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# Functional Characterization of Two PLP-Dependent Enzymes Involved in Capsular Polysaccharide Biosynthesis from *Campylobacter jejuni*

Alexander S. Riegert, Tamari Narindoshvili, Adriana Coricello, Nigel G. J. Richards, and Frank M. Raushel\*

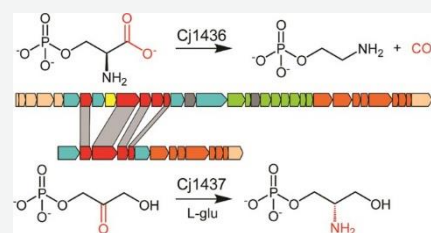
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**ABSTRACT:** *Campylobacter jejuni* is a Gram-negative, pathogenic bacterium that causes campylobacteriosis, a form of gastroenteritis. *C. jejuni* is the most frequent cause of food-borne illness in the world, surpassing *Salmonella* and *E. coli*. Coating the surface of *C. jejuni* is a layer of sugar molecules known as the capsular polysaccharide that, in *C. jejuni* NCTC 11168, is composed of a repeating unit of D-glycero-L-gluco-heptose, D-glucuronic acid, D-*N*-acetyl-galactosamine, and D-ribose. The D-glucuronic acid moiety is further amidated with either serinol or ethanolamine. It is unknown how these modifications are synthesized and attached to the polysaccharide. Here, we report the catalytic activities of two previously uncharacterized, pyridoxal phosphate (PLP)-dependent enzymes, Cj1436 and Cj1437, from *C. jejuni* NCTC 11168. Using a combination of mass spectrometry and nuclear magnetic resonance, we determined that Cj1436 catalyzes the decarboxylation of L-serine phosphate to ethanolamine phosphate. Cj1437 was shown to catalyze the transamination of dihydroxyacetone phosphate to (*S*)-serinol phosphate in the presence of L-glutamate. The probable routes to the ultimate formation of the glucuronamide substructures in the capsular polysaccharides of *C. jejuni* are discussed.



## INTRODUCTION

*Campylobacter jejuni* is a Gram-negative, zoonotic organism that causes campylobacteriosis, the leading food-borne illness in the world.<sup>1</sup> In the United States, campylobacteriosis accounts for 1.3 million cases of gastroenteritis each year.<sup>2</sup> *C. jejuni* is a commensal organism in chickens that can easily spread to other farm animals and pets through unsanitary drinking water, meat processing facilities, unpasteurized milk, and pet feces.<sup>3</sup> Symptoms of campylobacteriosis include nausea, cramps, fever, and bloody diarrhea.<sup>4</sup> Additionally, *Campylobacter* infections recede ~40% of all Guillain-Barré syndrome diagnoses.<sup>5,6</sup> It is estimated that *Campylobacter* causes over 25 000 deaths annually in children under five years of age, primarily affecting Asia and Africa.<sup>7</sup> There is no known FDA-approved vaccine for *Campylobacter* infection, making it a significant public health concern. The best vaccine candidates to date have been glycoconjugate vaccines, which are based on the surface-exposed sugars of *C. jejuni*.<sup>7</sup>

Coating the exterior surface of *C. jejuni* is a polymeric layer of carbohydrates known as the capsular polysaccharide (CPS). This polysaccharide is quite diverse among different strains of *C. jejuni* and is used to serotype ~60 different variants of *C. jejuni*.<sup>8</sup> The CPS is known to aid in evasion of the host immune system and adherence to epithelial tissue.<sup>9</sup> The CPS of *C. jejuni* NCTC 11168 (HS:2) is composed of a repeating unit of D-glycero-L-gluco-heptose, D-glucuronic acid, D-*N*-acetyl-galactosamine, and D-ribose.<sup>10,11</sup> The capsule is further modified by methylation,

phosphoramidation, and amidation. The glucuronic acid moiety of the CPS from *C. jejuni* serotype HS:2 is found amidated with either ethanolamine or serinol. In the HS:19 serotype, the glucuronate is amidated only with serinol.<sup>12</sup> The biosynthetic pathways for the formation of these two modifications to the glucuronate moiety are currently unknown. The structures of the capsules found in serotypes HS:2 and HS:19 are provided in Figure 1.

The DNA cluster responsible for the formation of the CPS in *C. jejuni* NCTC 11168 is composed of 35 genes, as illustrated in Figure S1.<sup>13</sup> Previous investigations have functionally characterized the genes necessary for the biosynthesis of the D-glycero-L-gluco-heptose moiety and modification (Cj1152, Cj1423, Cj1424, Cj1425, Cj1427, Cj1428, and Cj1430).<sup>14–19</sup> In addition, the enzymes Cj1415, Cj1416, Cj1417, and Cj1418 have been shown to be required for the biosynthesis of the phosphoramidate modification, and gene knockout experiments have identified the enzymes (Cj1422 and Cj1421) required for the transfer of the phosphoramidate modifications to specific

