Identification of the Final Two Genes Functioning in Methanofuran Biosynthesis in *Methanocaldococcus jannaschii*

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ABSTRACT

All methanofuran structural variants contain a basic core structure of 4-[*N*-(γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-(aminomethyl)furan (APMF-Glu), but have different side chains depending on the source organism. Recently, we identified four genes (MfnA, MfnB, MfnC, and MfnD) that are responsible for the biosynthesis of the methanofuran precursors γ-glutamyltyramine and 5-(aminomethyl)-3-furanmethanol-phosphate (F1-P) from tyrosine, glutamate, glyceraldehyde-3-P, and alanine in *Methanocaldococcus jannaschii*. How γ-glutamyltyramine and F1-P couple together to form the core structure of methanofuran was previously unknown. Here, we report the identification of two enzymes encoded by the genes *mj0458* and *mj0840* that catalyze the formation of F1-PP from ATP and F1-P and the condensation of F1-PP with γ-glutamyltyramine, respectively, to form APMF-Glu. We have annotated these enzymes as MfnE and MfnF, respectively, representing the fifth and sixth enzymes in the methanofuran biosynthetic pathway to be identified. Although MfnE was previously reported as an archaeal adenylate kinase, our present results show that MfnE is a promiscuous enzyme and its possible physiological role is to produce F1-PP. Unlike other enzymes catalyzing coupling reactions involving pyrophosphate as the leaving group, MfnF exhibits a distinctive α/β two-layer sandwich structure. By comparing MfnF with thiamine synthase and dihydropteroate synthase, an S_N-1 reaction mechanism is proposed for MfnF. With the identification of MfnE and MfnF, the biosynthetic pathway for the methanofuran core structure APMF-Glu is complete.

IMPORTANCE

This work describes the identification of the final two enzymes responsible for catalyzing the biosynthesis of the core structure of methanofuran. The gene products of *mj0458* and *mj0840* catalyze the formation of F1-PP and the coupling of F1-PP with γ-glutamyltyramine, respectively, to form APMF-Glu. Although the chemistry of such a coupling reaction is widespread in biochemistry, this work provides the first evidence that such a mechanism is employed in methanofuran biosynthesis. MfnF belongs to the hydantoinase-A family (PF01968) and exhibits a unique α/β two-layer sandwich structure different from the enzymes catalyzing similar reactions. Our results show that MfnF catalyzes the formation of an ether bond during methanofuran biosynthesis. Therefore, this work further expands the functionality of this enzyme family.

62 **M**ethanofuran is the first in a series of coenzymes involved in the biochemical reduction of
63 carbon dioxide to methane (1-3). This process, known as methanogenesis, is carried out only by
64 the methanogenic archaea, which produce more than 400 million tons of methane each year as an
65 essential part of the global carbon cycle (4). Methanofuran is the initial C1 acceptor molecule
66 involved in the first two-electron reduction of carbon dioxide to produce the formamide
67 derivative of methanofuran, where the formate is attached to the amino group of methanofuran
68 through methanogenesis (5). This is one of the few known pathways for carbon dioxide fixation
69 in Nature. In addition, methylotrophic bacteria also use methanofuran as a coenzyme to oxidize
70 formaldehyde to formic acid (6, 7). The chemical structure of methanofuran varies among
71 different methanogens (8); each currently known methanofuran molecule contains the basic core
72 structure of 4-[*N*-(γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(amino-methyl)furan
73 (APMF-Glu), but isolated analogs have different attached side chains (8). Recently, our
74 laboratory identified a new methanofuran structure in *Methanocaldococcus jannaschii*, which
75 contains a long γ-glutamyl tail with 7-12 γ-linked glutamates (9) (Fig 1). Although the function
76 of methanofuran has been known for many years, its biosynthetic pathway has not been fully
77 elucidated.

78 Recently, we discovered the route for the production of 4-(hydroxymethyl)-2-
79 furancarboxaldehyde-P (4-HFC-P) from glyceraldehyde-3-P (10). The resulting 4-HFC-P
80 undergoes a transamination reaction to produce 5-(aminomethyl)-3-furanmethanol-phosphate
81 (F1-P) (10, 11), a precursor of the furan moiety of methanofuran (Fig 1). We previously
82 demonstrated that the *mj0050* gene encodes a tyrosine decarboxylase that produces tyramine
83 from tyrosine (12). A tyramine-glutamate ligase (the gene product of *mj0815*) catalyzes the ATP-
84 dependent addition of one glutamate to tyramine via a γ-linked amide bond (13) (Fig 1). To

85 produce APMF-Glu, we propose that one enzyme is required to catalyze the conversion of F1-P
86 to F1-PP, where the pyrophosphate group serves as a better leaving group in the subsequent
87 condensation reaction. Another enzyme then catalyzes the condensation between F1-PP and γ-
88 glutamyltyramine.

89 The homologs of *mj0458* and *mj0840* are widely distributed among the methanofuran-
90 containing organisms, including all methanogenic archaea and some methylotrophic bacteria.
91 Comparative genomic analysis shows that in some methanogens and methylotrophs, the
92 homologs of the *mj0458* and *mj0840* genes appear in the neighborhood of *mj0815* (Fig 2), which
93 encodes the enzyme that catalyzes the ATP-dependent addition of one glutamate to tyramine,
94 producing γ-glutamyltyramine. Therefore, the gene products of *mj0458* (MJ0458) and *mj0840*
95 (MJ0840) are likely to be involved in methanofuran biosynthesis. We reported that *mj0458*
96 encodes a second type of archaeal adenylate kinase that catalyzes phosphoryl group transfer from
97 one molecule of ATP to one molecule of AMP, generating two molecules of ADP (14). A similar
98 phosphoryl transfer reaction is expected to generate F1-PP from F1-P and ATP. It is possible that
99 the gene product of mj0458 is a promiscuous enzyme, catalyzing both reactions. The gene
100 product of *mj0840* is an uncharacterized protein. PSI-blast analysis shows that it belongs to the
101 hydantoinase A family (PF01968) and contains a sugar kinase domain. However, the four
102 histidines (15) that are found in hydantoinase, which are catalytically essential, are not conserved
103 in the proteins encoded by *mj0840* and its homologues, indicating that the gene product of
104 *mj0840* is unlikely to function as a hydantoinase.

