# Identification and Characterization of the 2-Phospho-L-lactate Guanylyltransferase Involved in Coenzyme $F_{420}$ Biosynthesis†

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ABSTRACT: Coenzyme $F_{420}$ is a hydride carrier cofactor functioning in methanogenesis. One step in the biosynthesis of coenzyme $F_{420}$ involves the coupling of 2-phospho-L-lactate (LP) to 7,8-didemethyl-8-hydroxy-5-deazaflavin, the $F_{420}$ chromophore. This condensation requires an initial activation of 2-phospho-L-lactate through a pyrophosphate linkage to GMP. Bioinformatic analysis identified an uncharacterized archaeal protein in the *Methanocaldococcus jannaschii* genome, MJ0887, which could be involved in this transformation. The predicted MJ0887-derived protein has domain similarity with other known nucleotidyl transferases. The MJ0887 gene was cloned and overexpressed, and the purified protein was found to catalyze the formation of lactyl-2-diphospho-5′-guanosine from LP and GTP. Kinetic constants were determined for the MJ0887-derived protein with both LP and GTP substrates and are as follows: $V_{max} = 3$ $\mu$mol min$^{-1}$ mg$^{-1}$, GTP $K_M^{app} = 56$ $\mu$M, and $k_{cat}/K_M^{app} = 2 \times 10^4$ M$^{-1}$ s$^{-1}$ and LP $K_M^{app} = 36$ $\mu$M, and $k_{cat}/K_M^{app} = 4 \times 10^4$ M$^{-1}$ s$^{-1}$. The MJ0887 gene product has been designated CofC to indicate its involvement in the third step of coenzyme $F_{420}$ biosynthesis.

The biosynthesis of coenzyme $F_{420}$ has been a focus of our laboratory for a number of years. $F_{420}$ is a 7,8-didemethyl-8-hydroxy-5-deazaflavin (Fo)[1] containing coenzyme that is found in a number of archaea and bacteria. $F_{420}$ is a hydride carrier coenzyme involved in processes such as methanogenesis (*1*), the reduction of sulfite (*2*), oxygen detoxification (*3*), and electron transport (*4*) in the archaea. $F_{420}$ is also an important coenzyme in many Gram-positive bacteria such as *Streptomyces* and *Mycobacteria* where it is involved in the biosynthesis of several antibiotics (*5–7*) and nitrophenol biodegradation (*8*). An $F_{420}$-dependent glucose-6-phosphate dehydrogenase has also been identified in several *Mycobacteria* and *Nocardia* species and shown to be required for the activation of the antitubercular prodrug PA-824 (*9–11*).

The structure of coenzyme $F_{420}$ found in these various organisms is not fixed but varies on the basis of the number and mode of attachment of glutamate residues to the carboxylic acid of the 2-phospho-L-lactate (LP) moiety of the core $F_{420}$ structure ($F_{420}$-0, $F_{420}$ with no glutamates). The enzymes responsible for the formation of lactate (*12*), the biosynthesis of Fo (*13*), and the condensation of lactyl-2-diphospho-5′-guanosine (LPPG) and Fo to form $F_{420}$-0 (*14*), as well as the enzymes involved in the formation of the polyglutamate moiety (*15–17*), have been characterized. Those involved in the formation of 2-phospho-L-lactate and the subsequent activation of 2-phospho-L-lactate as LPPG have not been identified, however.