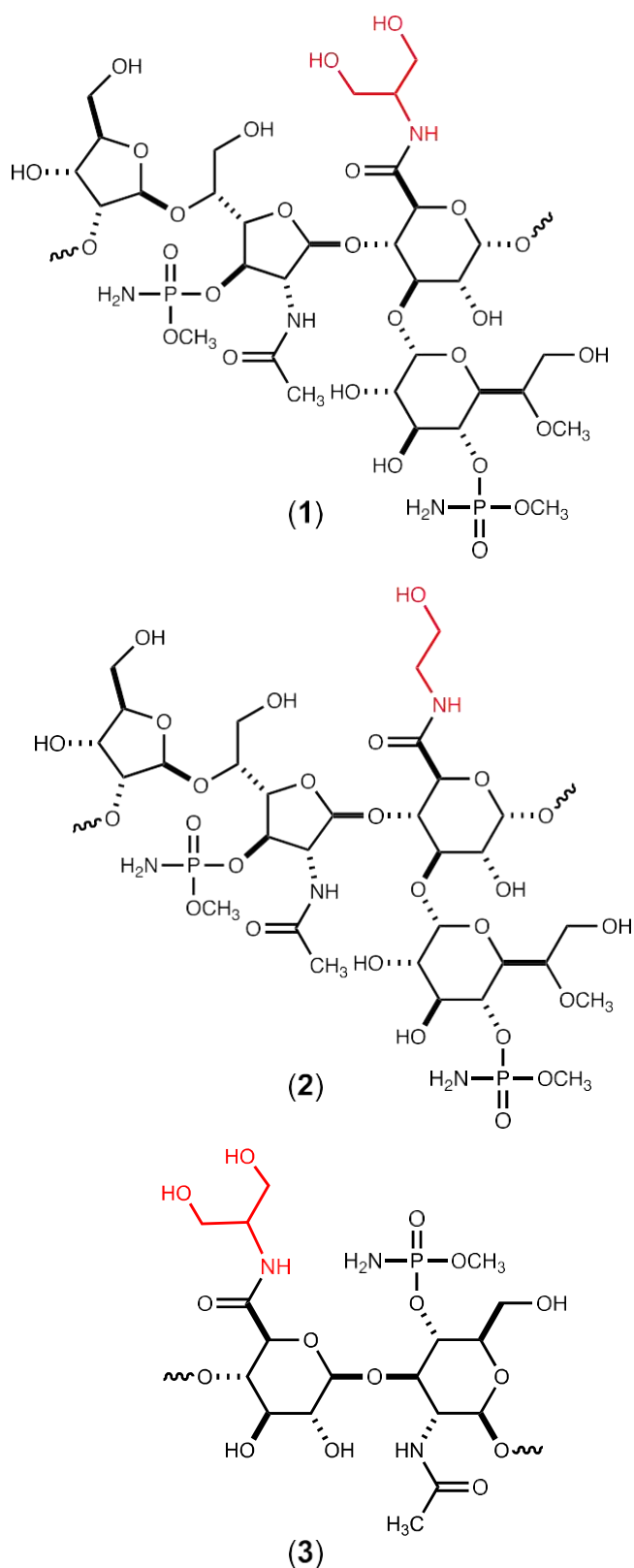


Figure 1. Structures of the capsular polysaccharides found in *C. jejuni* NCTC 11168 (HS:2) (1 and 2) and *C. jejuni* strain RM1285 (HS:19) (3). The amide moiety in each structure is highlighted in red.

sugar receptors.<sup>8,20–25</sup> More recently, we have shown that Cj1441 is required for the conversion of uridine diphosphate (UDP)-glucose to UDP-glucuronate and that this enzyme is

unable to catalyze the formation of an amide bond via the attack of an amine substrate with the putative thioester intermediate formed during the oxidation of UDP-glucose.<sup>26</sup> The enzymes required for the biosynthesis of the two amines (serinol and ethanolamine) found in the HS:2 capsule are currently unknown.

The three most likely enzymes required for the biosynthesis of serinol and ethanolamine in the HS:2 capsule of *C. jejuni* are Cj1435, Cj1436, and Cj1437. Cj1435 is predicted to be a HAD phosphatase, while Cj1436 and Cj1437 are anticipated to be pyridoxal phosphate (PLP)-dependent enzymes. Close homologs to Cj1435 and Cj1437 are found in the gene cluster for the HS:19 serotype, but none to Cj1436. A sequence similarity network (SSN) for the closest 1000 protein sequences to Cj1436 is presented in Figure 2a, at a threshold of 39% sequence identity.<sup>27,28</sup> The nearest functionally characterized enzyme is *L*-serine phosphate decarboxylase (SMUL\_1544), with an overall sequence identity of 46%. This enzyme has been shown to catalyze the formation of ethanolamine phosphate during the biosynthesis of norcobamides.<sup>29,30</sup> The SSN with Cj1437 for the nearest 1000 sequences is presented in Figure 2b, at a sequence identity of 41%. The nearest functionally characterized enzyme to Cj1437 is histidinol phosphate aminotransferase, which catalyzes the transamination of imidazole acetol phosphate, in the presence of *L*-glutamate, during the biosynthesis of *L*-histidine. Here, we have purified Cj1436 and Cj1437 and have functionally characterized these two enzymes. Cj1436 is found to catalyze the decarboxylation of *L*-serine phosphate to generate ethanolamine phosphate and CO<sub>2</sub>, while Cj1437 catalyzes the transamination of dihydroxyacetone phosphate to (*S*)-serinol phosphate in the presence of *L*-glutamate.

## MATERIALS AND METHODS

**Cloning, Expression, and Purification of Cj1436 and Cj1437.** The genes for Cj1436 (UniProt id: Q0P8H8) and Cj1437 (UniProt id: Q0P8H7) from serotype HS:2 were cloned from the genomic DNA of *C. jejuni* NCTC 11168 obtained from ATCC. The genomic DNA served as the starting template for PCR using Phusion DNA polymerase (New England Biolabs). Primers were designed that incorporated *Nde*I and *Xho*I restriction sites, and the resulting fragments were digested at 37 °C and ligated into a pET31b expression vector, which carried a C-terminal hexahistidine tag. The gene encoding the homolog to Cj1437 (HS19.10) from the HS:19 serotype in *C. jejuni* strain RM1285 (Uniprot id: Q5M6M3) was codon-optimized for expression in *E. coli* and ordered from Twist Biosciences in a pET28a expression vector with an N-terminal hexahistidine tag.