105 To test whether the gene products of *mj0458* and *mj0840* are involved in methanofuran
106 biosynthesis, we cloned and heterologously expressed both genes in *Escherichia coli*. Here, we
107 report that the purified protein expressed from the *M. jannaschii* gene locus *mj0458* catalyzes the

ATP-dependent phosphoryl transfer reaction to generate F1-PP from F1-P. The purified protein expressed from the *M. jannaschii* gene locus *mj0840* catalyzes the condensation reaction between F1-PP and γ-glutamyltyramine, likely via a S_N-1 reaction mechanism. These are the fifth and sixth enzymes that we have identified in the methanofuran biosynthetic pathway; therefore, we have annotated the gene products of *mj0458* and *mj0840* as MfnE and MfnF, respectively.

MATERIALS AND METHODS

Chemicals. All reagents were purchased from Sigma-Aldrich unless otherwise specified.

Chemical Synthesis of F1-P from 4-HFC-P. The chemical synthesis of F1-P started from 4-HFC-P, whose synthesis has been previously described (11), with the following changes. To synthesize 4-HFC-P, 4-HFC (68 mg, 0.53 mmol) was dissolved in 2 mL of acetonitrile to which was added 120 µl of trichloroacetonitrile (1.2 mmol) and tetrabutylammonium phosphate (300 mg, 0.88 mmole). This procedure was patterned after a previously described method (16). After four hours (at room temperature), solvent was evaporated with a stream of nitrogen gas and the sample mixed with 2 mL of water and cooled overnight at 3°C. The resulting trichloroacetamide crystals were separated by filtration and the resulting solution was passed through a Dowex 50 NH_4^+ column (0.5 × 2 cm) to remove the tetrabutylammonium cation. Portions of the resulting solution were purified by preparative thin layer chromatography (TLC) as described before (11). The reaction mixture containing 4-HFC-P was then evaporated to dryness and dissolved in 3 mL of concentrated NH_4OH to which 11 mg of $NaBH_4$ was dissolved. At first, the sample was clear, but after a few minutes it became cloudy. After 2 hours at room temperature the solution became clear again and there was no borohydride present, as detected by a lack of hydrogen production

upon acidification of a small volume of the reaction mixture (2-3 µL). The reaction mixture was then evaporated to remove ammonia and placed in 2 mL of water followed by pH adjustment to < 4 using 1 M HCl. The resulting sample was then placed on a Dowex 50W8-H^+ column (0.8 x 4 cm) that was washed with 2 mL of 20 mM HCl. The F1-P was retained on the column under these conditions. The elution of F1-P was begun by passing 2 mL water through the column and completed with an additional elution using 3 M NH_4OH. Thin-layer chromatography (TLC) analysis with ninhydrin detection of amines showed that the water fraction contained F1-P as the only detectable compound. The F1-P was further purified by preparative TLC using the solvent system (acetonitrile, water, and 88% formic acid (40:20:10 vol/vol/vol)) where it had an R_f = 0.21. The ammonia fraction contained most of the F1-P and also ammonium-containing salts that was detected by the ninhydrin. The total yield of F1-P was 18%. Both F1 and F1-P showed an absorbance maximum at 215 nm, similar to 2,5-dimethylfuran. The concentration of F1-P was estimated based on the extinction coefficient of 2,5-dimethylfuran (ε_{215} = 7900 M^{-1} cm^{-1}) (17).

Analysis of F1 and its Derivatives. Direct analysis of F1 by HPLC is difficult due to the short wavelength at which F1 absorbs (215 nm), which is common to many compounds, and the fact that F1 is not retained by reverse-phase HPLC columns. In this study, F1, F1-P, and F1-PP were each converted to the 7-nitrobenzofurazan (NBD)-derivative, as previous reported (10). NBD-F1-P was purified by preparative TLC using the solvent system (5% formic acid in acetonitrile), where NBD-F1-P had an R_f = 0.15. After removal from the TLC plate, the structure of the NBD-derivatives were then confirmed using HPLC (see below) and LC- electrospray ionization-MS (LC-ESI-MS) analysis (10).

HPLC Analysis of NBD-F1, NBD-F1-P, and NBD-F1-PP Derivatives. Chromatographic separation of NBD derivatives was performed on a Shimadzu HPLC System (UFLC) equipped

with a C18 reverse phase column (Kromasi 100-5-C_{18}, 4.6 × 250 mm). The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM, pH 6.0, in presence of 0.02% NaN_3) and 5% MeOH followed by a linear gradient to 45% sodium acetate buffer/55% MeOH over 35 min at 1.0 mL/min. F1-NBD derivatives were detected by fluorescence using an excitation wavelength of 480 nm and an emission wavelength of 542 nm. Under these conditions, NBD derivatives were eluted in the following order (min): NBD-F1-PP (14.5), NBD-F1-P (17.0), and NBD-F1 (20.9).

HPLC Analysis of ATP, ADP, and AMP. The separation and analysis of ATP, ADP, and AMP in the sample were measured using a gradient ion pairing method performed on a Shimadzu UFLC System equipped with a C_{18} reverse phase column (ODS 250 × 4.6 mm, 5 µm particle size) and a photodiode array detector (PDA). The elution profile consisted of 5 min at 100% Buffer A (0.1 M KH_2PO_4, 10 mM $[CH_3(CH_2)_3]_4N(Br)$, pH 3.0) and 0% methanol followed by a linear gradient to 25% Buffer A/75% methanol over 20 min at 1.0 mL/min. ATP, ADP, and AMP were detected by absorbance at 260 nm. Under these conditions, ATP eluted at 10.8 min, ADP eluted at 5.7 min, and AMP eluted at 4.1 min.

