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The *cobY* Gene of the Archaeon *Halobacterium* sp. Strain NRC-1 Is Required for De Novo Cobamide Synthesis

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Genetic and nutritional analyses of mutants of the extremely halophilic archaeon *Halobacterium* sp. strain NRC-1 showed that open reading frame (ORF) Vng1581C encodes a protein with nucleoside triphosphate: adenosylcobinamide-phosphate nucleotidyltransferase enzyme activity. This activity was previously associated with the *cobY* gene of the methanogenic archaeon *Methanobacterium thermoautotrophicum* strain ΔH, but no evidence was obtained to demonstrate the direct involvement of this protein in cobamide biosynthesis in archaea. Computer analysis of the *Halobacterium* sp. strain NRC-1 ORF Vng1581C gene and the *cobY* gene of *M. thermoautotrophicum* strain ΔH showed the primary amino acid sequence of the proteins encoded by these two genes to be 35% identical and 48% similar. A strain of *Halobacterium* sp. strain NRC-1 carrying a null allele of the *cobY* gene was auxotrophic for cobinamide-GDP, a known intermediate of the late steps of cobamide biosynthesis. The auxotrophic requirement for cobinamide-GDP was corrected when a wild-type allele of *cobY* was introduced into the mutant strain, demonstrating that the lack of *cobY* function was solely responsible for the observed block in cobamide biosynthesis in this archaeon. The data also show that *Halobacterium* sp. strain NRC-1 possesses a high-affinity transport system for corrinoids and that this archaeon can synthesize cobamides de novo under aerobic growth conditions. To the best of our knowledge this is the first genetic and nutritional analysis of cobalamin biosynthetic mutants in archaea.

Cobamides are complex molecules belonging to the family of cyclic tetrapyrroles, which includes hemes, chlorophylls, and coenzyme F_{430}. Unlike other members of the family, cobamides have an upper and a lower ligand, both of which play important roles in the chemistry catalyzed by cobamides (12). The upper ligand forms a labile, covalent bond with the cobalt ion of the corrin ring, while the lower ligand interacts with the cobalt ion via a coordination bond. The best-known cobamide is cobalamin (i.e., B_{12}), which in its biologically active form has a 5′-deoxyadenosyl group as an upper ligand, hence the name adenosylcobalamin (AdoCbl) or coenzyme B_{12} (Fig. 1). What distinguishes cobamides among each other is the chemical nature of the nucleotide base (25), which in the case of AdoCbl is 5,6-dimethylbenzimidazole.

Cobamides are involved in processes central to prokaryotic and eukaryotic cell physiology, including deoxyribonucleotide synthesis, synthesis of modified tRNAs, amino acid biosynthesis, and energy generation (14). In spite of its broad use in nature, the ability to synthesize cobamides de novo appears to be restricted to prokaryotes. A better understanding of the biochemistry underpinning the synthesis of this fascinating molecule has been pursued for several decades (3, 23, 25, 26, 28). While most of the understanding of this process has been derived from work with bacteria, knowledge of how archaea may synthesize cobamides is very limited (5, 35). It is clear, however, 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 (9). Interestingly, methanogenic archaea can also salvage corrinoids from their environment (31). Genome sequence analysis suggests that some archaea may have a cobamide-dependent ribonucleotide reductase required for DNA synthesis. This was confirmed by the purification of active cobamide-dependent ribonucleotide reductases from *Thermoplasma acidophilum* and *Pyrococcus furiosus* (24, 34). The availability of archaeal genome sequences has also provided insights into the extent to which a given archaeon can synthesize cobamides. That is, whether the organism can synthesize the entire molecule de novo, or whether it can assimilate precursors present in its environment.

