the loss of the plasmid (20). Colonies resistant to 5-FOA were screened by PCR to identify the desired recombinant (ΔcbiP). DNA sequencing was used to confirm the in-frame deletion of the cbiP gene in the chromosome of strain JE6738.

(ii) Construction of a ΔcbiB mutant strain. An in-frame deletion of cbiB in the chromosome of strain MPK414 was generated by using the same strategy as mentioned above. Strain JE6791 (ΔcbiB ΔcbiP) was constructed with strain MPK414 and plasmid pVNG15782. DNA sequencing was used to confirm the in-frame deletion of the cbiB gene in the chromosome of strain JE6791.

(iii) Construction of a cbi<sup>P</sup> complementation strain. Complementation studies were performed with a single copy of the wild-type allele of the gene in question placed at the ara locus. For cbi<sup>P</sup> complementation studies, a wild-type allele of cbiP<sup>+</sup> was placed at the chromosomal ara<sup>P</sup> locus of strain JE6738. Plasmid pCBP2 was transformed into strain JE6738, and strains carrying the cbi<sup>P</sup>-<sup>P</sup> allele at the chromosomal ara<sup>P</sup> locus (strain JE7001 [ΔcbiP ara<sup>P</sup>: cbi<sup>P</sup> <sup>P</sup>]) were isolated by using the same ara<sup>P</sup>-based gene replacement method for the isolation of deleted genes. PCR and DNA sequencing verified the presence of cbiP<sup>+</sup> at the ara<sup>P</sup> locus.

(iv) Construction of a cbi<sup>B</sup> complementation strain. For cbi<sup>B</sup> complementation studies a wild-type allele of cbi<sup>B</sup> was placed at the chromosomal ara<sup>B</sup> locus of strain JE6791. Plasmid pVNG15782-3 was transformed into strain JE6791, and a strain carrying the cbi<sup>B</sup>-<sup>B</sup> allele at the chromosomal ara<sup>B</sup> locus (strain JE6930 [ΔcbiB ara<sup>B</sup>: cbi<sup>B</sup> <sup>B</sup>]) was isolated. PCR and DNA sequencing verified the presence of cbi<sup>B</sup> at the ara<sup>B</sup> locus.

RESULTS

Rationale used to probe into corrinoid salvaging in Halobacterium. Because the growth of Halobacterium in defined medium requires cobamides, the growth of a corrinoid-deficient mutant in medium supplemented with incomplete cobamide precursors would be indicative of precursor salvaging. To block corrin ring biosynthesis in Halobacterium, in-frame deletions were introduced in the second-to-last step or in the last step of corrin ring biosynthesis. In S. enterica, these steps of the pathway are catalyzed by the AdoCby synthase (CbyP) enzyme and the AdoCbi-P synthase (CbiB) enzyme, respectively (38). It was hypothesized that a block in either one of these steps would render a strain dependent on exogenous Cby or Cbi precursors. The mutation in cbiP would block salvaging of cobyrinic acid, a,c-diamide but should not interfere with Cby or Cbi salvaging. A mutation in cbiB would address the question of what the point of entry of Cbi is in the Halobacterium genome sequence. That is, if a cbiB mutation does not prevent Cbi salvaging, then an unidentified kinase may be responsible for the activation of Cbi to Cbi-P (the substrate of the CobY enzyme). Alternatively, the inability of a cbiB mutant to salvage Cbi would suggest the existence of a new pathway for the activation of Cbi in this archaeon.

Identification of the cbiP and cbiB genes of Halobacterium. ORF Vng1576G (gene identification [gi] number 15790548) of the Halobacterium strain NRC-1 genome sequence (17) was identified as the putative cbiP gene of this archaeon based on the 45% identity and 53% similarity of the predicted gene product to the CbiP protein of S. enterica. In the Halobacterium genome, the cbiP (ORF Vng1576G) gene is located at the 3′ end of a putative operon containing ORF Vng1574G and ORF Vng1573G, which encode the putative orthologs of the bacterial ATP:co(T)riinoid adenosyltransferase (CoBα in S. enterica) and the cobyrinic acid a,c-diamide synthase (CoBα in S. enterica), respectively (Fig. 2A). These two proteins are believed to modify the corrinoid immediately preceding the CbiP-catalyzed step (38).