LC-MS Analysis of the 4-[*N*-(γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(amino-methyl)furan (APMF-Glu). Analysis was performed with an AB Sciex 3200 Q TRAP mass spectrometer system with an Agilent 1200 Series liquid chromatograph. A Zorbax (100 × 4.0 mm, 2.6 µm particle size) column was used and the injection volume was 15 µL. Solvent A was water with 25 mM ammonium acetate and solvent B was methanol. The flow rate was 0.5 mL/min. Gradient elution was employed in the following manner (t (min), %B): (0.01, 5), (10, 65), (15, 65), (15.01, 5). Column effluent was passed through a variable wavelength detector set from 200 - 800 nm and then into the Turbo Spray ion source. Electrospray ionization (ESI) was

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177 employed at -4500 volts and a temperature of 600 °C. Curtain gas, gas 1, and gas 2 flow
178 pressures were 35, 70, and 60 psi, respectively. Desolvation, entrance, and collision cell entrance
179 potentials were -40, -12, and -22.5 volts, respectively.

180 **Cloning of *M. jannaschii mj0458* and *mj0840* Genes and Expression of their Gene**
181 **Products.** The *mj0458 and mj0840* genes were amplified from *M. jannaschii* genomic DNAs.
182 The primers used for *mj0458* were *mj0458*-Fwd: 5'-
183 GGTGGTCATATGCATATAGTAAAAATTGG-3' and *mj0458*-Rev: 5'-
184 GATCGGATCCTTATATTTTATCTATTCC-3'. The primers used for *mj0840* were *mj0840*-
185 Fwd: 5'-GTGTTTGATGTTAATGGGAATTTTTTAACTTCAGAAG-3' and *mj0840*-Rev: 5'-
186 CTTCTGAAGTTAAAAAATTCCCATTAACATCAAACAC-3'. PCR amplifications were
187 performed at 55 °C as the annealing temperature. The PCR products were purified, digested with
188 *Nde*1 and *Bam*H1 restriction enzymes and then ligated into compatible sites in plasmid pT7-7 to
189 make the recombinant plasmid *pmj0458* and *pmj0840*. The sequences were verified by
190 sequencing at the University of Iowa DNA core facility. The resulting plasmids were used to
191 transform the *E. coli* BL21-Codon Plus (DE3)-RIL (Stratagene). Transformed cells were grown
192 in Lysogeny broth (LB)-medium (200 mL) supplemented with 100 µg/mL ampicillin at 37 °C
193 with shaking until an OD_{600} of 1.0. Recombinant protein production was induced by addition of
194 lactose to a final concentration of 28 mM. After an additional 4 hours of culture at 37°C, the
195 cells were harvested by centrifugation (4000*g*, 5 min) and frozen at -20 °C. SDS-PAGE analysis
196 of total cellular proteins confirmed the induction of the gene products of *mj0458* and *mj0840* by
197 the appearance of intense protein bands at the expected 24 kDa and 37 kDa molecular weights,
198 respectively.

199 **Purification of the Gene Products of Recombinant *mj0458* and *mj0840*.** The frozen *E. coli*

cell pellet (~0.4 g wet weight from 200 mL of growth medium) was suspended in 3 mL of extraction buffer (50 mM N-[tris(hydroxymethyl)methyl]2-amionoethanesulfonic acid (TES), 10 mM $MgCl_2$, 20 mM Dithiothreitol (DTT) at pH 7.0) and lysed by sonication. The crude lysate was then treated by benzonase nuclease (250 U). The protein products from *mj0458* and *mj0840* were found to remain soluble after heating to 80 °C. Therefore, purification of the gene products of *mj0458* and *mj0840* started by heating the resulting cell extracts for 10 min at 80 °C followed by centrifugation (16000g, 10 min). This process allowed purification of the desired enzymes from the majority of *E. coli* proteins, which denature and precipitate under these conditions. Supernatant of the gene product of *mj0458* was then pooled and dialyzed against buffer containing 50 mM TES and 10 mM $MgCl_2$, at pH 7.0. Further purification of the gene product of *mj0840* was performed by anion-exchange chromatography of the 80 °C soluble fractions on a MonoQ HR column (1 × 8 cm; Amersham Bioscience) using a linear gradient from 0 to 1 M NaCl in 25 mM Tris buffer (pH 7.5), over 82 min at a flow rate of 1 mL/min.

Enzymatic Assay of MfnE (gene product of *mj0458*). To test whether MfnE could catalyze the formation of F1-PP from F1-P and ATP, 80 µM F1-P and 500 µM ATP in the presence of 3.7 µM MfnE was incubated at 70°C for 60 min in 50 mM TES buffer in the presence of 5 mM Mg^{2+} and 5 mM K^+ at pH 7.0. The reaction mixture was then converted to the NBD-derivatives and analyzed by HPLC as described above.

To measure the MfnE activity, the reaction was conducted in a 100 µL reaction volume containing 1.9 µM of MfnE, 200 µM F1-P or AMP, and 500 µM ATP in 50 mM 4-morpholineethanesulfonic acid (MES) buffer in the presence of 5 mM Mg^{2+} and 5 mM K^+ at pH 7.0 for 20 min at 80 °C. Following incubation, the reactions were quenched by the addition of 10

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µL 1 M HCl, the precipitated protein was removed by centrifugation (16000*g*, 10 min), and the resulting sample was neutralized by adding 8.3 µL 1.5 M, pH 8.8, Tris buffer.

Metal and pH-Dependence of the MfnE Catalyzed Reaction. For the metal-dependent study, the standard enzymatic assays were carried out including 5 mM of one of the following cations: Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, K^{+} or 10 mM EDTA in the standard assay. To investigate the influence of pH on catalytic ability, the specific activity at varying pH's was measured. The standard enzymatic assay was conducted in 25 mM citrate buffer (pH 4.0 - 6.0), 25 mM MES buffer (pH 6.0 - 7.0), and 25 mM tricine/3-(cyclohexylamino)1-propanesulfonic acid (CAPS)/TES buffer (pH 6.7 to 11.5).