Analysis of archaeal genomes revealed the absence of a gene encoding one of the key enzymes involved in the late steps of cobamide biosynthesis in bacteria (35). The enzyme in question was the ATP:Ado-cobinamide (AdoCbi) kinase/GTP: AdoCbi-phosphate (AdoCbi-P) guanylyltransferase (encoded by the *cobU* gene in *Salmonella enterica*; *cobP* in *Pseudomonas denitrificans*) (4, 19, 36–38). The CobU enzyme is present in all cobamide-producing bacteria and plays a key role in both de novo AdoCbl biosynthesis and salvaging of unphosphorylated cobinamide (Cbi) (6, 35).

In vitro and in vivo evidence supports the idea that a conserved archaeal gene, referred to as *cobY*, is the nonorthologous replacement of the *S. enterica cobU* gene (35). The CobY protein of *Methanobacterium thermoautotrophicum* ΔH has nucleoside triphosphate (NTP):AdoCbi-P nucleotidyltransferase activity in vitro, but the enzyme lacks AdoCbi kinase activity (35). Although these data provided biochemical evidence that the CobY protein can catalyze the guanylylation of AdoCbi-P in vitro, they did not directly address the question of whether *cobY* encodes a function dedicated to cobamide biosynthesis in archaea.

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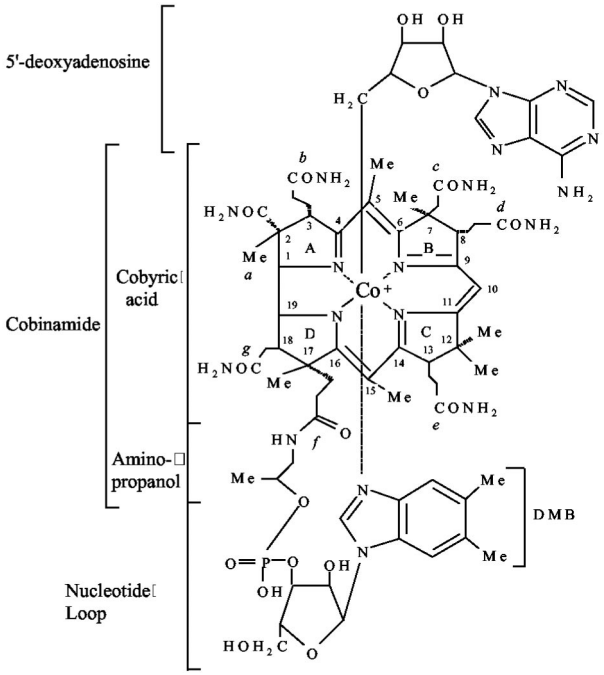

FIG. 1. Structure of AdoCbl (coenzyme B_{12}).

This work reports the first in vivo evidence showing the *cobY* gene product is involved in de novo cobamide biosynthesis in the extremely halophilic archaeon *Halobacterium* sp. strain NRC-1, which was used as a genetic model for archaeal cobamide biosynthesis. Data reported herein demonstrate that this archaeon synthesizes cobamides de novo under aerobic conditions and has the ability to assimilate corrinoid precursors and complete cobamides. To the best of our knowledge this is the first genetic analysis of cobamide biosynthesis in archaea.

MATERIALS AND METHODS

Strains and plasmids. The genotypes of strains and plasmids used in this work are described in Table 1.