ORF Vng1578H (gi number 15790550) of the Halobacterium genome sequence was identified as the putative cbiB gene of this archaeon based on the 30% identity and 43% similarity of the predicted gene product to the CbiB of S. enterica. In the Halobacterium genome, the cbiB gene is the promoter-distal gene in a putative operon containing one other ORF of unknown function (Fig. 2A).

cbiP (ORF Vng1576G) is a cobamide biosynthetic gene in Halobacterium. To determine if strain JE6738 (ΔcbiP) was deficient in cobamide biosynthesis, growth was assessed in defined medium where cobamides were essential for growth. Unlike strain MPK414 (cbiP<sup>+</sup>), strain JE6738 (ΔcbiP) failed to grow in the defined medium lacking corrinoids (Fig. 3A). To
determine if the observed lack of growth of JE6738 was caused by the inability to synthesize cobamides de novo, the medium was supplemented with Cby (the nonadenosylated product of the CbiP-catalyzed reaction). The addition of Cby restored wild-type growth of JE6738 (Fig. 3A) but did not significantly enhance the growth of the wild-type strain (data not shown). The doubling times of strains MPK414 and JE6738 in medium supplemented with Cby were very similar (30 and 27 h, respectively), whereas doubling times could not be calculated for the strains that displayed extremely poor growth. These data strongly suggested that the absence of cbiP function correlated with the predicted phenotype of a strain lacking AdoCby synthase activity under conditions that demand de novo synthesis of cobamides. This finding led to the proposal that ORF Vng1576G was the archaeal ortholog of the CbiP.

**Halobacterium can salvage Cbi.** Having a *Halobacterium* mutant blocked before the late steps of cobamide biosynthesis allowed us to test if this archaean can salvage Cbi. In bacteria, AdoCby is not an intermediate of the de novo pathway (8, 36, 39) (Fig. 1), and it is also not predicted to be an intermediate in archaea, based on the presence of CbiB. The salvaging of Cbi, therefore, would require additional enzymes or functions. The addition of Cbi to the medium allowed wild-type growth (i.e., 24-h doubling time) of strain JE6738 (ΔcbiP) (Fig. 3A) but did not significantly enhance the growth of the wild-type strain (data not shown). The ability of *Halobacterium* to salvage Cbi suggested the existence of an enzyme that can convert Cbi to a true intermediate of the de novo pathway. A mutation in the CbiB enzyme would block the pathway at a point that would allow us to ascertain whether the entry point for Cbi salvaging in archaea occurred via AdoCbi-P (as in bacteria) or via a new metabolic route.

**cbiB (ORF Vng1578H) is a cobamide biosynthetic gene in *Halobacterium.*** Unlike strain MPK414, strain JE6791 (ΔcbiB) cannot grow in the defined medium lacking corrinoids (Fig. 3B). To test if the lack of growth was due to the inability to synthesize cobamides, Cbi-GDP (a pathway intermediate downstream of the CbiB-catalyzed reaction) (Fig. 1) was added to the medium. Cbi-GDP restored the growth of strain JE6791 (30-h doubling time) (Fig. 3B) but did not significantly enhance growth of the wild-type strain MPK414 (data not shown). The addition of Cby (a pathway intermediate prior to the CbiB-catalyzed reaction), however, failed to restore growth of strain JE6791 (Fig. 3B). These results were consistent with a block in the synthesis of AdoCbi-P and led us to propose that ORF Vng1578H in *Halobacterium* encodes the archaeal ortholog of *S. enterica* CbiP enzyme.

**CbiB activity is required for Cbi salvaging.** As mentioned above, strain JE6738 (ΔcbiP) can salvage Cbi; however, the addition of Cbi to the medium did not restore the growth of strain JE6791 (ΔcbiB) (Fig. 3B). These results confirmed that in *Halobacterium* Cbi must enter the de novo pathway at an entry point prior to the CbiB-catalyzed step. This finding is also consistent with the observation that Cbi and AdoCbi are not intermediates of the archaean de novo pathway. If they were, strain JE6791 would be predicted to be able to salvage Cbi.

**Complementation of cbiP and cbiB mutants of *Halobacterium.*** The observed AdoCby auxotrophy of JE6738 (ΔcbiP) and the AdoCbi-GDP auxotrophy of JE6791 (ΔcbiB) were corrected when the cbiP+ and cbiB+ alleles were reintroduced into the appropriate strains. Strain JE7001 (ΔcbiP ura3::cbiP+) and strain JE6930 (cbiB+ ura3::cbiB+) grew in the defined medium without any corrinoid supplementation (Fig. 3) with a doubling time of 26 and 34 h, respectively. The growth rate of these strains was similar to the rates of strains JE6738 (ΔcbiP) and JE6791 (ΔcbiB) growing on medium supplemented with the correct corrinoid supplements. These results showed that the cbiP+ or cbiB+ functions were necessary and sufficient to restore de novo cobamide synthesis in the mutant strains.