The formation of the phosphodiester linkage between 7,8-didemethyl-8-hydroxy-5-deazaflavin (Fo) and 2-phospho-L-lactate is the last step in the biosynthesis of the $F_{420}$-0 core. Previous studies with cell extracts from *Methanocaldococcus jannaschii* and *Methanosarcina thermophilia* demonstrated that the phosphodiester linkage in coenzyme $F_{420}$ was formed through the activation of 2-phospho-L-lactate by GTP to form LPPG. Subsequent condensation of LPPG with Fo forms $F_{420}$-0 concurrent with the release of GMP (Figure 1) (*18*). LPPG contains a pyrophosphodiester linkage that is a common structural moiety in several coenzymes including NAD, FAD, coenzyme A, and molybdopterin guanine dinucleotide. In addition, the biosynthesis of both cobamide and $F_{420}$ includes the formation of a phosphodiester moiety from a pyrophosphodiester-containing intermediate. The condensation of GTP and LP in $F_{420}$ biosynthesis is analogous to the formation of the adenosylcobinamide guanosine diphosphate intermediate in cobalamin biosynthesis. Bioinformatic analysis of organisms known to produce coenzyme $F_{420}$ was used to identify potential genes for the 2-phospho-L-lactate guanylyltransferase. One gene, *M. jannaschii* MJ0887, was found to be clustered with $F_{420}$-0 biosynthetic genes in *Methanosarcina acetivorans*, *Methanosarcina mazei*, and *Methanococcoides burtonii*. The predicted MJ0887 protein was found to have similarity to the conserved domains of GTP:adenosylcobinamide-phosphate guanylyltransferase (CobY), molybdopterin−guanine dinucleotide

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[1] Abbreviations: GTP, guanosine 5′-triphosphate; GMP, guanosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; DTT, dithiothreitol; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; coenzyme $F_{420}$, the *N*-(*N*-L-lactyl-$\gamma$-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazaflavin; Fo, 7,8-didemethyl-8-hydroxy-5-deazaflavin; $F_{420}$-0, $F_{420}$ with no glutamic acids; LP, 2-phospho-L-lactate; LPPG, lactyl-2-diphospho-5′-guanosine; LPPA, lactyl-2-diphospho-5′-adenosine; $\gamma$-thiolGTP, guanosine 5′-[$\gamma$-thio] triphosphate; $PP_i$, pyrophosphate.

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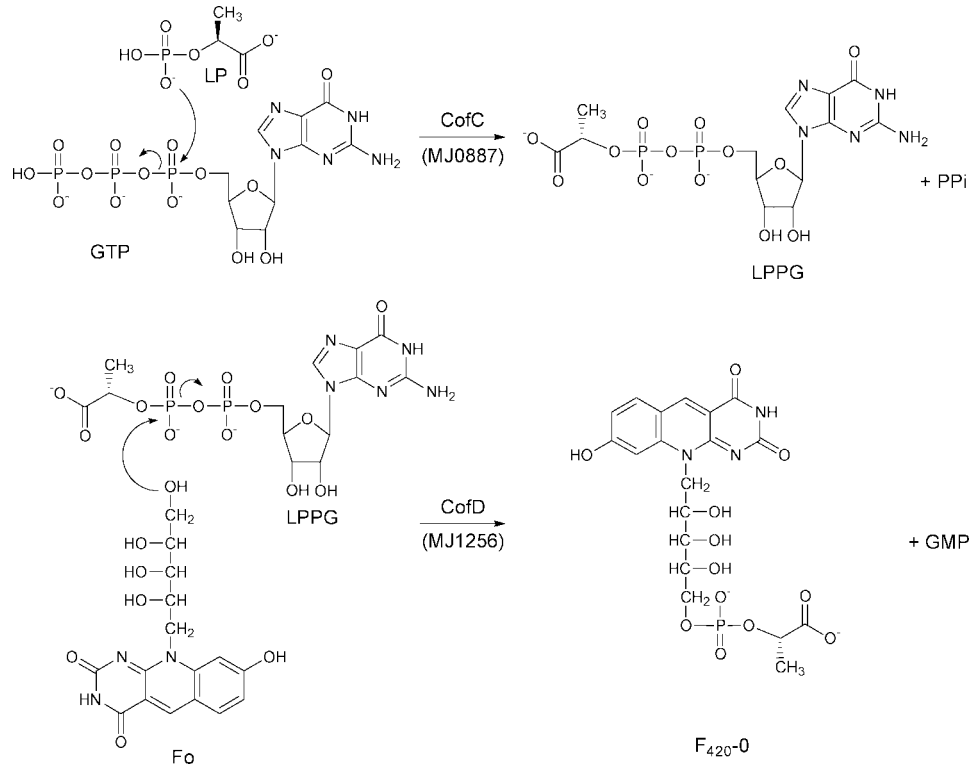