Standard Enzymatic Assay of MfnF (gene product of *mj0840*). The standard assay for measuring MfnF enzymatic activity was conducted at 50 °C for 40 min in a 120 µL reaction volume containing 5.2 µg of MfnF (40 µL), 40 µL MfnE catalyzed reaction mixture (containing ~ 100 µM F1-PP), and 40 µL MfnD reaction mixture (13) (containing ~ 1 mM γ-glutamyltyramine and ~4 mM tyramine), in 50 mM TES buffer in the presence of 5 mM Mg^{2+} and 5 mM K^{+} at pH 7.0. Following incubation, the reactions were quenched by the addition of 10 µL 1 M HCl, the precipitated protein was removed by centrifugation (16000*g*, 10 min), and the resulting sample was neutralized by adding 8.3 µL 1.5 M, pH 8.8, Tris buffer. The samples were analyzed by LC-ESI-MS in the positive ion mode.

RESULTS

Synthesis and Analysis of F1-P Derivatives. F1-P was chemically synthesized from 4-HFC-P and converted into the NBD-derivative. HPLC with fluorescence detector analysis showed that NBD-F1-P eluted as a single peak at 17.0 min (Fig 3). After treatment with 1 µL

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phosphatase (0.2 U/L), NBD-F1-P was converted to NBD-F1 (20.9 min), which co-eluted with NBD-F1 (10). LC-ESI-MS analysis of NBD-F1-P showed a single peak with MH^+ = 371.4 *m/z* and $(M - H)^-$ = 369.4 *m/z*. MS/MS of the $(M - H)^-$ = 369.4 ion showed fragments at 79, 97, 179, 289, and 323 (Fig 4A).

Recombinant Expression, Purification, and Analysis of the Gene Products of *mj0458* and *mj0840*. Two genes at the loci of *mj0458* and *mj0840* were cloned and overexpressed in *E. coli*. Their gene products were purified as described above. The purified proteins migrated as single bands and were greater than 90% pure with an apparent molecular mass of 24 kDa (gene product of *mj0458*) and 37 kDa (gene product of *mj0840*) (Fig 5). The identities of the purified proteins were also confirmed by matrix-assisted laser desorption/ionization (MALDI)-MS analysis of the tryptic-digested protein band from the SDS gel based on a previously described procedure (18).

The Promiscuity of the Gene Product of *mj0458* (MfnE). We had reported that the gene product of *mj0458* encodes an adenylate kinase that catalyzes the transfer of a phosphoryl group from ATP to AMP, producing two molecules of ADP (14). In addition to the gene product of *mj0458* (MfnE), it is known that another archaeal adenylate kinase in *M. jannaschii* is the product of the *mj0479* gene (19, 20). However, it was unexpected that methanogens would contain two distinct adenylate kinases. Incubation of the gene product of *mj0458* with F1-P in the presence of ATP clearly showed the formation of F1-PP and ADP. A control experiment containing the same concentration of substrates in the absence of MfnE showed none of the expected product (data not shown). Generation of ADP from the MfnE reaction was detected by ion pairing HPLC, and the formation of F1-PP was confirmed using HPLC and LC-ESI-MS after conversion to NBD-F1-PP. The NBD-F1-PP was observed as a new HPLC peak eluting at 14.5 min with a corresponding decrease in the intensity of the NBD-F1-P (17.0 min) peak (Fig 3).

LC-ESI-MS analysis of the NBD-derivative of the MfnE reaction mixture showed a single peak with MH^+ = 451.5 *m/z* and $(M - H)^-$ = 449.5 *m/z*. MS/MS of the $(M - H)^-$ = 449.5 ion showed fragments at 79, 159, 177, 385, 403, and 431 (Fig 4B), which are consistent with NBD-F1-PP's molecular weight and structure. These results clearly showed MfnE to catalyze the phosphoryl-transfer reaction between ATP and F1-P producing ADP and F1-PP.

The pH-dependent study of MfnE activity showed a pH optimum at 7.0. We also observed that MfnE exclusively employed Mg^{2+} as a cofactor to facilitate phosphoryl group transfer. Addition of 10 mM EDTA to the reaction mixture resulted in complete loss of MfnE activity. The activity of MfnE at 80°C was 0.16 μmol/mg/min using F1-P as substrate and 0.072 μmol/mg/min when AMP was employed as substrate (both of these values are the extent of substrate conversion to product for each incubation). The adenylate kinase activity of MfnE 80°C is 500 fold less than that of the gene product of *mj0479, which* encodes the first archaeal adenylate kinase identified in *M. jannaschii* (19). In addition, The homologs of *mj0458* in some methanogens and methylotrophs cluster with MfnD (*mj0815*), which encodes the enzyme that catalyzes γ-glutamyltyramine formation during methanofuran biosynthesis (13). These results strongly suggest that the possible physiological function of MfnE is to catalyze the formation of F1-PP involved in methanofuran biosynthesis.

The Gene Product of *mj0840* (MfnF) Catalyzes the Formation of APMF-Glu. The gene product of *mj0840* was annotated as a hypothetical protein in *M. jannaschii* (21). Incubation of the purified gene product of *mj0840* (MfnF) with γ-glutamyltyramine and F1-PP, which are generated from the MfnE and MfnD catalyzed reactions, respectively, clearly showed the formation of APMF-Glu. This was confirmed with mass spectral data showing a single peak with the expected $(M - H)^-$ = 374.2 *m/z* and MH^+ = 376.2 *m/z*. The extracted ion chromatograph

(XIC) of the product with MH^+ = 376.2 *m/z* eluted as a single peak at 18 min (Fig 6A and 6B). The identity of APMF-Glu was also supported by MS/MS data (Fig 6C) with the MH^+ = 376.2 ion producing expected fragments at 110, 213, 230, and 267. Also, incubation of γ-glutamyltyramine plus F1-PP in the absence of MfnF showed that no APMF-Glu was formed under the same conditions (data not shown). When incubating γ-glutamyltyramine and F1-P in the presence of MfnF, a trace of APMF-Glu was observed in LC/MS (Fig 6A), but the intensity was less than 2% of that when F1-PP was used as substrate. In addition, MfnF could not use tyramine as a substrate to condense with F1-PP, since no APMF ($(M - H)^-$ = 245.1 *m/z* and MH^+ = 247.1 *m/z*) was detected from the mass spectral data, consistent with our previous hypothesis that condensation of the tyramine moiety with F1-PP must occur after formation of γ-glutamyltyramine (13).

