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Discovery of a Glutamine Kinase Required for the Biosynthesis of the *O*-Methyl Phosphoramidate Modifications Found in the Capsular Polysaccharides of *Campylobacter jejuni*

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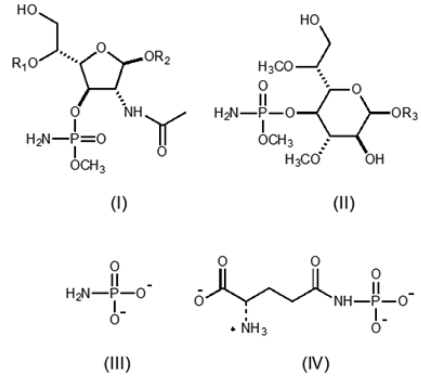
Supporting Information

ABSTRACT: Bacterial capsular polysaccharides (CPS) are complex carbohydrate structures that play a role in the overall fitness of the organism. *Campylobacter jejuni*, known for being a major cause of bacterial gastroenteritis worldwide, produces a CPS with a unique *O*-methyl phosphoramidate (MeOPN) modification on specific sugar residues. The formation of P−N bonds in nature is relatively rare, and the pathway for the assembly of the phosphoramidate moiety in the CPS of *C. jejuni* is unknown. In this investigation we discovered that the initial transformation in the biosynthetic pathway for the MeOPN modification of the CPS involves the direct phosphorylation of the amide nitrogen of L-glutamine with ATP by the catalytic activity of Cj1418. The other two products are AMP and inorganic phosphate. The L-glutamine-phosphate product was characterized using ^{31}P NMR spectroscopy and mass spectrometry. We suggest that this newly discovered enzyme be named L-glutamine kinase.

Campylobacter jejuni is a Gram-negative bacterium that causes foodborne gastroenteritis in humans worldwide.[1] It is commonly found in chickens, and as a consequence contaminated poultry are a significant reservoir for human disease. Whereas infection with *C. jejuni* is typically self-limiting, in rare cases it can lead to the subsequent development of Guillian-Barré syndrome, a devastating acute polyneuropathy.[2] Like many Gram-positive and Gram-negative organisms, *C. jejuni* produces capsular polysaccharides, which are composed of chains of sugars that form extensive layers surrounding the outer surface of the bacterium. In some cases, these chains can be composed of more than 200 sugars.[3] The capsular polysaccharides, hereafter referred to as CPS, protect the organism from the environment and from complement-mediated phagocytosis and killing.[4] It is now well documented that, in *C. jejuni*, the CPS is important for colonization and invasion of the host organism.[5] More than 40 serological strains of *C. jejuni* have been identified, and each strain is likely to produce structural variations to the CPS.[6,7] These modifications are involved in a complex strategy for evasion of both bacteriophage predation and host defense systems.[4,8] In *C. jejuni* strain NCTC11168, a cluster of 35 genes has been identified as being responsible for the synthesis and export of the CPS.[9]

By far the most unusual modification to the CPS of *C. jejuni* is the addition of *O*-methyl phosphoramidate groups (MeOPN) attached to the polysaccharide backbone. For example, in *C. jejuni* strain NCTC11168, C3 of a 2-acetamido-2-deoxy-β-D-galactofuranose (**I**) moiety is decorated with an *O*-methyl phosphoramidate group, and the CPS of the hypermotile variant of this strain (11168H) has an additional MeOPN modification at C4 of a derivative of D-*glycero*-α-L-*gluco*-heptopyranose (**II**) as illustrated in Scheme 1.[6,7] The occurrence of P−N bonds in biological systems is relatively rare (creatine phosphate and arginine phosphate are notable exceptions), and the presence of the *O*-methyl phosphoramidate groups in the capsular polysaccharides of *C. jejuni* plays