Chemicals, growth media, growth conditions, and assessment of viability. All chemicals used in this work were commercially available, high-purity compounds. When added to the medium, corrinoids were present at 15 nM. All corrinoids were added in their cyano form. $(CN)_2$Cbi and CNCbl were purchased from Sigma (St. Louis, Mo.). $(CN)_2$Cbi-GDP was synthesized as described elsewhere (35). Cobyric acid [$(CN)_2$Cby] was a gift from Paul Renz (Institut für Biologische Chemie und Ernahrungswissenschaft, Universität-Hohenheim, Stuttgart, Germany). 5-Fluoroorotic acid (5-FOA) was purchased from Sigma. Mevinolin was a gift from A. W. Alberts (Merck, Whitehouse Station, N.J.). Strains were grown in liquid peptone (Oxoid Bacteriological Peptone, Hampshire, England) medium (18) lacking trace metals. Cultures were grown at 37°C with shaking for 4 days to stationary phase. Cells used as inoculum were harvested by centrifugation (10,000 × *g* for 2 min using a Beckman-Coulter [Fullerton, Calif.] Microfuge 18 centrifuge) and washed once with chemically defined Grey and Fitt medium, pH 6.6 (13). Cells were diluted 100-fold and used to inoculate the defined medium containing the appropriate corrinoid supplements. Growth was monitored every 24 h by plating cells onto solid peptone medium (6.6% agar, wt/vol) to determine cell viability (reported as CFU). Growth was also assessed on defined solid medium. Four independent colonies of each strain were patched onto solid peptone medium (6.6% agar, wt/vol), grown for 7 days at 37°C, replica printed onto solid Grey and Fitt medium (6.6% Noble agar [Difco Laboratories Detroit, Mich.], wt/vol) supplemented with trace mineral mix (1) with and without Cbi-GDP, and grown for 8 days at 37°C. Plates with solid medium were incubated in sealed plastic bags to prevent desiccation. In all cases, media were supplemented with uracil (450 μM).

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 from cells from 1 ml of a dense cell culture grown in peptone medium (10,000 × *g* for 2 min using a Microfuge 18 centrifuge [Beckman-Coulter]), resuspending the cell pellet in 1 ml of double-distilled H_2O, and boiling for 10 min. All primers were purchased from Integrated DNA Technologies Inc. (Coralville, Iowa). Underlined portions of the primer sequences (see below) indicate introduced restriction site(s).

Plasmid pCOBY29. The 5′ primer *Xba*ITop#2 (5′-GGTGAGCTTCTAGACGCGGCTGC-3′) and 3′ reverse primer *Nco*IDel1 (5′-CGACCTCGAACCATGGCTTCTCG-3′) were used to amplify a 783-bp PCR fragment from strain MPK414 genomic DNA. The fragment was digested with *Xba*I/*Nco*I restriction enzymes (unless otherwise noted, underlined portion of sequence is restriction enzyme site), gel purified, and cloned into the *Xba*I/*Nco*I restriction site of plasmid pMPK428, which contains the wild-type allele of *Halobacterium* sp. strain NRC-1 *ura3* and a mevinolin resistance determinant (22). The resulting plasmid is referred to as pCOBY29.

Plasmid pCOBY30. Plasmid pCOBY30 (Δ*cobY ura3*$^+$) carries an in-frame deletion of the *Halobacterium* sp. strain NRC-1 *cobY* gene, and was constructed as follows. The 5′ primer *Hin*dIIICobYbottom (5′-AAGCTTAAGCTTAACAGCTTGGTGAGCGAGC-3′) and reverse 3′ primer *Nco*IDel2 (5′-GAGGCCACCCCATGGAGCTTCGAC-3′) were used to amplify a 747-bp PCR fragment from MPK414 genomic DNA. The fragment was digested with *Nco*I/*Hin*dIII restriction enzymes, gel purified, and cloned into the *Nco*I/*Hin*dIII restriction site

TABLE 1. *Halobacterium* sp. strain NRC-1 strains and plasmids used in this study

Strain	Marker(s)[a]	Relevant genotype	Description	Reference
MPK414		Δ*ura3 cobY*$^+$	Strain with de novo cobamide biosynthetic capability	This study
JE6736		Δ*ura3* Δ*cobY*	Strain with in-frame deletion of *cobY*	This study
JE6737		Δ*ura3* Δ*cobY ura3*::*cobY*$^+$	Strain used to test for complementation studies	This study
pMPK410	Mev^r	Δ*ura3*	Plasmid transformed into *Halobacterium* sp. strain NRC-1 to delete *ura3*	20
pMPK428	5-FOA^s Mev^r	*ura3*$^+$	Plasmid used to generate in-frame deletions of targeted genes	21
pCOBY30	5-FOA^s Mev^r	*ura3*$^+$ Δ*cobY*	Plasmid transformed into MPK414 to delete *cobY*	This study
pMPK424	5-FOA^s Mev^r	*ura3*$^+$	Plasmid contains flanking sequence to *ura3* to allow recombination at the chromosomal *ura3* locus	22
pCOBY33	5-FOA^s Mev^r	*ura3*$^+$ *cobY*$^+$	Plasmid used to recombine *cobY* into *ura3* locus	This study