FIGURE 1: Formation of $F_{420}$-0 from 2-phospho-L-lactate, GTP, and Fo.

biosynthesis protein A (MobA), and *N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU). In addition, the distribution of MJ0887 orthologues was correlated with organisms known to produce coenzyme $F_{420}$. We have cloned and overexpressed the MJ0887 gene, characterized the MJ0887 gene product, and confirmed its involvement in coenzyme $F_{420}$ biosynthesis. We have designated the MJ0887 gene product CofC to indicate its involvement in the third step of coenzyme $F_{420}$ biosynthesis.

## MATERIALS AND METHODS

*Chemicals.* 2-Phospho-L-lactate was prepared as previously reported (*18*). Fo was prepared according to the method of Ashton and Brown from D-ribose, 3-aminophenol, and 6-chlorouracil (*19*). All other chemicals and reagents were obtained from Sigma/Aldrich.

*Cloning and Expression of MJ0887 and Purification of the Recombinant Gene Product, CofC.* The *M. jannaschii* MJ0887 gene (SwissProt Q58297) was amplified by PCR from genomic DNA using oligonucleotide primers: MJ0887F, 5′-GGTCATATGAATTGTGGGATAAAAATG-3′; MJ0887R, 5′-GCTGGATCCTTATCTCCTCTTTAC-3′. PCR was performed as described previously using a 50 °C annealing temperature (*20*). The amplified PCR product was purified by a QIAquick spin column, digested with restriction enzymes *Nde*I and *Bam*HI, and ligated into compatible sites in plasmid pT7-7. Plasmid DNA sequences were verified by sequencing. The resulting plasmid, pMJ0887, was transformed into *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). The transformed cells were grown in Luria–Bertani medium (200 mL) supplemented with 100 $\mu$g/mL ampicillin at 37 °C with shaking until they reached an $OD_{600}$ of 1.0. Recombinant protein production was induced by addition of lactose to a final concentration of 28 mM. After an additional 2 h of culture, the cells were harvested by centrifugation (4000*g*, 5 min) and frozen at −20 °C. SDS−polyacrylamide gel electrophoresis of total cellular proteins confirmed induction of the desired protein. *E. coli* cells expressing recombinant protein were resuspended in 4 mL of extraction buffer [50 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), pH 7.0, 10 mM $MgCl_2$, 20 mM DTT] and lysed by sonication. After the majority of *E. coli* proteins were precipitated by heating the cell lysate to 80 °C for 10 min, the MJ0887-derived protein was purified by anion-exchange chromatography on a MonoQ HR column (1 × 8 cm; Amersham Bioscience) with a linear gradient from 0 to 1 M NaCl in 25 mM TES, pH 7.5, over 55 mL at 1 mL/min. Protein concentration was determined by Bradford analysis (*21*).

*Coupled Assay for Lactyl-Phosphate Guanylyltransferase Activity. M. jannaschii* 2-phospho-L-lactate guanylyltransferase activity was determined in a coupled assay with 2-phospho-L-lactate transferase (CofD, MJ1256 gene product) as shown in Figure 1. CofD was cloned, overexpressed, and purified as previously described (*14*). The standard assay was in a final volume of 50 $\mu$L and contained 100 mM TES, pH 7.5, 2 mM GTP, 5 mM 2-phospho-L-lactate, 240 $\mu$M Fo, 2 mM $MgCl_2$, and 3 $\mu$g CofD and was initiated with the addition of 0.08 $\mu$g of 2-phospho-L-lactate guanylyltransferase. Following incubation at 70 °C for 10 min, the reactions were quenched by adding 1 $\mu$L of 0.5 M EDTA and 200 $\mu$L of 100% methanol and centrifuged for 10 min. The resulting supernatant was brought to a final volume of 1 mL with water and analyzed by HPLC as described below.