Scheme 1



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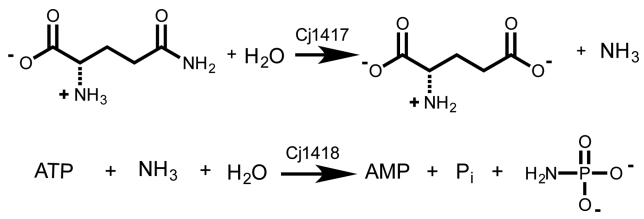
a significant role in its pathogenicity.[5] In *C. jejuni* 11168H, genes with the locus tags *cj1418c*, *cj1417c*, *cj1416c*, and *cj1415c* have been implicated in the biosynthesis of the phosphoramidate moiety (**III**) of the CPS but the pathway leading to the formation of the P−N bond in this organism has not been elucidated.[10]

The focus of this investigation is on the catalytic functions of Cj1418 and Cj1417. Cj1418 is a member of cog0574, and this enzyme is currently annotated as a putative PEP synthase or a pyruvate phosphate dikinase.[11,12] Structurally characterized enzymes in this family are composed of three distinct protein segments, including an ATP-grasp domain, a PEP/pyruvate binding region, and a phosphohistidine domain.[9] The amino acid sequence of Cj1418 suggests that it has an *N*-terminal ATP-grasp domain (residues 1−219) and a *C*-terminal phosphohistidine domain (residues 694−767). However, its central domain (residues 220−693) does not appear to be homologous to any of the known PEP/pyruvate binding regions. The closest structurally characterized homologue to Cj1418 with a known catalytic activity is rifampin phosphotransferase (23% sequence identity) from *Listeria monocytogenes* (PDB id: 5FBS, 5FBT, and 5FBU).[13] This enzyme catalyzes the ATP-dependent phosphorylation of the antibiotic rifampin via a mechanism that involves the pyrophosphorylation of His-825, hydrolysis of this intermediate to generate a phosphorylated histidine intermediate, and subsequent phosphoryl transfer to rifampin.[13,14]

Cj1417 is a member of cog2071 and is annotated as a type I glutamine amidotransferase.[11,15] This class of enzymes catalyzes the hydrolysis of glutamine (or structurally similar glutamine analogs) via the formation of a thioester intermediate with an active site cysteine residue.[15] In many cases, such as in carbamoyl phosphate synthetase, the hydrolysis of glutamine is coupled to an ATP-dependent phosphorylation of a second substrate by an associated synthetase domain/subunit.[16] We thus initially proposed that the combined activities of Cj1418 and Cj1417 would likely be required for the *in vivo* formation of the putative phosphoramidate intermediate (**III**) during the biosynthesis of the *O*-methylphosphoramidate groups. In our proposed mechanism, the first reaction is initiated by the Cj1417 dependent hydrolysis of glutamine to form glutamate and ammonia. This step is subsequently followed by the phosphorylation of ammonia via the catalytic activity of Cj1418 (Scheme 2).

Scheme 2. Predicted Functions of Cj1417 and Cj1418



The gene for the expression of Cj1418 with an *N*-terminal hexa-histidine purification tag was cloned from the genomic DNA of *C. jejuni* 11168H into a modified form of the pET-28b expression vector.[17] This vector was subsequently used to transform Rosetta (DE3) *Escherichia coli*, and the cells were subsequently grown in a medium of lysogeny broth at 30 °C. Following induction with 1.0 mM IPTG, the cells were allowed to grow at 16 °C for 16 h. After cell lysis and centrifugation, Cj1418 was purified using Ni-affinity chromatography and the excess imidazole was removed by dialysis. The purified protein was concentrated and stored at −80 °C. Approximately 4 mg of Cj1418 were purified from 1.0 L of the original cell culture.