[a] Abbreviations: Mev^r, resistance to mevinolin; 5-FOA^s, sensitivity to 5-fluoroorotic acid.

of plasmid pCOBY29 to create plasmid pCOBY30. The latter contained an in-frame deletion of *cobY* that replaced bases 49 to 471 with a 6-bp *Nco*I restriction site, thus deleting 141 of the 190 amino acids. Plasmid pCOBY30 also carries the mevinolin resistance determinant and a wild-type allele of the *ura3* gene.

Plasmid pCOBY31. The 5′ primer cobYCompEcoRI5′ (5′-GAATTCGAATTCCGCTGGCTCACGGGACTGC-3′) and the reverse primer cobYComp BglII3′ (5′-AGATCTAGATCTAAAAGCCGCGCCGGTTGCGTCAGGGTGCATTGTCG-3′) were used to amplify a 740-bp PCR product from strain MPK414 genomic DNA. The fragment was digested with *Eco*RI/*Bgl*II restriction enzymes, gel purified, and cloned into the *Eco*RI/*Bgl*II restriction site of plasmid pT7-7 (33) to produce plasmid pCOBY31.

Plasmid pCOBY32. The 5′ primer cobYCompXbaI5′ (5′-TCTAGATCTAGATCGCGTACGCGCTCACTGC-3′) and reverse primer cobYCompEcoRI3′ (5′-GAATTCGAATTCGAACGCGACCGTTCCGTG-3′) were used to amplify a 240-bp PCR product from strain MPK414 genomic DNA. The fragment was digested with *Xba*I/*Eco*RI restriction enzymes, gel purified, and cloned into the *Xba*I/*Eco*RI restriction site of plasmid pCOBY31 to yield plasmid pCOBY32.

Plasmid pCOBY33. The two fragments carried on plasmid pCOBY32 were excised from plasmid DNA prepared from the mutant strain GM2163 *dam* (New England Biolabs, Manchester, Mass.) as a single 970-bp fragment with a *Xba*I/*Bgl*II digest and cloned into the *Xba*I/*Bgl*II restriction site of plasmid pMPK424 (21) (prepared from the mutant strain GM2163 *dam*) to yield plasmid pCOBY33 (*ura3*$^+$ *cobY*$^+$). The latter contains the fragment cloned flanked by sequence that would allow recombination at the *Halobacterium* sp. strain NRC-1 *ura3* locus. The resulting plasmid carried a wild-type copy of the *cobY* gene, including 110 bases upstream of the putative start codon and 180 bases upstream of the putative operon. To include these sequences part of Vng1580H was also cloned, but it carried an in-frame deletion spanning from residue 151 of residue 200. Including these sequences should preserve the regulation of *cobY* in its own operon without including other genes. Flanking the 3′ end was a 16-bp sequence derived from the *bop* transcription terminator sequence (7) to ensure transcriptional termination of the *cobY* mRNA transcript.

Strain constructions. (i) Construction of a Δ*ura3* strain of *Halobacterium* sp. strain NRC-1. Strain MPK414 (Δ*ura3*) was constructed by transforming *Halobacterium* sp. strain NRC-1 with plasmid pMPK410 (20), which contained a mevinolin resistance determinant and sequence flanking the *Halobacterium* sp. strain NRC-1 *ura3* gene, but with the *ura3* ORF missing (20). Mevinolin-resistant transformants were selected as described previously (15) and plated on medium containing 5-FOA to select for loss of the chromosomal *ura3* gene. The absence of the *ura3* gene in the chromosome was confirmed by PCR and Southern blot analysis (29, 30).