*Assay for the Formation of Pyrophosphate.* Pyrophosphate was assayed by enzymatic coupling to the oxidation of NADH utilizing pyrophosphate-dependent fructose-6-phosphate kinase, fructose-1,6-diphosphate aldolase, triosephos-

phate isomerase, and glycerophosphate dehydrogenase. The enzymes and substrates necessary for the coupling were provided in pyrophosphate reagent for the enzymatic determination of pyrophosphate (Sigma P7275). Incubation mixtures included 150 mM TES, pH 7.5, 2 mM $MgCl_2$, 0.11 $\mu$g CofC, and concentrations of 100–500 $\mu$M 2-phospho-L-lactate and 25–200 $\mu$M GTP, respectively. Following incubation at 70 °C, the assay mixture (200 $\mu$L) was cooled on ice, centrifuged, and combined with 100 $\mu$L of pyrophosphate reagent at 30 °C in a 100 $\mu$L cuvette. The oxidation of NADH was monitored at 340 nm.

*Characterization of CofC.* Kinetic parameters were determined using the pyrophosphate assay. Kinetic parameters were estimated from the secondary plots of the intercepts from the corresponding Lineweaver–Burk plots for varying concentrations LP and GTP, respectively, using Microsoft Excel software.

The metal dependency of *M. jannaschii* 2-phospho-L-lactate guanylyltransferase was determined by incubating the enzyme under the coupled assay conditions with 1 mM divalent metal ions including $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Co^{2+}$, and $Zn^{2+}$. CofC was incubated with lactate and ATP in place of 2-phospho-L-lactate in the coupled assay to test for lactate kinase activity. The temperature stability of CofC was determined by incubating the enzyme in assay buffer at 70, 80, 90, or 100 °C for 10 min in sealed tubes. Following heating, the enzyme mixture was cooled on ice and centrifuged to return the entire contents to the bottom of the tube, and the other assay components were added. The final composition and volume of the reaction mixture were as described for the standard assay above.

The native molecular mass of CofC was determined by size exclusion chromatography on a Superose 12HR column (10 × 300 mm) with a buffer containing 50 mM TES, pH 7.5, and 300 mM NaCl at 0.5 mL/min and detection at 280 nm. Protein standards used to generate the standard curve included alcohol dehydrogenase (150 kDa), conalbumin (77 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and $B_{12}$ (1.4 kDa).

*HPLC Detection and Measurement of Fo and $F_{420}$-0.* Chromatographic separation of Fo and $F_{420}$-0 was performed on a Shimadzu HPLC system with a C18 reverse-phase column (Varian PursuitXRs 250 × 4.6 mm, 5 $\mu$m particle size). The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, 0.02% $NaN_3$) and 5% MeOH followed by a linear gradient to 20% sodium acetate buffer/80% MeOH over 40 min at 0.5 mL/min. Fo and $F_{420}$-0 were detected by fluorescence using an excitation wavelength of 420 nm and an emission wavelength of 480 nm. GTP and GMP were detected by UV absorbance at 256 nm. Under these conditions GTP eluted at 5.5 min, GMP at 9.9 min, $F_{420}$-0 at 24.3 min, and Fo at 28.5 min.

## RESULTS

*Characteristics of the Recombinant MJ0887 Gene Product (CofC).* The recombinant MJ0887 gene product (CofC) was purified to >99% purity and ran at approximately 25 kDa as judged by SDS−polyacrylamide electrophoresis with Coomassie staining. This corresponded well with the predicted molecular mass of 26 kDa from the encoding gene sequence. CofC was found to have an apparent molecular mass of 50 kDa by size exclusion chromatography, which is consistent with existing in solution as a dimer.