The pET31b vector was used to transform BL21 *E. coli* cells (Novagen) via electroporation. Cells harboring the pET31b-Cj1436, pET31b-Cj1437, and pET28a-HS19.10 plasmids were cultured in lysogeny broth with 100 mg/L ampicillin for the pET31b constructs and 50 mg/L kanamycin for the pET28a construct. The cells were grown at 37 °C with shaking and induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density reached 0.8 at 600 nm. The cells were allowed to express protein at 21 °C for 18 h after induction and then harvested by centrifugation at 15000rcf at 4 °C. The cell pellet was resuspended in the loading buffer (50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES)/K<sup>+</sup>, 300 mM KCl, 20 mM imidazole, pH 8.0) and lysed with sonication. The sonicated cells were centrifuged at 25000rcf at 4 °C before the lysate was passed through a 0.45 μm



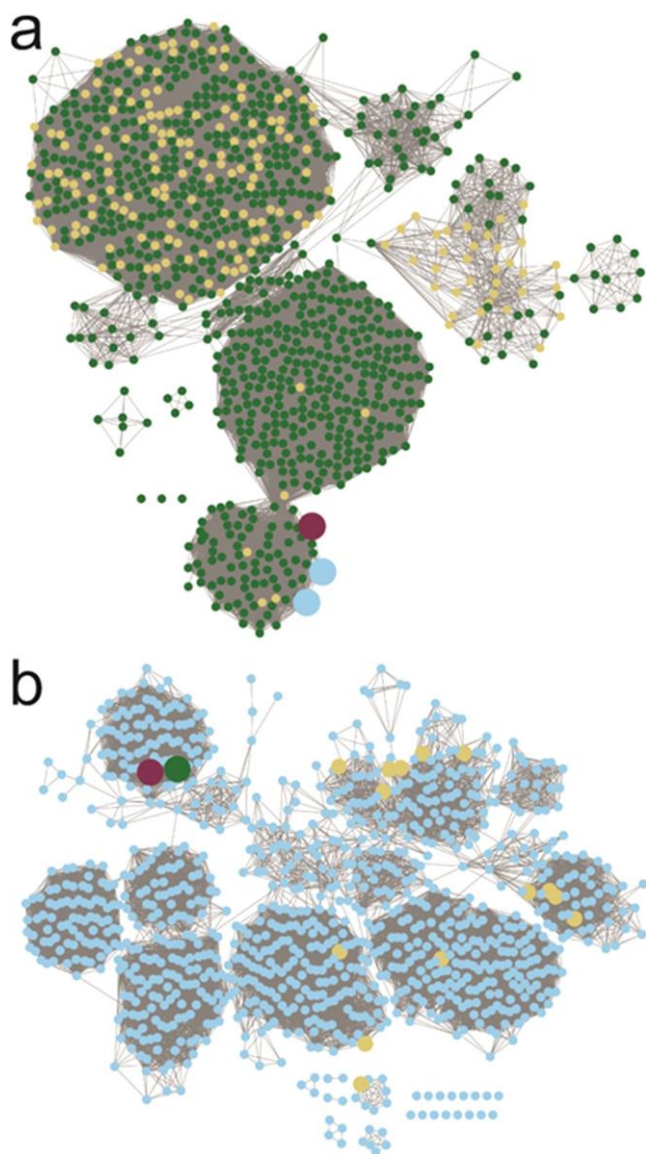


Figure 2. Sequence similarity networks for Cj1436 and Cj1437 using the 1000 nearest protein sequences. (a) The SSN for Cj1436 clustered at a sequence identity of 39%. Maroon node is Cj1436. Blue nodes are enzymes sharing 99% sequence identity to the functionally characterized *L*-serine phosphate decarboxylase (SMUL\_1544 from *Sulfur-ospirillum multivorans*). Yellow nodes are annotated as *L*-threonine phosphate decarboxylases. Green nodes are described as amino-transferases. (b) SSN for Cj1437 clustered at a sequence identity of 41%. The maroon node is Cj1437, while the green node is HS19.10. Yellow nodes are proteins with a Swiss-Prot designation as histidinol phosphate aminotransferases. Blue nodes do not have a Swiss-Prot annotation but are generically described as aminotransferases.

filter (Whatman). The sample was loaded onto a prepacked 5 mL HisTrap (GE Healthcare) nickel affinity column. The protein was eluted from the column using 50 mM HEPES/K<sup>+</sup>, pH 8.0, 300 mM KCl, and 250 mM imidazole over a gradient of 25 column volumes. The protein was pooled and dialyzed against 10 mM HEPES/K<sup>+</sup>, pH 8.0 and 200 mM KCl. Cj1436 was concentrated to 10 mg/mL and flash-frozen using liquid nitrogen before being stored at -80 °C. Approximately 5 mg of protein was obtained per liter of cell culture. Cj1437 (~3 mg of protein per liter of cell culture) was concentrated to 3.5 mg/mL and flash-frozen before being stored at -80 °C. HS19.10 (~10

mg of protein per liter of cell culture) was concentrated to 6 mg/mL and flash-frozen before being stored at -80 °C.