To test our initial prediction that Cj1418 was required for the ATP-dependent phosphorylation of ammonia, we first incubated the enzyme (5.0 μM) in the presence of 2.0 mM $MgCl_2$ and 1.0 mM ATP in 100 mM HEPES buffer (pH 8.0) at 30 °C. This control experiment was monitored using anion exchange chromatography by measuring the changes in the concentration of ATP at 255 nm. After an incubation period of ∼60 min, the concentration of ATP (retention time of 8.2 min) did not change significantly but relatively small amounts of AMP (retention time of 5.3 min) and ADP (retention time of 7.1 min) could be detected (Figure 1A). The addition of 100 mM NH_4Cl did not change the amounts of AMP or ADP that were produced (Figure 1B). Since catalytic activity was not observed with ammonia, Cj1418 was next assayed in the presence of 5.0 mM L-glutamine. After an incubation period of ∼60 min, all of the ATP was converted to AMP (Figure 1C). The reaction mixture was subsequently examined by ^{31}P NMR spectroscopy, and resonances were observed for AMP at 4.36 ppm and at 3.03 ppm for inorganic phosphate (Figure 2A). Two additional resonances were observed at −3.57 and −4.06 ppm. Integration of the signal intensities for the sum of these two resonances equaled those observed for either AMP or P_i. The observed chemical shifts (−3.57 and −4.06 ppm) for the new phosphate containing product(s) did not match the ^{31}P NMR spectrum for authentic phosphoramidate (**III**) at 1.3 ppm.[18]

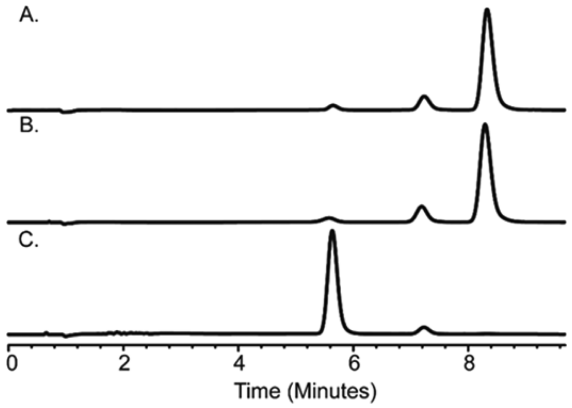


Figure 1. Anion exchange chromatograms of the reaction products when Cj1418 (5.0 μM) and 100 mM HEPES buffer (pH 8.0) were incubated for 60 min at room temperature with (A) 1.0 mM ATP and 2.0 mM $MgCl_2$. (B) 1.0 mM ATP, 2.0 mM $MgCl_2$ and 100 mM NH_4Cl. (C) 1.0 mM ATP, 2.0 mM $MgCl_2$, and 5.0 mM L-glutamine. Peak retention times correspond to the following: AMP (5.3 min), ADP (7.1 min), and ATP (8.2 min).

The most likely (but initially unexpected) product to form from the reaction catalyzed by Cj1418 is L-glutamine-phosphate (**IV**) where the amide nitrogen is phosphorylated. To test this conjecture, the reaction was repeated using L-glutamine with an ^{15}N-label exclusively at the amide nitrogen. The two ^{31}P resonances of the reaction product now appear as doublets, due to the apparent spin coupling with the ^{15}N-labeled amide nitrogen (Figure 2B). The observed coupling constants $J(^{15}N-^{31}P)$ are 18 Hz for the phosphorus resonance at −3.57 ppm and 21 Hz for the resonance at −4.06 ppm. The

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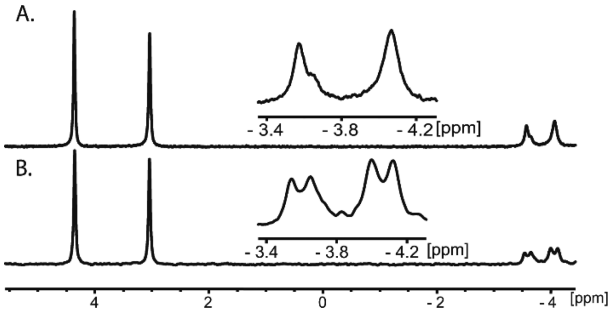

Figure 2. (A) ^{31}P NMR spectrum of the reaction products when Cj1418 was mixed with MgATP and L-glutamine. The resonance at 4.36 ppm is from AMP, and the resonance at 3.03 ppm is from inorganic phosphate. The resonances at −3.57 and −4.06 ppm correspond to L-glutamine phosphate (**IV**). (B) ^{31}P NMR spectrum of the reaction products when Cj1418 was mixed with MgATP and L-glutamine-(amide-^{15}N). The phosphorus resonances at −3.57 and −4.06 are now doublets due to the apparent spin coupling with the adjacent ^{15}N-nucleus.