DISCUSSION

Methanofuran is the initial C1 acceptor molecule in the formation of methane through methanogenesis. Several structurally different methanofurans are currently known, with the nature of the differences residing in modifications of the side chain attached to the basic core APMF-Glu structure found in all methanofurans (9). With the discovery of MfnA, MfnB, MfnC, and MfnD, the possible biosynthetic pathway of APMF-Glu was proposed (Fig 1). In this pathway, at least two enzymes are required to condense F1-P with γ-glutamyltyramine moiety. One enzyme is required to catalyze the conversion of F1-P to F1-PP, where the pyrophosphate group serves as a better leaving group for the subsequent condensation reaction. The other enzyme catalyzes the condensation between F1-PP and γ-glutamyltyramine to produce APMF-

313 Glu. Such a coupling mechanism is a common strategy used in the biosynthesis of coenzymes
314 such as folate (22) and thiamine (23) as well as terpenes and steroids (24-26).

315 During thiamine biosynthesis, ThiD (4-amino-5-hydroxymethyl-2-methylpyrimidine
316 phosphate (HMP-P) kinase) phosphorylates HMP-P to 4-amino-5-hydroxymethyl-2-
317 methylpyrimidine pyrophosphate (HMP-PP), and then another enzyme, thiamin phosphate
318 synthase (ThiE), catalyzes the coupling reaction of HMP-PP and 4-methyl-5-
319 hydroxyethylthiazole phosphate (23). During folate biosynthesis, the formation of 6-
320 hydroxymethyl-7,8-dihydropterin pyrophosphate (H_2HMP-PP) is catalyzed via a one-step
321 pyrophosphoryl-transfer reaction directly from 6-hydroxymethyl-7,8-dihydropterin, then
322 dihydropteroate synthase catalyzes the condensation of H_2HMP-PP and *p*-aminobenzoic acid
323 (22). Similarly, to condense the F1-P moiety and γ-glutamyltyramine in the methanofuran
324 biosynthesis pathway, a kinase is likely involved in transferring a phosphoryl group to form F1-
325 PP before the condensation step. This assumption is based on the fact that 1) the pyrophosphate
326 group serves as a better leaving group compared to a phosphate group because pyrophosphate is
327 a stronger acid compared to a phosphate group and 2) pyrophosphate complexes to magnesium
328 ions to form a ubiquitous leaving group.

329 We previously reported that MfnE encodes a second type of archaeal adenylate kinase (14). In
330 addition to MfnE, it has been established that another archaeal adenylate kinase in *M. jannaschii*
331 is the product of the *mj0479* gene (19, 20). It is unlikely that methanogens would contain two
332 distinct adenylate kinases. Our results clearly show that the promiscuous MfnE also catalyzes the
333 formation of F1-PP from F1-P and ATP. The gene locus *mj0458* is in the neighborhood of MfnD
334 (encoded by *mj0815*) and MfnF (encoded by *mj0840*), which are known to be involved in
335 methanofuran biosynthesis. We propose that the physiological function of MfnE is to catalyze

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F1-PP formation during methanofuran biosynthesis. However, to approve our hypothesis, future work will focus on screening the F1-PP kinase activity in other *M. jannaschii* kinases and further characterization of MfnE.

Many promiscuous enzymes that have been found in archaea (27, 28). *M. jannaschii* is an autotrophic archaea with a small genome (21). Therefore, promiscuity of enzymes can provide an obvious advantage, allowing it to react with a broader range of substrates to maximize its catalytic versatility using limited enzyme resources (29). Thus, the promiscuous MfnE may possibly also perform a similar function to other adenylate kinases, which regulate the ATP/ADP balance in the cell (14).

MfnF catalyzes the formation of an ether bond (C-O), instead of the C-N or C-C bond as found in folate, thiamin, terpene, and steroid biosynthesis. The other well-known enzyme that catalyzes C-O bond formation is geranylgeranylglycerol phosphate synthase (GGGPS) (30). This enzyme catalyzes the formation of an ether bond between glycerol-1-phosphate and polyprenyl diphosphates, which is essential for biosynthesis of archaeal membrane lipid (30). GGGPS exhibits an α/β TIM barrel structure (31), as does thiamine synthase (32) and dihydropteroate synthase (33, 34).

PSI-blast analysis shows that MfnF belongs to the hydantoinase A family (PF01968) and contains a sugar kinase domain. However, the catalytically essential histidines (15) in hydantoinase are not conserved in MfnF. The crystal structure of the MfnF homolog in *Methanococcus maripaludis*, which was solved by Kuzin, A. P. *et al.* (PDB: 3CET), exhibits a distinctive α/β two-layer sandwich structure (Fig 7), unlike the α/β TIM barrel structure observed in thiamine synthase (32), dihydropteroate synthase (33, 34) and GGGPS (31). In the active sites of both the thiamine synthase and dihydropteroate synthase, the Mg^{2+} used to stabilize the

359 leaving pyrophosphate is found to be ligated by two aspartic acid residues (33, 35). Such a
360 pyrophosphate stabilizing interaction is similar to that found in farnesyl pyrophosphate synthase
361 (24), aristolochene synthase (25), and pentalenene synthase (26), which catalyze the formation of
362 an allylic carbocation from a pyrophosphate ester. In addition, serine and/or threonine residues
363 are hydrogen-bonded to the oxygen atom of the pyrophosphate to activate the pyrophosphate as a
364 leaving group (35). Sequence alignment and structural analysis of MfnF show a highly conserved
365 motif, D_{135}XGSTTX$D_{142,}$ which is likely involved in metal and pyrophosphate binding during
366 catalysis. In addition, a strictly conserved Arg158 likely plays a role in stabilizing pyrophosphate
367 as a leaving group (Fig 7).

368 In all examples where pyrophosphate serves as a leaving group, it has been proposed that the
369 reaction mechanism follows an S_N-1 mechanism that proceeds via formation of a cationic
370 intermediate before nucleophilic attack by the other substrate (24-26, 33, 35). Therefore, it is
371 reasonable to assume that MfnF catalyzes a reaction following a similar mechanism (Fig 8). In
372 this mechanism, pyrophosphate is first removed from F1-PP, stabilized by the metal ion (likely
373 Mg^{2+}) and Arg158 (Fig 7), where the resulting cationic intermediate species ($F1^{+}$) is resonance
374 stabilized with the positive charge delocalized over the furan ring. The hydroxyl group of γ-
375 glutamyltyramine finally attacks $F1^{+}$ at the C6 carbon atom to generate the product APMF-Glu.