**Bioinformatic Analysis of Cj1436 and Cj1437.** The amino acid sequences for Cj1436 and Cj1437 were obtained from the Uniprot database. Individually, the protein sequences for the two genes were input as the search queries for the BLAST section of the EFI-EST webtool to generate the sequence similarity networks (SSNs).<sup>27,28</sup> The parameters were set such that the BLAST search would retrieve the 1000 closest sequences to the search query. Sequence identities for Cj1436 and Cj1437 were set at 39 and 41%, respectively. The networks were created using the yFiles organic layout, and all images were created using Cytoscape version 3.7.2.

**Determination of Kinetic Constants for Cj1437 and HS19.10.** The kinetic constants for Cj1437 and HS19.10 were determined using an *L*-glutamate dehydrogenase coupled assay to monitor the formation of  $\alpha$ -ketoglutarate. The reaction mixtures contained 150 nM enzyme, 250  $\mu$ M PLP, 250  $\mu$ M reduced nicotinamide adenine dinucleotide phosphate (NADPH), 15 mM ammonium chloride, 2.0 mM *L*-glutamate, 5.0 units of *L*-glutamate dehydrogenase, varying levels of dihydroxyacetone phosphate (DHAP) (0.005–2.0 mM), and 50 mM HEPES/K<sup>+</sup> buffer at pH 8.0. The reaction was monitored spectrophotometrically at 340 nm and 25 °C with a SpectraMax340 UV-visible spectrophotometer. The kinetic parameters were determined by fitting the initial rates to eq 1 using GraFit5, where  $v$  is the initial velocity of the reaction,  $E_t$  is the enzyme concentration,  $k_{cat}$  is the turnover number,  $A$  is the substrate concentration, and  $K_m$  is the Michaelis constant.

$$v/E_t = k_{cat}(A) / (K_m + A) \quad (1)$$

**Determination of Reaction Products for Cj1436.** The reaction catalyzed by Cj1436 was determined by following the decarboxylation of *L*-serine phosphate via <sup>1</sup>H and <sup>31</sup>P NMR spectroscopy. The reaction was conducted in 50 mM phosphate buffer, pH 8.0, in D<sub>2</sub>O and initiated by the addition of 1.0  $\mu$ M Cj1436 with a fixed level of PLP (250  $\mu$ M) and a fixed concentration of *L*-serine phosphate (5.0 mM). The catalytic activity of Cj1436 was also tested with *L*-serine, *L*-threonine phosphate, *L*-aspartate, and *L*-glutamate at a fixed concentration of 5.0 mM. The reaction was also monitored using <sup>31</sup>P NMR spectroscopy by monitoring the phosphorus resonance for the product (ethanolamine phosphate) relative to the <sup>31</sup>P resonance for substrate (serine phosphate) as a function of time. The reaction was conducted in 50 mM HEPES/K<sup>+</sup> at pH 8.0 containing 250  $\mu$ M PLP, 5.0 mM *L*-serine and initiated by the addition of 1.0  $\mu$ M enzyme.

**Membrane Inlet Mass Spectrometry (MIMS) for the Determination of the Kinetic Constants for Cj1436.** Steady-state kinetic constants for Cj1436 were determined by following the decarboxylation of *L*-serine phosphate using MIMS.<sup>31–33</sup> In these experiments, assay mixtures contained 0.1, 0.5, 1.0, 5.0, and 10 mM *L*-serine phosphate dissolved in 100 mM HEPES/K<sup>+</sup> buffer, pH 8.0. Reactions were initiated at 25 °C by the addition of an enzyme/PLP mixture in 100 mM HEPES/K<sup>+</sup> buffer, pH 8.0, to give final concentrations of 1.0  $\mu$ M Cj1436 and 250  $\mu$ M PLP (total volume 2.0 mL). The catalytic activity of Cj1436 was also tested with *L*-serine at a fixed concentration of 5.0 mM.

**Reaction Product Confirmation Using Mass Spectrometry.** Cj1436 (1.0  $\mu$ M) was incubated with 5.0 mM *L*-serine phosphate, 250  $\mu$ M PLP, and 50 mM ammonium bicarbonate at pH 8.0. The reaction was monitored using <sup>31</sup>P NMR

spectroscopy until the reaction was complete. The solution was filtered through a GE Healthcare Vivaspin 500 10 kDa molecular weight cutoff (MWCO) filter, and 100  $\mu$ L of the flow-through was submitted for mass spectrometry analysis using a Thermo Scientific Q Exactive Focus mass spectrometer. Cj1437 (1.0  $\mu$ M) was incubated with 5.0 mM DHAP, 15 mM  $L$ -glutamate, and 250  $\mu$ M PLP in 50 mM ammonium bicarbonate buffer at pH 8.0 until the reaction reached equilibrium as determined by  $^{31}$ P NMR spectroscopy. The solution was filtered through a GE Healthcare Vivaspin 500 10 kDa MWCO filter, and 100  $\mu$ L of the flow-through was analyzed using a Thermo Scientific Q Exactive Focus mass spectrometer.

**Chemical Synthesis of (*R/S*)-Serinol Phosphate and (*S*)-Serinol Phosphate.** The synthesis of racemic and (*S*)-serinol phosphate is described in the [Supporting Information](#).