(ii) Construction of a Δ*cobY* mutant strain of *Halobacterium* sp. strain NRC-1. An in-frame deletion of *cobY* in the chromosome of strain MPK414 (Δ*ura3*) was generated by using the *ura3*-based gene replacement method using *ura3* as a counterselectable marker (20). Briefly, strain JE6736 (Δ*ura3* Δ*cobY*) was constructed by transforming strain MPK414 with plasmid pCOBY30 as described previously (15). Flanking sequences of over 700 bases on each side of the deleted *cobY* 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 (Δ*cobY*). DNA sequencing was used to confirm the in-frame deletion of the *cobY* gene in the chromosome of strain JE6736.

Complementation studies. Complementation studies were performed with a single copy of the wild-type allele of *cobY* placed at the chromosomal *ura3* locus of strain JE6736. Plasmid pCOBY33 was transformed into strain JE6736 as described previously (39), and a strain carrying the *cobY*$^+$ allele at the chromosomal *ura3* locus (strain JE6737) was isolated using the same *ura3*-based gene replacement method for the isolation of the Δ*cobY* allele. The presence of *cobY*$^+$ at the *ura3* locus was verified by PCR and DNA sequencing.

RESULTS AND DISCUSSION

Identification of the *cobY* gene of *Halobacterium* sp. strain NRC-1. ORF Vng1581C of the *Halobacterium* sp. strain NRC-1 genome sequence (17) was identified as the putative *cobY* gene of this archaeon based on the 35% identity and 48% similarity of the predicted gene product to the NTP:AdoCbi-P nucleotidyltransferase (CobY) of *M. thermoautotrophicum* ΔH. In the *Halobacterium* sp. strain NRC-1 genome, the *cobY* (Vng1581C) gene is located in a putative operon between ORF Vng1580H (the putative ortholog encoding *S. enterica*'s CobS protein) and ORF Vng1582G (the putative ortholog encoding *S. enterica*'s CobD protein) (data not shown).

***cobY* is a cobamide biosynthetic gene.** Unlike strain MPK414 (*cobY*$^+$), strain JE6736 (Δ*cobY* [in-frame deletion]) failed to grow in defined medium lacking corrinoids (Fig. 2 A and C). To determine whether the observed lack of growth of strain JE6736 was due to an inability to synthesize cobamides, the medium was supplemented with either CNCbl or $(CN)_2$Cbi-GDP. Addition of CNCbl to the medium restored wild-type growth of strain JE6736 (Fig. 2A) but did not significantly enhance the growth response of strain MPK414 (data not shown). These data suggested that *Halobacterium* sp. strain NRC-1 synthesized cobamides de novo under aerobic growth conditions, that cobamides were essential for growth of this archaeon in the defined medium, and that *cobY* function was required for de novo cobamide biosynthesis.

Addition of $(CN)_2$Cbi-GDP also restored wild-type growth of strain JE6736 (Fig. 2 A and D), but the addition of $(CN)_2$Cby, a Cbl precursor that enters the pathway prior to the proposed CobY catalyzed reaction, failed to restore growth of strain JE6736 (Fig. 2A). Control experiments with a strain deficient in Cby synthesis showed that *Halobacterium* sp. strain NRC-1 has the ability to transport Cby; thus, the observed lack of responsiveness of strain JE6736 (Δ*cobY*) to Cby was not due to a lack of transport of Cby into the cell (J. D. Woodson and J. C. Escalante-Semerena, unpublished results). The calculated doubling times in the defined liquid medium of the cells which grew were similar (9 to 12 h), whereas the doubling time of JE6736 without corrinoids or with Cby was too long to calculate. These data demonstrated that the absence of *cobY* function correlated with the predicted phenotype of an strain lacking NTP:AdoCbi-P nucleotidyltransferase under conditions that demanded de novo cobamide biosynthesis. These data also showed that *Halobacterium* sp. strain NRC-1 has the ability to salvage nonadenosylated intermediates and complete cobamides from the environment. This leads to the proposal that *Halobacterium* sp. strain NRC-1 also has an ATP:Co(I)rrinoid adenosyltransferase (CobA in *S. enterica*) (2, 10, 11, 32) and a specific transport system for corrinoids, although at present it is not clear whether such a system resembles the *btuCD* system found in bacteria (8, 16).