376 In summary, this work describes two enzymes, MfnE and MfnF, that are responsible for the
377 formation of APMF-Glu with the first enzyme (MfnE) catalyzing the phosphorylation of F1-P to
378 F1-PP and the second enzyme (MfnF) catalyzing the coupling of F1-PP with γ-glutamyltyramine
379 to produce the core structure of methanofuran. Although such coupling reactions are ubiquitous
380 in biochemistry, this work provides the first evidence that such a mechanism is employed in
381 methanofuran biosynthesis. Since methanofuran in *M. jannaschii* contains 7-12 γ-linked

glutamates, we propose that once the core structure APMF-Glu is synthesized, another enzyme catalyzes polyglutamylation, producing the final methanofuran molecule (Fig 1). However, we have yet to identify the enzyme catalyzing this polyglutamylation.

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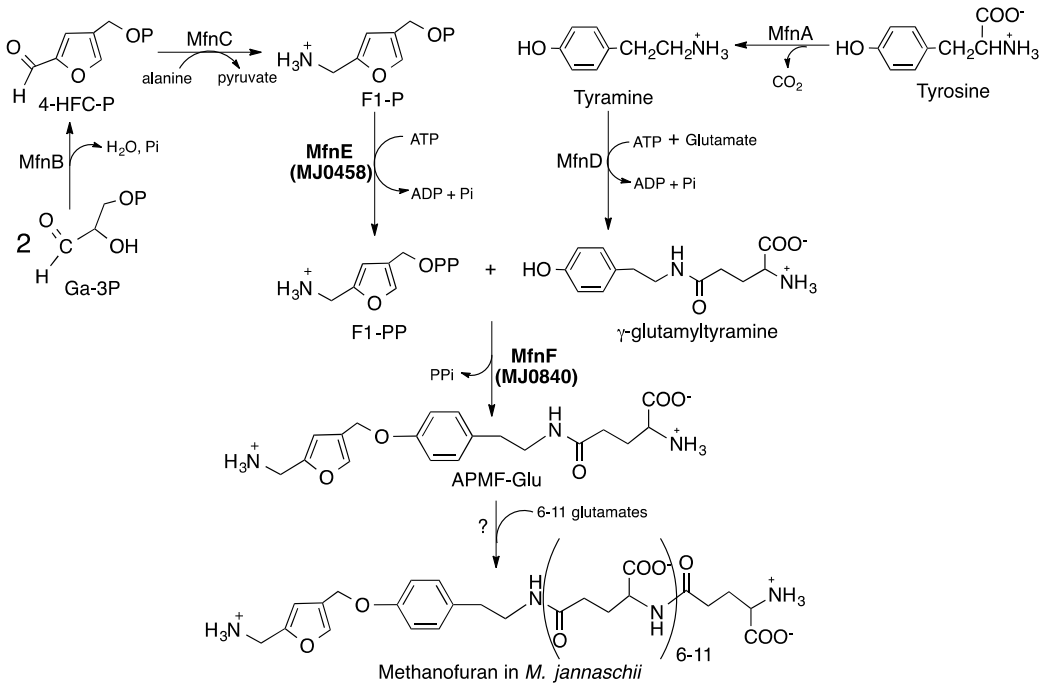

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510 **FIG 1** The proposed biosynthetic pathway of methanofuran in *M. jannaschii*.

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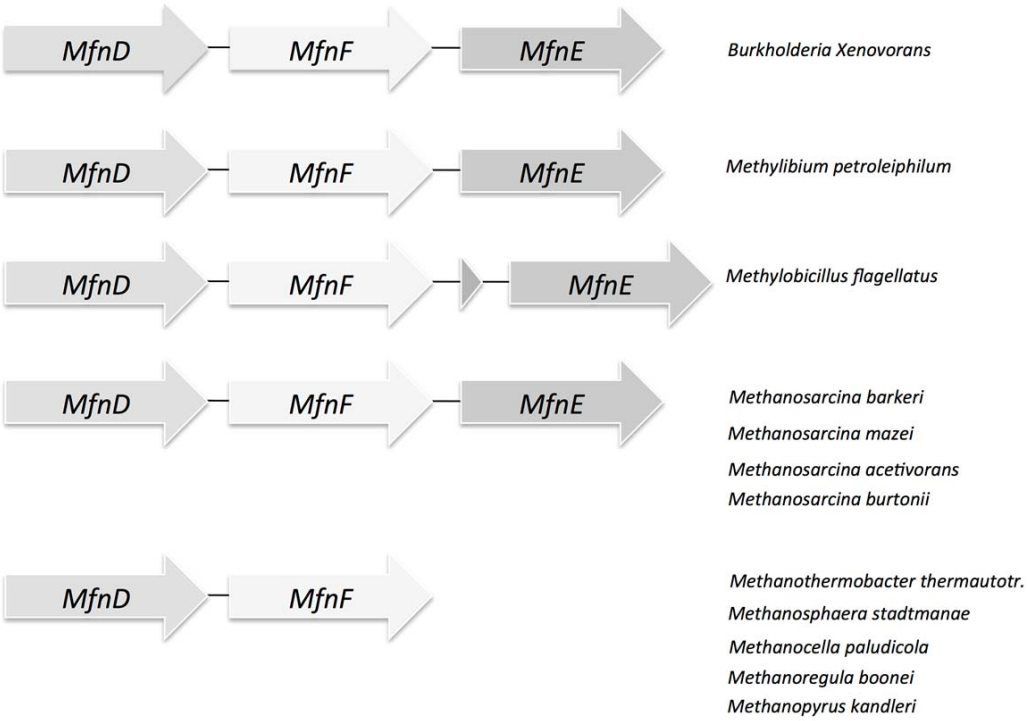


FIG 2 Clustering of MfnE (*mj0458*) and MfnF (*mj0840*) genes with the methanofuran biosynthetic related gene MfnD (*mj0815*) in some methanogen and methylotroph genomes.