**Stereochemical Analysis of Serinol Phosphate Produced by Cj1437 and HS19.10.** The stereochemistry of the chiral center at C2 of the serinol phosphate produced by Cj1437 and HS19.10 was determined by monitoring the exchange of the hydrogen at C2 of (*R/S*)-serinol phosphate and (*S*)-serinol phosphate with solvent deuterium. Cj1437 or HS19.10, at a concentration of 3.0  $\mu$ M, was allowed to incubate with 3.0 mM (*R/S*)-serinol in 50 mM phosphate buffer ( $D_2O$ ) at pH 8.0. The exchange of the hydrogen at C2 of serinol phosphate with deuterium was monitored using  $^1H$  NMR spectroscopy (Avance III 400 MHz). A similar experiment was conducted with the (*S*)-serinol phosphate enantiomer. The exchange reactions were also conducted in the presence of 250  $\mu$ M PLP and 250  $\mu$ M  $\alpha$ -ketoglutarate using the HS19.10 enzyme.

## RESULTS

**Determination of the Reaction Catalyzed by Cj1436.** The closest functionally characterized enzyme to Cj1436 is SMUL\_1544 from *S. multivorans*, and this enzyme has been shown to catalyze the decarboxylation of  $L$ -serine phosphate to ethanolamine phosphate and  $CO_2$ .<sup>29,30</sup>  $^1H$  NMR spectroscopy was used to monitor the reaction catalyzed by Cj1436 in  $D_2O$  with  $L$ -serine phosphate, and the results are presented in [Figure 3](#), showing that the product is ethanolamine phosphate. The two

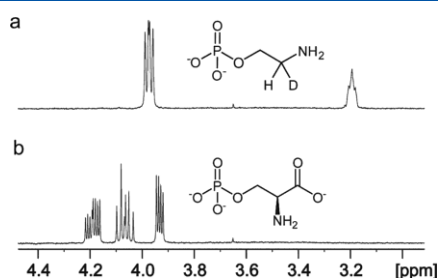


Figure 3.  $^1H$  NMR spectrum of the reaction catalyzed by Cj1436 in  $D_2O$ . (a) The product of the reaction catalyzed by Cj1436 in the presence of  $L$ -serine phosphate is ethanolamine phosphate. (b) The substrate of the reaction,  $L$ -serine phosphate, prior to the addition of enzyme. Additional details are provided in the text.

hydrogens from C2 resonate at 3.96 ppm and appear as a doublet of doublets due to coupling with the adjacent phosphate and the single proton from C1 with coupling constants of 1.89 and 12.14 Hz, respectively ([Figure 3a](#)). The single proton from C1 appears as a broadened triplet at 3.19 ppm. The  $^1H$  NMR spectrum of the substrate prior to the addition of the enzyme is provided in [Figure 3b](#). In the  $^{31}P$  NMR spectrum, the substrate

exhibits a single resonance at 3.89 ppm ([Figure 4b](#)), whereas the product resonates at 3.82 ppm ([Figure 4a](#)). The formation of

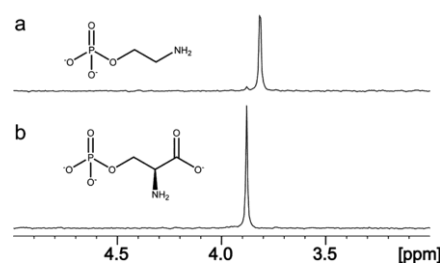


Figure 4.  $^{31}P$  NMR spectrum of the reaction catalyzed by Cj1436. (a) Ethanolamine phosphate resonates at 3.82 ppm. A small amount of the remaining substrate appears at 3.89 ppm. (b) Control reaction in the absence of enzyme showing the substrate,  $L$ -serine phosphate, at 3.89 ppm. Additional details are provided in the text.

ethanolamine phosphate was confirmed using electrospray Ionization (ESI) negative mode mass spectrometry. The substrate of the reaction,  $L$ -serine phosphate, is present at an  $m/z$  of 184.0 for the  $M-H$  ion ([Figure 5a](#)). The product of the reaction, ethanolamine phosphate, is found at an  $m/z$  of 140.01 when the reaction is conducted in  $H_2O$  ([Figure 5b](#)) and at 141.0 when the reaction is conducted in  $D_2O$  ([Figure 5c](#)).

**Determination of the Reaction Catalyzed by Cj1437.** The sequence similarity network for Cj1437 indicates that this enzyme will most likely catalyze a transamination reaction since the closest functionally characterized enzyme is histidinol phosphate aminotransferase. Since the pathway for the biosynthesis of the CPS in serotype HS:2 must eventually make serinol, the most probable reaction catalyzed by Cj1437 is the transamination of DHAP, with an amino donor such as  $L$ -glutamate to make serinol phosphate. When tested with dihydroxyacetone (DHA) as a potential substrate, the rate was less than 3% of the rate with DHAP. The reaction catalyzed by Cj1437 was initially characterized using  $^1H$  NMR spectroscopy using 3.0 mM DHAP and 15 mM  $L$ -glutamate. A portion of the  $^1H$  NMR spectrum of the mixture of DHAP and  $L$ -glutamate in  $D_2O$  is presented in [Figure 6a](#). After the addition of Cj1437, deuterated serinol phosphate is formed as indicated by the pair of doublet of doublets for the two protons at C1 centered at 3.95 ppm and the doublet of doublets for the two protons at C3 centered at 3.80 ppm ([Figure 6b](#)). The NMR spectrum of the chemically synthesized racemic serinol phosphate is shown for comparison in [Figure 6c](#), where the hydrogen at C2 resonates at 3.51 ppm, thus confirming the formation of serinol phosphate.