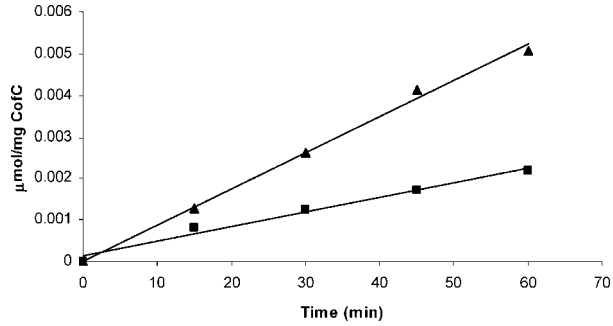


FIGURE 2: Production of $F_{420}$-0 (■) and pyrophosphate (▲) with time by the coupled CofC−CofD system. The incubation mixture included 150 mM TES buffer, pH 7.5, 2 mM $MgCl_2$, 5 mM GTP, 10 mM LP, 0.32 mM Fo, 30 nM CofC (MJ0887), and 400 nM CofD (MJ1256).

The MJ0887 gene product was found to catalyze the condensation of 2-phospho-L-lactate with GTP to form $PP_i$ and LPPG. Due to the instability of LPPG (*18*), the reaction was monitored indirectly through a coupled assay with Fo catalyzed by 2-phospho-L-lactate transferase (CofD, MJ1256) to form $F_{420}$-0. The reaction product was found to have an identical HPLC retention time, fluorescence, and UV–vis spectrum as authentic $F_{420}$-0 with both having an observed $\lambda_{max}$ of 421 nm at pH 10 and observed excitation and emission wavelengths of 425 and 466 nm, respectively. These values are consistent with those reported by Eirich et al. (*22*). The formation of LPPG, detected as the coupled product $F_{420}$-0, and $PP_i$ was found to be linear with respect to time (Figure 2) and proportional to enzyme concentration (data not shown). The formation of GMP was also time and enzyme dependent; however, the amount of GMP produced was significantly higher than the amount of $F_{420}$-0 produced (data not shown). This discrepancy is likely due to the instability of the LPPG, which decomposes to LP and GMP with a half-life of only 10 min at 50 °C (*18*). This is also likely to account for the lower amounts of $F_{420}$-0, as opposed to $PP_i$, observed in Figure 2.

In order to confirm that the coupling enzyme, 2-phospho-L-lactate transferase, was not catalyzing the formation of LPPG, it was tested for guanylyltransferase activity, and none was observed. This was in agreement with the previous report that LPPG was the required substrate for *M. jannaschii* 2-phospho-L-lactate transferase (*14*). Taken together, these results indicate that the MJ0887 gene product is the 2-phospho-L-lactyl guanylyltransferase involved in coenzyme $F_{420}$ biosynthesis. We propose that MJ0887 be annotated as CofC to reflect its involvement in the third step of coenzyme $F_{420}$ biosynthesis.

Steady-state kinetic analysis of the CofC reaction was used to determine the kinetic parameters for CofC. The determined kinetic parameters were as follows: $V_{max} = 3$ $\mu$mol $min^{-1}$ $mg^{-1}$, GTP $K_M^{app} = 56$ $\mu$M, and $k_{cat}/K_M^{app} = 2 \times 10^4$ $M^{-1}$ $s^{-1}$ and for 2-phospho-L-lactate $K_M^{app} = 36$ $\mu$M and $k_{cat}/K_M^{app} = 4 \times 10^4$ $M^{-1}$ $s^{-1}$. Reciprocal plots of CofC velocity versus GTP or LP concentration indicated that the reaction likely proceeds through a sequential mechanism (Figure 3).