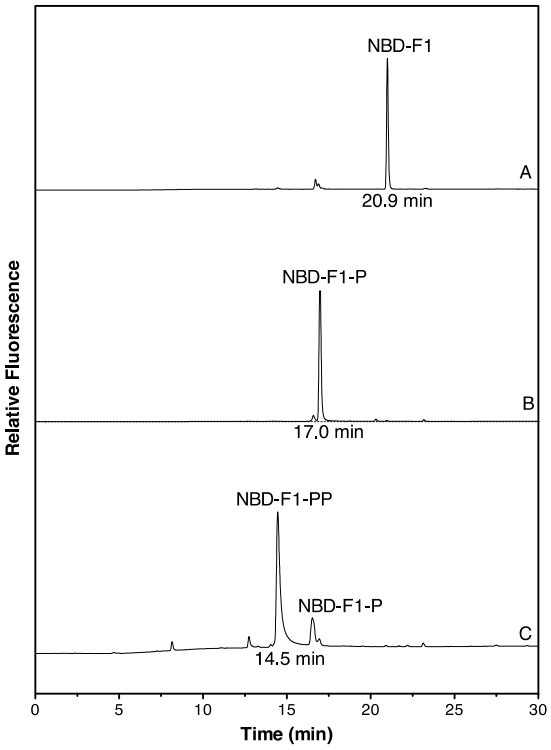


FIG 3 HPLC analysis of NBD-F1, NBD-F1-P, and NBD-F1-PP. A) 80 μM sample of synthetic F1-P was converted to NBD-F1-P treated with phosphatase (37°C, 20 min) to produce NBD-F1 to confirm the structure of F1-P; B) the NBD derivatives of a reaction mixture including 80 μM F1-P and 500 μM ATP in the absence of MfnE (70°C, 60 min); C) the NBD derivative of a reaction mixture containing 80 μM F1-P and 500 μM ATP in the presence of 3.7 μM MfnE (70°C, 60 min). Reactions in B and C are carried out in 50 mM TES buffer in the presence of 5 mM Mg^{2+} and 5 mM K^{+} at pH 7.0. The NBD derivatives were detected by fluorescence using an excitation wavelength of 480 nm and an emission wavelength of 542 nm.

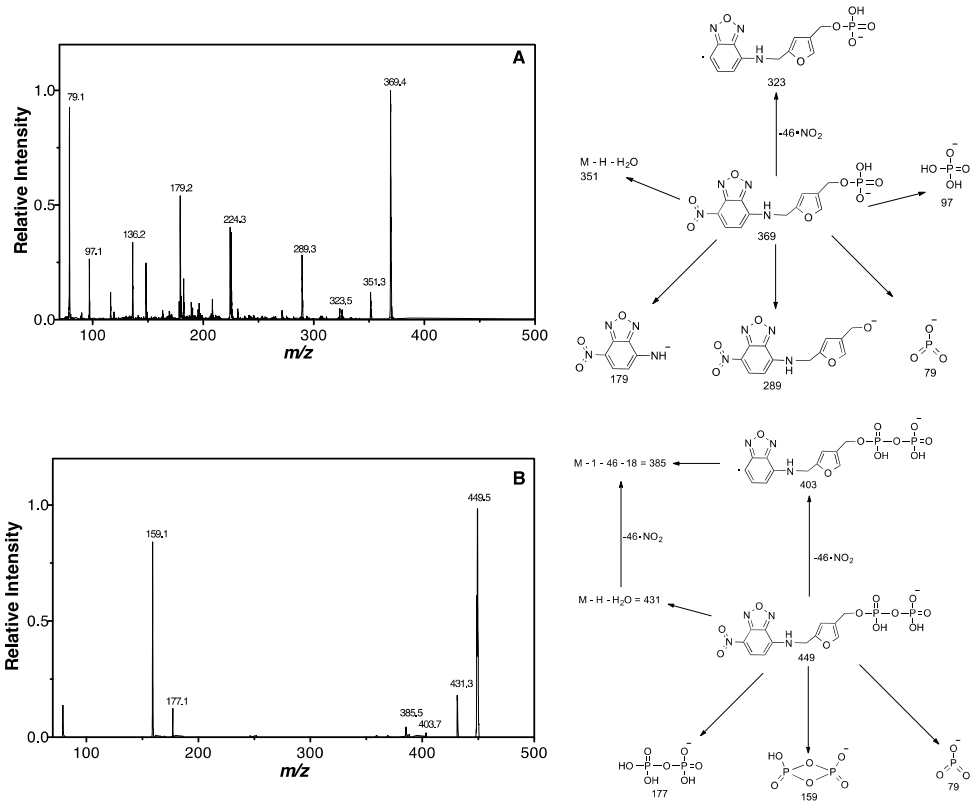


FIG 4 A) The MS/MS spectrum of NBD-F1-P $(M-H)^-$ = 369.4 *m/z* ion with the expected fragments at 79, 97, 179, 289, and 323 *m/z*. The proposed structures of the expected fragments are shown on the right. B) The MS/MS spectrum of NBD-F1-PP $(M-H)^-$ = 449.5 *m/z* ion generated from the phosphoryl transfer reaction between ATP and F1-P catalyzed by MfnE with the expected fragments at 79, 159, 177, and 403 *m/z*. The proposed structures of the expected fragment are shown on the right.