The product of the reaction catalyzed by Cj1437 has a chiral center at C2. To determine the stereochemistry at C2, the enzyme was incubated with racemic serinol phosphate (3.0 mM) and  $\alpha$ -ketoglutarate (250  $\mu$ M), and the exchange of the hydrogen at C2 with solvent deuterium was monitored by  $^1H$  NMR spectroscopy. Prior to the addition of the enzyme (3.0  $\mu$ M), the integrated ratio of the resonances for the hydrogens at C1 and C3 relative to the hydrogen at C2 was 4.0:1.04. After the addition of the enzyme, the ratio gradually changed to 4.0:0.55 ([Figure 6d](#)), where the mixture of the serinol phosphate with hydrogen and deuterium at C2 is now clearly visible. When the same reaction was conducted using chemically synthesized (*S*)-serinol phosphate ([Figure 6e](#)), the hydrogen at C2 completed exchange with solvent deuterium after 2 h ([Figure 6f](#)), thus demonstrating that Cj1437 catalyzes the synthesis of (*S*)-serinol phosphate from DHAP and  $L$ -glutamate.

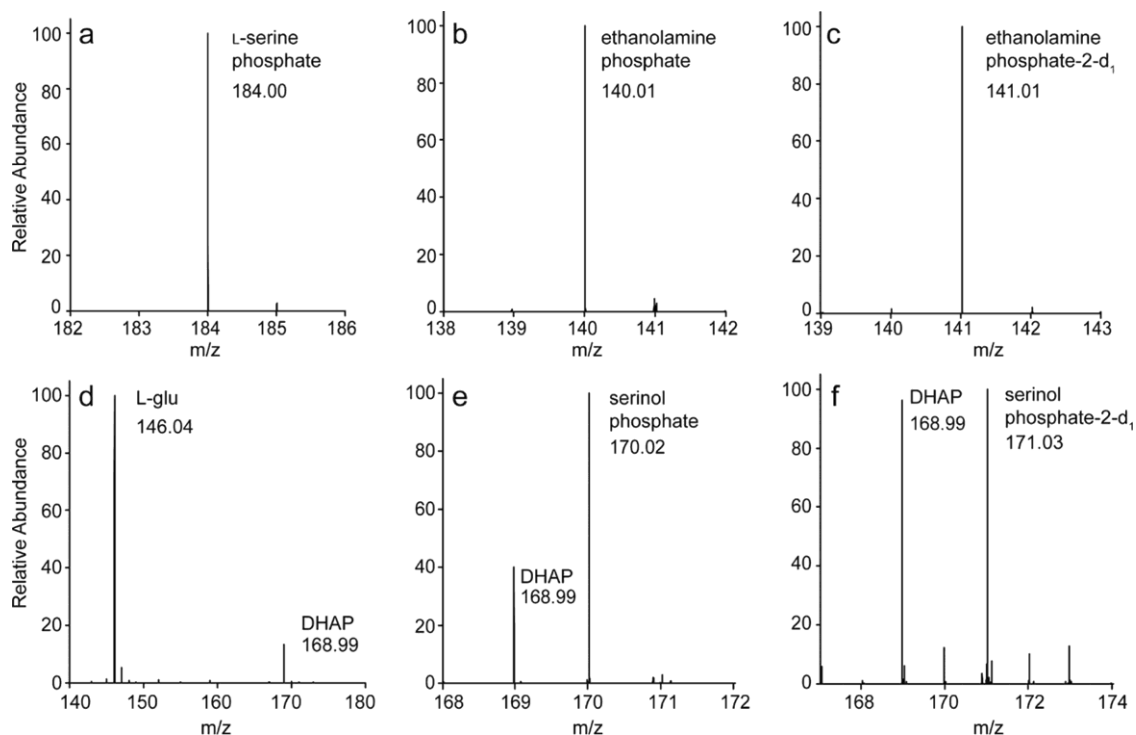


Figure 5. Mass spectrometry data for Cj1436 and Cj1437. (a) L-Serine phosphate, the substrate for Cj1436, appears in a no-enzyme control at an  $m/z$  of 184.00 for the M-H ion. (b) Product of the reaction catalyzed by Cj1436. Ethanolamine phosphate appears at an  $m/z$  of 140.01 for the M-H ion. (c) Product of the reaction catalyzed by Cj1436 in the presence of  $D_2O$ . The deuterated product, (2- $d_1$ )-ethanolamine phosphate, appears at an  $m/z$  of 141.01 for the M-H ion. (d) L-glutamate and DHAP, substrates for the reaction catalyzed by Cj1437, appear in a no-enzyme control at  $m/z$  of 146.04 and 168.99, respectively, for the M-H ions. (e) Product of the reaction catalyzed by Cj1437. The product serinol phosphate appears at an  $m/z$  equal to 170.02, while the leftover DHAP substrate appears at 168.99 for the M-H ions. (f) The product of the reaction catalyzed by Cj1437 in the presence of  $D_2O$ . (2- $d_1$ )-Serinol phosphate appears at an  $m/z$  of 171.03, while the leftover DHAP substrate appears at 168.99 for the M-H ions.

The reaction products formed after the incubation of Cj1437 with DHAP and L-glutamate were confirmed to be serinol phosphate and  $\alpha$ -ketoglutarate by ESI mass spectrometry (negative mode). In the control experiment, in the absence of enzyme, the substrates DHAP and L-glutamate are identified at  $m/z$  of 146.05 and 168.99, respectively, for the M-H ions (Figure 5d). The product, serinol phosphate, is found at 170.02 (Figure 5e). Furthermore, when the reaction is conducted in  $D_2O$ , the serinol phosphate product incorporates a single deuterium, as demonstrated by the appearance of the signal at an  $m/z$  of 171.03 (Figure 5f).