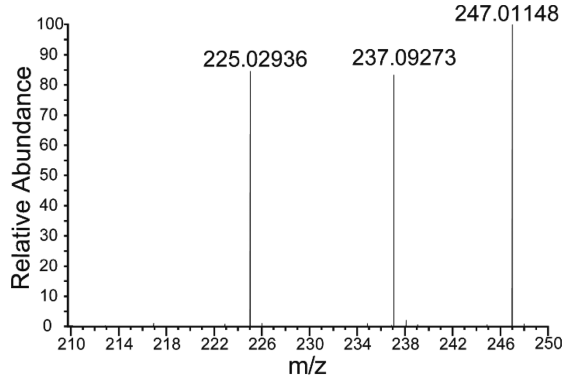

Figure 3. Negative ESI mass spectrum of the reaction mixture when Cj1418 was mixed with 2.0 mM ATP and 5.0 mM L-glutamine at pH 8.0 in 100 mM sodium bicarbonate buffer (pH 8.0). The identified ions correspond to L-glutamine phosphate (m/z = 225.03 for M − H and m/z = 247.01 for M − 2H + Na), and HEPES (m/z = 237.09 for M − H). The HEPES buffer was introduced with the preparation of Cj1418.

magnitude of this coupling constant is consistent with that previously observed for phosphocreatine, which exhibits a $J(^{15}N-^{31}P)$ coupling constant of 18−20 Hz.[19] The most likely explanation for the observation of two distinct ^{31}P NMR signals for this compound is the restricted rotation of the amide functional group thereby giving rise to separate resonances for the *syn* and *anti* conformations of the L-glutamine-phosphate product. This conclusion is further supported by the direct chemical synthesis of L-glutamine phosphate.[20] Two phosphorus resonances for the sodium salt of this compound are observed in D_2O at −3.5 and −3.7 ppm. A single resonance is observed at −5.10 ppm for the free acid where the rate of rotation about the amide bond is expected to increase. The chemical protocol for the synthesis of L-glutamine phosphate and the associated NMR (Figures S1 to S3) and mass spectra (Figure S4) for the chemically synthesized compound are found in the Supporting Information.

The formation of L-glutamine-phosphate after incubation of Cj1418, ATP, and L-glutamine is further supported by the mass spectrum (ESI negative mode) of the unfractionated reaction mixture. A peak that corresponds to the mass of the expected L-glutamine phosphate is observed with an m/z of 225.03 for the $(M - H)^-$ species and at an m/z of 247.01 $(M - 2H + Na)^-$ for the sodium adduct (Figure 3). Several other major peaks are observed that correspond to the known compounds in the unfractionated reaction mixture including phosphate (m/z = 96.96), HEPES (m/z = 237.09), and AMP (m/z = 346.05). The full-width mass spectrum is presented in Figure S5.

The kinetic parameters for the phosphorylation of L-glutamine by ATP as catalyzed by Cj1418 at pH 8.0 and 30 °C were determined spectrophotometrically at 340 nm using a coupled enzyme assay that measures the formation of AMP. The assay contained adenylate kinase (8 units/mL), pyruvate kinase (8 units/mL), and lactate dehydrogenase (8 units/mL) in the presence of 11 mM $MgCl_2$, 0.40 mM NADH, and 2.0 mM PEP.[21] Under these conditions the apparent kinetic constants for Cj1418 are k_{cat} = 2.5 ± 0.3 s^{-1}, K_{ATP} = 340 ± 70 μM, k_{cat}/K_{ATP} = 7400 ± 1700 M^{-1} s^{-1}, K_{Gln} = 640 ± 60 μM, and k_{cat}/K_{Gln}= 3900 ± 800 M^{-1} s^{-1}. No catalytic activity was observed (<1% of the rate with L-glutamine) in the presence of either L-glutamate (10 mM) or L-asparagine (10 mM).