One bacterial enzyme involved in cobalamin biosynthesis, *Salmonella typhimurium* CobU, has been shown to possess kinase activity in addition to nucleotidyl transferase activity

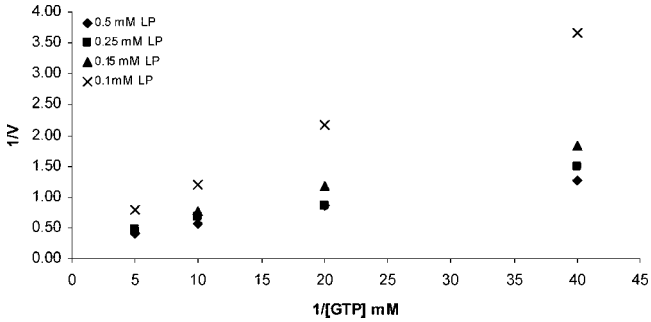


FIGURE 3: Reciprocal plots of CofC activity at varying LP concentration versus GTP concentration. The reaction velocity was measured in $\mu$mol min$^{-1}$ mg$^{-1}$.

Table 1: Specific Activity of CofC with Purine Nucleotides

| | specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$)[a] | | specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$)[a] |
|---|---|---|---|
| GTP | 3.5 | GDP | 0.2 |
| dGTP | 3.3 | ATP | 0.1 |
| ITP | 2.3 | ADP | <0.03 |
| $\gamma$-thiolGTP | 1.3 | | |

[a] Specific activities were determined using the coupled assay system with CofD and included 2 mM final concentrations of the indicated purine nucleotide.

(*23*). If CofC were such a bifunctional enzyme, it would be able to catalyze the formation of 2-phospho-L-lactate and then catalyze the guanylyltransferase reaction to form LPPG. CofC was tested for lactate kinase activity by incubating the enzyme with L-lactate, ATP, and GTP in the standard coupled assay. Neither phosphorylation of L-lactate nor the formation of LPPG was detected.

CofC was able to utilize other purine nucleotides including dGTP and ITP as cosubstrates in place of GTP but with a lower specific activity (Table 1). Although we observed only minimal activity with ATP, this may be due to the selectivity of the coupling enzyme, CofD, for LPPG over LPPA as was previously reported (*14*). The substrate specificity of CofD may also be contributing to the decrease in observed specific activity of CofC with the other purines tested. As expected from its thermophilic origins, CofC retained all activity when heated up to 90 °C for 10 mins and retained 38% activity after 10 min at 100 °C.

The GTP used by CofC would be expected to be bound as a divalent metal ion complex during catalysis. In order to establish this divalent metal ion dependency, we incubated CofC with 2-phospho-L-lactate and GTP in the presence or absence of several divalent metal ions and measured the activity. CofC exhibited much higher activity when 1 mM divalent metal was present in the incubation mixture. Although maximal activity was seen when $Mg^{2+}$ was added to the incubation mixture, significant activity was also observed with $Co^{2+}$ and $Fe^{2+}$ (Table 2).

*Cloning and Purification of MJ1117 and Activity of the Recombinant Gene Product.* The MJ1117 gene was cloned and overexpressed and the gene product purified as described for CofC (data not shown). The purified gene product was tested for 2-phospho-L-lactate guanylyltransferase activity in the coupled assay and found to have a $V_{max}$ of 115 nmol min$^{-1}$ mg$^{-1}$ and a $K_M^{app}$ of 6 mM for 2-phospho-L-lactate. We also tested the *Methanothermobacter thermautotrophicus* homologue of MJ1117 (MTH1152, CobY) for 2-phospho-L-lactate guanylyltransferase activity, and none was observed (unpublished data).

Table 2: Specific Activity of CofC with Different Divalent Metals

| metal added[a] | specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$) | metal added[a] | specific activity ($\mu$mol min$^{-1}$ mg$^{-1}$) |
|---|---|---|---|
| $Mg^{2+}$ | 2.3 | $Mn^{2+}$ | 0.6 |
| $Co^{2+}$ | 1.6 | $Zn^{2+}$ | 0.3 |
| $Fe^{2+}$ | 1.0 | $Cu^{2+}$ | 0.1 |
| $Ni^{2+}$ | 0.8 | no metal | 0.1 |

[a] Specific activities were determined using the coupled assay system with CofD and included 1 mM final concentrations of the indicated metal.