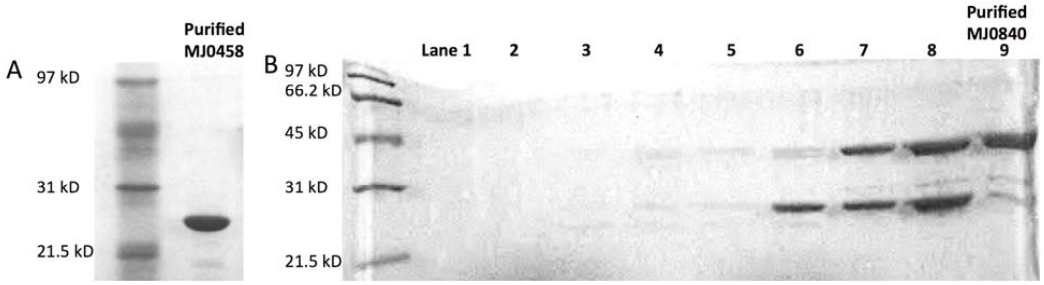
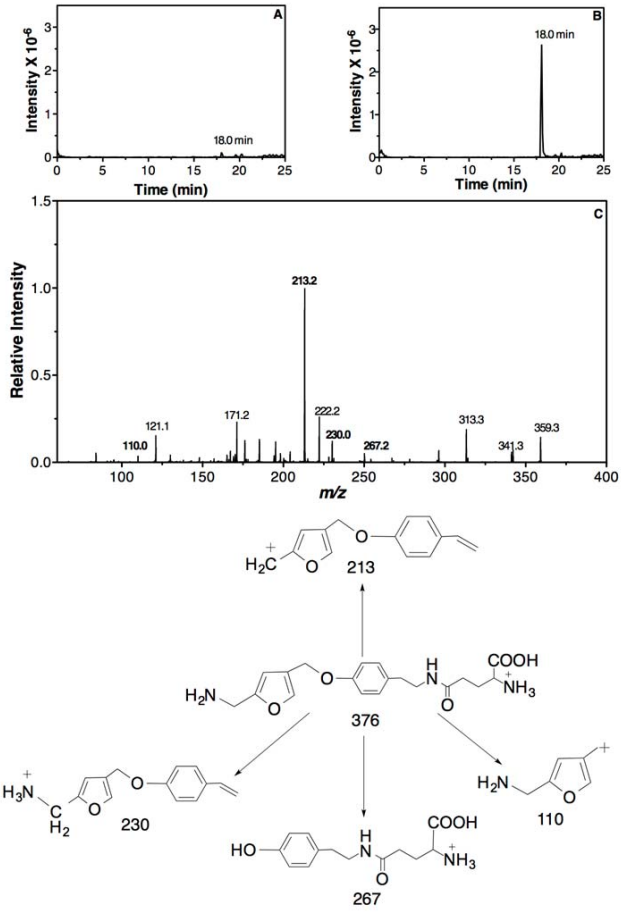


FIG 5 Purification of the *mj0458* gene product (A) and *mj0840* gene product (B). In panel B, lane 7 - 9 represents the target protein in the fractions eluting at 350 - 460 mM NaCl from a MonoQ anion exchange column. The target protein was about 90 % pure in the fraction in lane 9.



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555 **FIG 6** LC/MS/MS analysis of APMF-Glu in the positive ion mode. The extracted ion
556 chromatograph (XIC) of MH^+ = 376.2 *m/z* ion from the control experiment (A) (incubate F1-P
557 with γ-glutamyltyramine in presence of MfnF) and (B) from the reaction mixture of MfnF
558 containing F1-PP generated from the MfnE reaction and γ-glutamyltyramine. (C) The MS/MS
559 spectrum of MH^+ = 376.2 *m/z* ion with the expected fragments at 110, 213, 230, and 267 *m/z*.
560 The proposed structures of observed fragments are shown in the bottom of the figure.

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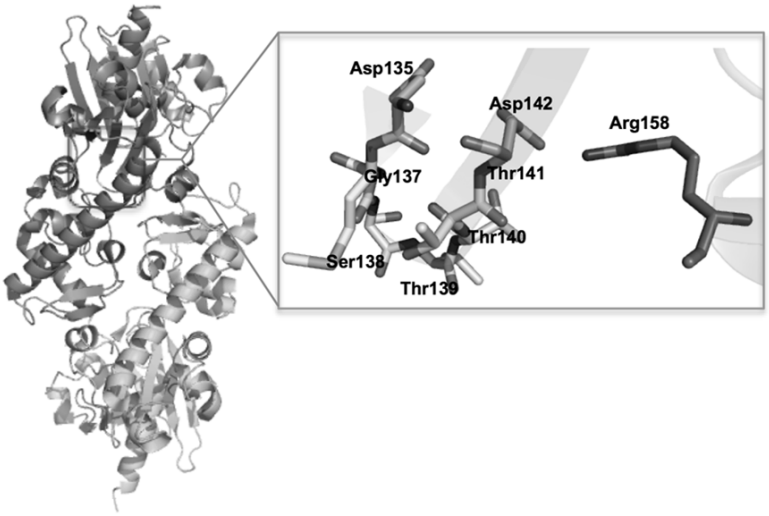

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563 **FIG 7** The overall dimer structure of MfnF (left) and the possible pyrophosphate and metal-

564 binding pocket (right).

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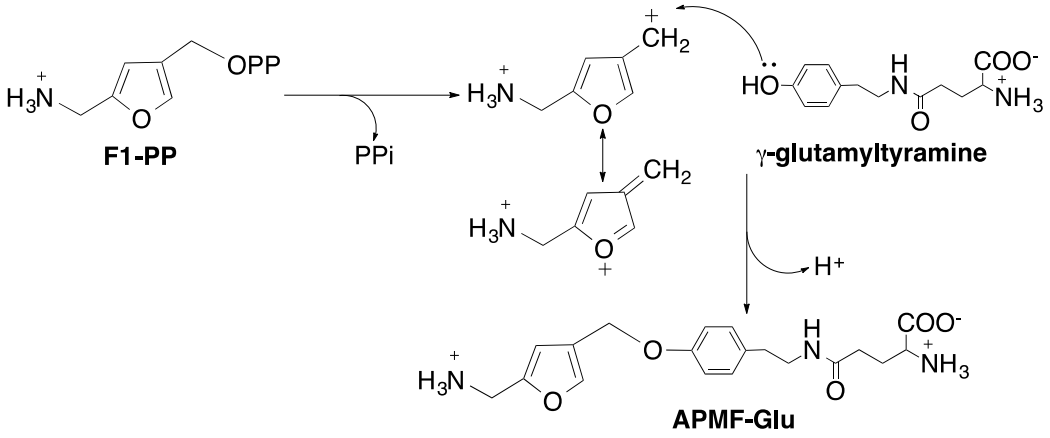

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573 **FIG 8** The proposed catalytic mechanism of MfnF.

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