**The archaeal cbiP and cbiB genes complement *S. enterica* cbiP and cbiB mutants.** To further support the conclusion that the archaeal orthologs of cbiP and cbiB do function as AdoCby and AdoCbi-P synthases in vivo, we tested the ability of archaeal cbiP and cbiB orthologs to complement *S. enterica* cbiP and cbiB mutants. To investigate this possibility, the cbiP and cbiB orthologs from the archaeal methanogen *M. mazei* strain Go1 were cloned. Previous work in the laboratory has shown that *Halobacterium* genes do not express well in *S. enterica,* whereas genes from archaeal methanogens are well expressed (36). *M. mazei* ORF Mm0093 (gi number 21226195) showed 42% identity and 58% similarity to the Halobacterium cbiP gene, and ORF Mm2059 (gi number 21228161) showed 28% identity and 45% similarity to the cbiP gene of *Halobacterium.* For this purpose, *S. enterica* strains carrying null alleles of *metE* and either cbiP or cbiB were used. The mutation in *metE* inactivates the cobamide-independent methionine synthase (MetE) enzyme, thus demanding cobamide-dependent methylation of homocysteine to yield methionine by the action of the MetH enzyme (35). An insertion in either cbiP or cbiB eliminated de novo cobamide synthesis.

For cbiP complementation, the positive control plasmid pCBIP9 (containing a wild-type allele of *S. enterica* cbiP+) or plasmid pMmCBIP1 (M. mazei cbiP+) was introduced into the *S. enterica* cbiP *metE* mutant strain JE588.

For cbiB complementation, a plasmid containing a wild-type allele of either *S. enterica* cbiB (the positive control plasmid pSeCBIB4) or *M. mazei* cbiB (plasmid pMmCBIB1) was introduced into the *S. enterica* cbiB *metE* mutant strain JE6368. Residual expression of the cbiP or cbiB genes in the absence of the T7 RNA polymerase allowed us to assess complementation. In both cases, plasmid pT7-7 was used as a vector-only negative control.
To test cbP complementation, S. enterica was grown anaerobically, where the cells can synthesize cobamides de novo. Complementation of cobamide biosynthesis was observed when either S. enterica or M. mazei cbP was provided in trans to JE588 but not with the control vector (Fig. 4A). Growth was similar for all strains when (CN)2Cby was added (Fig. 4B). These results were consistent with the archaeal CbiP enzyme having AdoCby synthase activity in vivo.

cbB complementation was tested under aerobic conditions, where S. enterica must salvage cobamide precursors. In this case Cby was added to the medium. Cby salvaging requires a functional CbiB synthase enzyme (Fig. 1); hence, growth on this intermediate would indicate restoration of the de novo pathway of cbiB mutant strain JE6368. Complementation of Cby salvaging was observed when either S. enterica cbiB (pSeCBIB4) or M. mazei cbiB (pMmCBIB1) was provided in trans but not when the control vector was provided (Fig. 5). These data support the conclusion that the archaeal CbiB enzyme has AdoCbi-P synthase activity in vivo.

DISCUSSION

The contributions of this work are twofold. First, the functions encoded by two putative ORFs in two archaea are supported by in vivo evidence. Second, evidence for the existence of the pathway for salvaging the cobamide precursor Cbi in archaea has been obtained. The latter pathway is distinct from the one used by bacteria.

Biochemical roles of two archaeal genes in cobamide biosynthesis. The results of the nutritional analysis of mutants of the extremely halophilic archaeon Halobacterium sp. strain NRC-1 showed that ORFs Vng1576G and Vng1578H were necessary for de novo cobamide biosynthesis and that ORF Vng1578H was necessary for salvaging cobyric acid from the environment. The conclusions drawn from these analyses were fully supported by complementation analyses of bona fide S. enterica mutants lacking either CbiP or CbiB activities by M. mazei strain Goe1 genes. On the basis of this work, we propose that Halobacterium ORF Vng1578H be annotated as encoding the AdoCbi-P synthase enzyme and that the putative annotation of Vng1576G as encoding the AdoCby synthase enzyme is correct. ORF Vng1578H should be renamed as cbiB to reflect its involvement in cobamide biosynthesis in archaea. This nomenclature should be extended to the ORFs Mm0093 (cbiP) and Mm2059 (cbiB) of M. mazei strain Goe1.

In this study, corrinoid intermediates have been assumed to be adenosylated in vivo. Although this fact has been established in bacteria (12), it is unknown if the corrinoids are adenosylated in archaea. Because archaea possess a putative
orthodox of CobA and archaeal genes can complement S. enterica cob mutants, it is assumed that the corrinoid substrates for the archaeal enzymes are adenosylated.

The archaean pathway for salvaging Cbi is different from the bacterial pathway. The requirement for Cbi enzyme activity for the salvaging of Cbi by *Halobacterium* is key to the proposal that the archaean pathway for salvaging this precursor is different from the one that operates in bacteria (Fig. 1 and 6). In bacteria, Cbi is not required for Cbi salvaging because the NTP:AdoCbi kinase activity of CobU directly converts AdoCbi to AdoCbi-P, the substrate for the Cbi salvage pathway observed in this archaeon is AdoCby, which can then be converted to the archaeal enzymes are adenosylated.

M. mazei chromosomal DNA. 7200 WOODSON ET AL. J. BACTERIOL.

REFERENCES