Complementation studies. The observed Cbi-GDP auxotrophy of strain JE6736 was corrected when the *cobY*$^+$ allele was reintroduced into the strain. Strain JE6737 was able to grow in the defined liquid medium without any corrinoids (Fig. 2B) with a doubling time of 10 h, which was similar to those of strains JE6736 (Δ*cobY*) with Cbl supplementation or MPK414 (*cobY*$^+$), suggesting that the presence of the *cobY*$^+$ allele in *trans* was necessary and sufficient to restore the ability of the *Halobacterium* sp. strain NRC-1 Δ*cobY* mutant (JE6736) to synthesize cobamides de novo. Complementation of the phenotype was also assessed on the solid medium lacking corrinoids (Fig. 2E).

Conclusions. The data reported herein support several conclusions regarding the role of the CobY protein in cobamide biosynthesis and about cobamide metabolism in the extreme halophilic archaeon *Halobacterium* sp. strain NRC-1. First, the CobY function is required for cobamide biosynthesis, and the

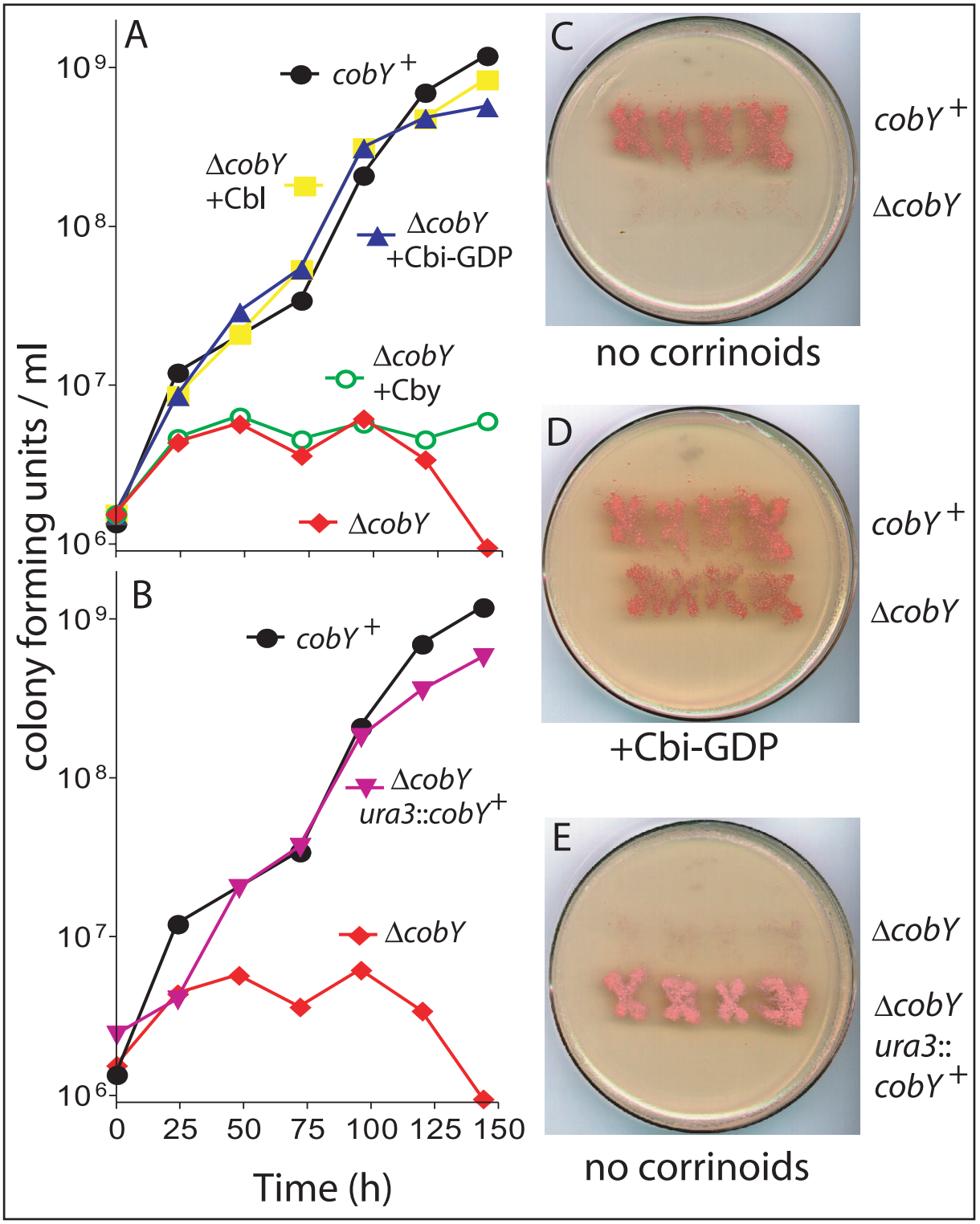

FIG. 2. Nutritional studies of *Halobacterium* sp. strain NRC-1 strains. Growth of *Halobacterium* sp. strain NRC-1 strains in the defined medium at 37°C is reported. (A and B) Growth in a liquid defined medium reported as CFU as a function of time. Strains are indicated by their genotype. Corrinoids added to the medium are indicated next to the genotype. (C, D, and E) Growth of cells seeded onto minimal agar plates and incubated for 8 days. Strains used were MPK414, *cobY*$^+$; JE6736, Δ*cobY*; and JE6737 Δ*cobY ura3*::*cobY*$^+$. Abbreviations: Cby, cobyric acid; Cbl, cobalamin. In all cases, corrinoids were added to 15 nM.