**Kinetic Analysis of the Reaction Catalyzed by Cj1436.** Steady-state kinetic parameters for Cj1436 were obtained by monitoring the rate of decarboxylation at various concentrations of L-serine phosphate using membrane inlet mass spectrometry. These experiments provided values for  $k_{cat}$ ,  $k_{cat}/K_m$ , and  $K_m$  of  $0.21 \pm 0.02 \text{ s}^{-1}$ ,  $350 \pm 120 \text{ M}^{-1} \text{ s}^{-1}$ , and  $0.6 \pm 0.2 \text{ mM}$ , respectively. The value of  $k_{cat}/K_m$  for Cj1436 is 3–4 fold lower than that determined for Cj1437 (see below). No activity ( $<0.01 \text{ s}^{-1}$ ) was observed when L-serine replaced L-serine phosphate in the assay.

**Kinetic Analysis of the Reaction Catalyzed by Cj1437.** The reaction catalyzed by Cj1437 and HS19.10 was followed using a glutamate dehydrogenase coupled assay to measure the rate of formation of  $\alpha$ -ketoglutarate. Using DHAP and L-glutamate as substrates, the values of  $k_{cat}$ ,  $k_{cat}/K_m$ , and  $K_m$  with Cj1437 were determined to be  $0.50 \pm 0.02 \text{ s}^{-1}$ ,  $1.2 (\pm 0.2) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , and  $42 \pm 6 \text{ }\mu\text{M}$ , respectively. Using the homolog to Cj1437 from the HS:19 serotype (HS19.10), the values of  $k_{cat}$ ,  $k_{cat}/K_m$ , and  $K_m$  were determined to be  $1.10 \pm 0.01 \text{ s}^{-1}$ , of  $2.7 (\pm$

$0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , and  $41 \pm 2 \text{ }\mu\text{M}$ , respectively. The turnover using L-aspartate (15 mM) was less than 50% of the rate exhibited by L-glutamate (15 mM) at a fixed concentration of DHAP (3.0 mM) as monitored by  $^1\text{H}$  NMR spectroscopy. Essentially, no activity could be detected using L-alanine.

## DISCUSSION

The HS:2 serotype of *C. jejuni* is surrounded by a capsular polysaccharide consisting of a repeating unit of D-glycero-L-gluco-heptose, D-glucuronic acid, D-N-acetyl-galactosamine, and D-ribose.<sup>10,11</sup> The glucuronic acid is amidated with either serinol or ethanolamine. This same modification can be found within the CPS of the *C. jejuni* serotype HS:19, except that the amide bond is formed only with serinol. The exact role of the amide bond-containing molecule within the CPS is unknown. Other modifications to the CPS are critical for the evasion of the immune system and adherence to epithelial tissue.<sup>9,34</sup> Additionally, the phase variable phosphoramidate modification is a target for recognition by bacteriophage F336.<sup>35</sup> Phase variation may play a role in the biosynthesis of glucuronamide. The gene, which encodes for Cj1437 appears to have a poly-G tract of 9 nucleotides (877–885). Poly-G tracts are common to *C. jejuni* and are known to introduce phase variation, which creates phenotypic heterogeneity in populations of *C. jejuni*. This allows *C. jejuni* to avoid the immune system as well as phage attack.<sup>37</sup>

To form the amide bond found in the CPS of *C. jejuni* HS:2, the bacterium must synthesize two primary amines, ethanolamine and serinol. Here, we have shown that the first step in the biosynthesis of the ethanolamine product is the decarboxylation of L-serine phosphate to ethanolamine phosphate catalyzed by



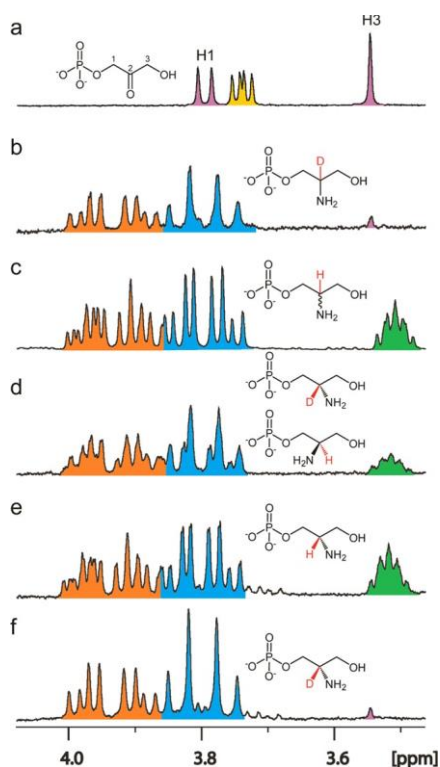


Figure 6.  $^1\text{H}$  NMR spectra of reactions catalyzed by Cj1437. (a) Control spectrum of a mixture of DHAP (pink) and *L*-glutamate (yellow). The resonance for the H1 hydrogen of DHAP is centered at 3.79 ppm, while the H3 hydrogen is centered at 3.55 ppm. The C $\alpha$  of *L*-glutamate appears as a doublet of doublets centered at 3.74. (b) Product of the reaction catalyzed by Cj1437. The H1 hydrogens (orange) are centered at 3.94 ppm. The C3 hydrogens (blue) are centered at 3.80 ppm. A small amount of DHAP is leftover, as shown by the pink singlet at 3.55 ppm. (c) Chemically synthesized (*R/S*)-serinol phosphate. The H1 protons are centered at 3.93 ppm, and the H2 hydrogen (green) is centered at 3.51 ppm. (d) Incubation of Cj1437 with (*R/S*)-serinol phosphate with 50  $\mu\text{M}$   $\alpha\text{KG}$  in  $\text{D}_2\text{O}$  for 2 h. (e) Chemically synthesized (*S*)-serinol phosphate. (f) Incubation of Cj1437 with (*S*)-serinol phosphate and 250  $\mu\text{M}$   $\alpha\text{-KG}$  for 2 h. Additional details are provided in the text.