## DISCUSSION

At the beginning of this work we sought to identify the enzyme responsible for the formation of LPPG, an intermediate in coenzyme $F_{420}$ biosynthesis. A search of the *M. jannaschii* genome identified one gene, MJ1117, which is a predicted guanyltransferase. The MJ1117 homologue in *M. thermautotrophicus* (formerly *Methanobacterium thermoautotrophicum* ΔH) is MTH1152, and the coded protein (CobY) was found to be an adenosylcobinamide guanosine transferase involved in cobalamin biosynthesis (*24*). Although we detected some 2-phospho-L-lactate guanylyltransferase activity with the MJ1117 protein, it had a high $K_M^{app}$ for 2-phospho-L-lactate and was also shown to possess adenosylcobinamide guanosine transferase activity (unpublished data). The latter activity, as well as the demonstrated involvement of MJ1117 homologues in cobalamin biosynthesis, conflicted with its assignment as the true CofC (*24*).

Bioinformatic analysis of $F_{420}$-0 biosynthetic genes using the STRING database (*25*) resulted in the identification of MJ0887 as having possible linkages to $F_{420}$ biosynthesis. In *M. acetivorans*, *M. mazei*, and *M. burtonii* the MJ0887 homologues were found in operons containing other $F_{420}$ biosynthetic genes including CofG and CofH that are involved in Fo formation, suggesting its involvement in the biosynthesis of coenzyme $F_{420}$. A search of the conserved domain database (*26*) with the MJ0887 protein revealed that it is a member of COG1920 that contains uncharacterized archaeal proteins and a domain of unknown function, DUF121. CofC also has limited similarity to several nucleotidyl transferase domains including COG2266 (GTP:adenosylcobinamide-phosphate guanylyltransferase, 4e-04) and COG0746, the conserved N-terminal domain of MobA (molybdopterin−guanine dinucleotide biosynthesis protein A, 6e-04), which has been shown to be involved in GTP binding (*27*). On the basis of the overall reaction catalyzed, CofC is most similar to MobA that catalyzes the formation of the pyrophosphodiester linkage between molybdopterin and GDP to form molybdopterin−guanine dinucleotide (*27*).

Gene threading of the translated MJ0887 gene further supported a proposed function as a nucleotidyl transferase (*28, 29*). The closest hits to the predicted structure of the MJ0887 gene product were a series of nucleotidyl transferases including citidyltransferase. CofC contains an imperfect sequence motif for nucleotide diphosphate sugar pyrophosphorylases LxxGxGTxMxxxxPK (*30, 31*). In CofC the sequence motif is PxxGxGTxxxxxxPK and is conserved in the archaeal homologues.

Here we have shown that the MJ0887 gene product from *M. jannaschii* is a nucleotidyl transferase, specifically a

2-phospho-L-lactate guanylyltransferase involved in coenzyme $F_{420}$ biosynthesis. With the identification of CofC, the enzymes and genes necessary for the formation of Fo and the assembly of $F_{420}$ from 2-phospho-L-lactate are all known. The only remaining enzyme to be identified in $F_{420}$ biosynthesis is the lactate kinase.

Recently, Guerra-Lopez et al. reported that a transposon mutant disrupted in the *Mycobacterium smegmatis* $mc^2$ homologue of CofC was unable to decolorize triphenylmethane dye (*32*). Further analysis of this mutant showed that it accumulated Fo to five times the wild-type levels but did not produce $F_{420}$. Their results further support the involvement of CofC in $F_{420}$ biosynthesis and are consistent with this enzyme being the 2-phospho-L-lactate guanylyltransferase.

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