Quite unexpectedly, we have shown that Cj1418, an enzyme involved in the biosynthesis of the *O*-methyl phosphoramidate groups in *C. jejuni*, catalyzes the phosphorylation of the amide nitrogen of L-glutamine, rather than ammonia. However, it has been shown previously that utilization of $^{15}NH_4Cl$ in the medium for growth of *C. jejuni* results in ^{15}N-labeling of the MeOPN groups in whole cells.[22] Our current results suggest that the ammonia must first be transformed to L-glutamine, presumably by the action of L-glutamine synthetase. To the best of our knowledge our results represent the first documented case of an enzyme-catalyzed phosphorylation of a simple amide nitrogen. However, similar compounds have been chemically synthesized as potential inhibitors of D-alanine:D-alanine ligase[20,23] and aspartate semialdehyde dehydrogenase.[24] Glutamine synthetase has also been demonstrated to catalyze the phosphorylation of L-methionine-*S*-sulfoximine on nitrogen.[25] The identity of L-glutamine phosphate was confirmed by ^{31}P NMR experiments, ^{15}N-labeling, and mass spectrometry. These results have further demonstrated that the initial series of steps as proposed in Scheme 2 for the biosynthesis of the *O*-methyl phosphoramidate capsule modification in *C. jejuni* is incorrect.

A more likely scenario for phosphoramidate biosynthesis is illustrated in Scheme 3. In this modified pathway, L-glutamine phosphate is hydrolyzed by Cj1417 to generate phosphoramidate (**III**). The closest functionally characterized homologue of Cj1417 is γ-L-glutamyl-γ-aminobutyrate hydrolase (PuuD) from *E. coli*. This protein has a 23% sequence identity with Cj1417, and thus homologous amidotransferase enzymes can catalyze the hydrolysis of substrates other than L-glutamine.[26] In the next step we postulate that Cj1416 catalyzes the displacement of pyrophosphate by phosphoramidate (**III**) from a nucleotide triphosphate (NTP) to form the phosphoramidate of NDP (**V**). Cj1416 is a member of cog1213, and homologous enzymes have been shown to catalyze similar reactions. For example, CTP:phosphocholine cytidylyltransferase from *Streptococcus pneumonia* (LicC) catalyzes the formation of CDP-choline from CTP and choline phosphate.[27] Alternatively, Cj1416 may catalyze the formation of NDP-glutamine (**VI**) through the displacement of pyrophosphate from NTP by L-glutamine-phosphate (**IV**). The NDP phosphoramidate (**V**) would then be formed by the catalytic activity of Cj1417. Experiments are currently underway

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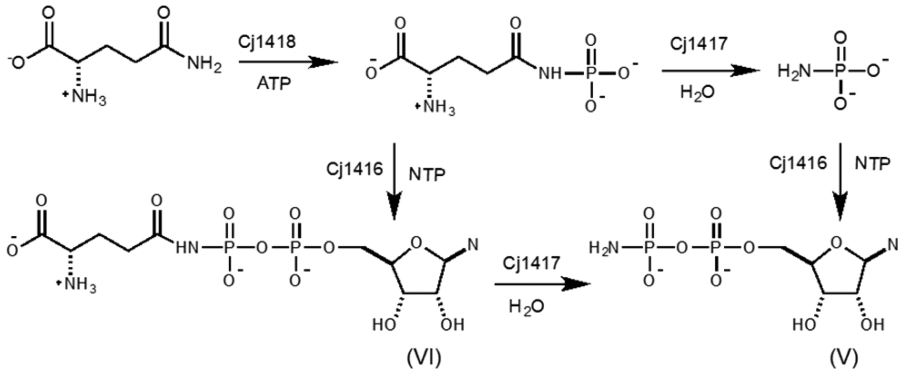
Scheme 3. Experimentally Determined Function of Cj1418 and Possible Catalytic Functions of Cj1417 and Cj1416



to firmly establish the catalytic activities of Cj1417, Cj1416, and the remaining transformations that lead to the biosynthesis of this fascinating modification to the capsular polysaccharides of *C. jejuni*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b04824.

Additional methods, NMR and mass spectral data (PDF)

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Notes

The authors declare no competing financial interest.

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