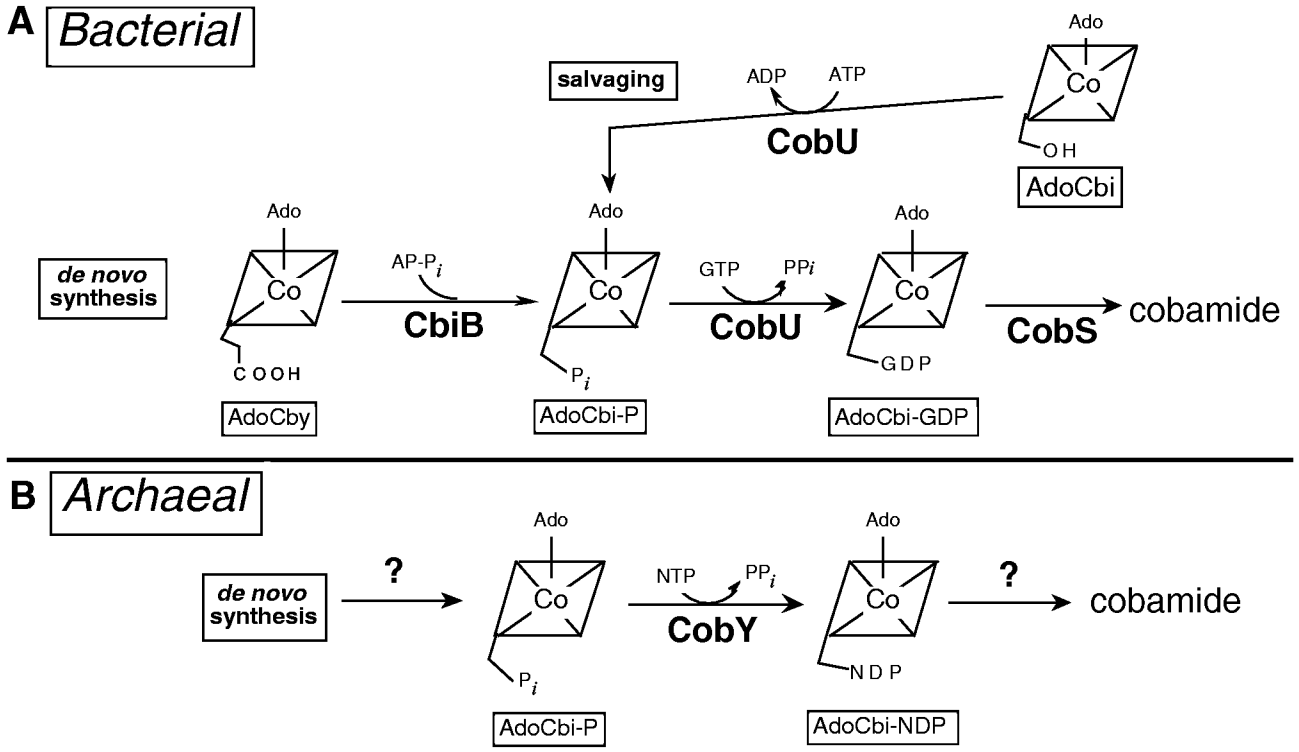

FIG. 3. Abbreviated view of the late steps of AdoCbl biosynthesis in bacteria (A) and archaea (B). Intermediates are boxed. Abbreviations: AP-P_i, aminopropanol phosphate; AdoCby, adenosylcobyric acid. (A) CbiB, putative AdoCbi-P synthase; CobU, AdoCbi:NTP kinase, GTP/AdoCbi-P guanylyltransferase; CobS, cobalamin (5′-P) synthase. (B) CobY, NTP:AdoCbi nucleotidyltransferase.

lack of it does not result in any additional discernible phenotypes. The ability of Cbi-GDP to restore growth of the *cobY* mutant in the defined medium supports the hypothesized role of the CobY protein as the archaeal NTP:AdoCbi-P nucleotidyltransferase. Second, it is clear that *Halobacterium* sp. strain NRC-1 synthesizes cobamides de novo under aerobic conditions, a fact that is somewhat surprising because the cobamide biosynthetic genes of this archaeon are homologous to those found in *S. enterica*, a bacterium known to use the anaerobic pathway of de novo corrin ring biosynthesis (27). How *Halobacterium* sp. strain NRC-1 protects de novo corrin ring biosynthesis from the deleterious effects of oxygen remains an open question. Given that oxygen solubility would be low in a high-salt medium, it is possible that microaerophilic environments where corrinoid biosynthesis can occur exist in the medium even when the culture is exposed to air. Third, *Halobacterium* sp. strain NRC-1 appears to have a corrinoid transport system that can effectively translocate corrinoids into the cell even when the latter are present in the environment at very low levels.

Previous studies have shown that methanogenic archaea can salvage Cbi from the environment (31). This result is of interest considering that archaea do not posses an ortholog to the ATP:AdoCbi kinase protein used by bacteria to salvage Cbi (Fig. 3A). The absence of an ortholog in archaea does not rule out the existence of an alternative function, which may be unique to archaea (Fig. 3B). This possibility is currently under investigation.

The results of the work reported here are important because they represent the first genetic analysis of any of the steps of the cobamide biosynthetic pathway in archaea. On the basis of the lack of similarity between CobY and CobU at the amino acid sequence level, the structure-function analysis of the CobY protein is likely to be different from its multifunctional bacterial counterpart (CobU in *S. enterica*) in both its catalytic mechanism and protein fold.

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