Cj1436. This result is consistent with the prior identification of an *L*-serine phosphate decarboxylase (SMUL\_1544), which was shown previously to catalyze the decarboxylation of *L*-serine phosphate.<sup>29</sup> SMUL\_1544 is the only other functionally characterized *L*-serine phosphate decarboxylase reported to date and is used in the biosynthesis of norpseudovitamin B<sub>12</sub> in *S. multivorans*.<sup>29,30</sup> *L*-Threonine phosphate is also a weaker substrate, but no activity could be detected with *L*-serine, *L*-threonine, *L*-aspartate, or *L*-glutamate. The phosphate group of the substrate is thus necessary for substrate recognition and is required for catalytic activity. The homologous three-dimensional structures of SMUL\_1544 and CobD of *S. enterica* have identified the residues used to bind the phosphate group of the substrate.<sup>29,38</sup> In SMUL\_1544, these residues are Arg-356, Arg-368, and Ser-25. A sequence alignment of Cj1436 with SMUL\_1544 reveals the conservation of those residues required to bind the phosphate group of the substrate (Figure S2).

Cj1437 participates in the first step in serinol formation, where it catalyzes the transamination of DHAP to serinol phosphate using *L*-glutamate as the amine donor. The stereochemistry of the reaction was shown to produce the (*S*)-enantiomer of serinol phosphate using solvent deuterium

exchange with chemically synthesized (*S*)-serinol phosphate. This stereochemical outcome is consistent with the known product formation of *L*-histidinol phosphate in the reaction catalyzed by histidinol phosphate transaminase.<sup>39</sup> Serinol phosphate has been shown to be an important precursor for serinol production.<sup>40</sup> Serinol is an intermediate in the formation of other chemicals, including the anti-cancer drugs *N*-palmitoyl-2-amino-1,3-propanediol, and the first orally administered treatment for multiple sclerosis, Gilenya (Novartis).<sup>41–43</sup>

The precise sequence of the enzymatic transformations required for the biosynthesis of the glucuronamide moiety in the capsular polysaccharide of *C. jejuni* HS:2 has not yet been elucidated. A previous bioinformatic investigation identified a probable candidate enzyme, Cj1438, which contains an N-terminal glycosyltransferase domain as well as a C-terminal ATP-grasp domain that could be used for amide bond formation. However, at this point, it is unclear whether the ATP-grasp domain would utilize serinol phosphate or serinol as the amine substrate or whether the acceptor carboxylate would be contributed by UDP-glucuronate or the growing polysaccharide chain. In either case, the putative phosphatase (Cj1435) will ultimately have to be used to remove the phosphoryl group from either serinol phosphate or the phosphorylated glucuronamide moiety. Efforts to unravel these possible scenarios are in progress.

Glucuronamides are found within the lipopolysaccharide O-antigen and CPS of many bacteria, including *Acinetobacter baumannii* G7, *E. coli* O143, *E. coli* L-19, *Vibrio cholerae* H11 (non-O1), *Shigella boydii* type 8, *Pseudomonas aeruginosa*, *Proteus mirabilis* O27, *Vibrio vulnificus* strain 6353, *Bacillus halodurans*, *Providencia stuartii* O25, and *Proteus penneri* strain 14.<sup>44–36</sup> In *A. baumannii* G7, *E. coli* O143, *E. coli* L-19, *S. boydii* type 8, and *B. halodurans*, there is an ATP-grasp enzyme that is located in the O-antigen or CPS biosynthetic gene cluster. In addition to the organisms listed, *C. jejuni* HS:19 contains a glucuronamide as well as an ATP-grasp-containing enzyme. An analysis of other *C. jejuni* genomes revealed that *C. jejuni* HS:22 also contains the genes necessary for the biosynthesis of a CPS modified with a glucuronamide. A comparison of the capsular polysaccharide biosynthetic gene clusters for HS:2, HS:19, and HS:22 is presented in Figure S1.

## CONCLUSIONS

Here, we report the functional characterization of two enzymes necessary for the formation of the *C. jejuni* HS:2 capsular polysaccharide, Cj1436 and Cj1437. Cj1436 is a PLP-dependent decarboxylase, which catalyzes the decarboxylation of *L*-serine phosphate to ethanolamine phosphate. The products were confirmed by mass spectrometry and  $^1\text{H}$  NMR spectroscopy. The rate and catalytic constants were measured using NMR spectroscopy and membrane inlet mass spectrometry. Cj1437 is a PLP-dependent transaminase, which catalyzes the transamination of DHAP using *L*-glutamate to form (*S*)-serinol phosphate. The product of the reaction was characterized using mass spectrometry and  $^1\text{H}$  NMR spectroscopy. The rate and catalytic constants were determined spectrophotometrically, using a glutamate dehydrogenase coupled assay.

## ASSOCIATED CONTENT

★ Supporting Information

Gene cluster alignment; pair-wise sequence alignment; synthesis of racemic serinol phosphate; and reaction scheme for the synthesis of (S)-serinol phosphate (PDF)

#### Accession Codes

Cj1436 (UniProt id: Q0P8H8) Cj1437 (UniProt id: Q0P8H7) HS19.10 (Uniprot id: Q5M6M3)

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The authors declare no competing financial interest.

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