Published in final edited form as:

J Mol Biol. 2008 December 12; 384(2): 364–376. doi:10.1016/j.jmb.2008.09.036.

Structure of a σ^{28} -regulated Non-flagella Virulence Protein from *Campylobacter jejuni*

Takeshi Yokoyama¹, Seonghee Paek¹, Cheryl P. Ewing², Patricia Guerry², and Hye-Jeong Yeo^{1,*}

¹ Department of Biology and Biochemistry, University of Houston, Houston, TX 77204

² Enteric Disease Department, Naval Medical Research Center, Silver Spring, MD 20910

Abstract

Campylobacter jejuni, a Gram-negative motile bacterium, is a leading cause of human gastrointestinal infections. Although the mechanism of *C. jejuni* mediated enteritis appears to be multi-factorial, flagella play complex roles in virulence of this human pathogen. Cj0977 is a recently identified virulence factor in *C. jejuni*, and is expressed by a σ^{28} promoter which controls late genes in the flagellar regulon. A Cj0977 mutant strain was fully motile, but significantly reduced in invasion of intestinal epithelial cells *in vitro*. Here we report the crystal structure of the major structural domain of Cj0977, which reveals a homodimeric “hot dog” fold architecture. Of note, the characteristic hot dog fold has been found in various coenzyme A (CoA) compound binding proteins with numerous oligomeric states. Structural comparison with other known hot dog fold proteins locates a putative binding site for an acyl-CoA compound in the Cj0977 protein. Structure-based site-directed mutagenesis followed by invasion assays indicates that key residues in the putative binding site are indeed essential for the Cj0977 virulence function, suggesting a possible function of Cj0977 as an acyl-CoA binding regulatory protein.

Keywords

crystal structure; hot dog fold; homodimer; virulence; *Campylobacter jejuni*

Introduction

Campylobacter jejuni is an important food-borne pathogen worldwide. *C. jejuni*, a member of the epsilon group of proteobacteria, is a Gram-negative, microaerophilic, spiral bacterium, common properties of the related gastric pathogen *Helicobacter pylori*. Remarkably little is understood about the details of molecular pathogenesis of *C. jejuni*, but the polar flagella play multiple, complex roles in virulence of this pathogen.¹ An updated sampling of flagella-imparted virulence includes motility, chemotaxis, autoagglutination, colonization, and secretion of virulence proteins.¹ Of note, in the absence of a specialized type III secretion system, the *C. jejuni* flagella filament secretes several non-flagellar proteins to the extracellular

*Corresponding author: Tel: (1) 713 743 8377; Fax: (1) 713 743 8351; Email: hyeo@uh.edu.

Accession Code:

Coordinates of the Cj0977_{p17} structure have been deposited at Protein Data Bank (ID code: 3BNV).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

milieu or host cells, some of which are involved in virulence.^{2–4} Flagella biosynthesis is energetically costly, and is regulated in an ordered manner. The majority of *C. jejuni* genes encoding protein components of the basal body, hook and minor flagellin are regulated by σ^{54} , while the major flagellin and other late genes in the flagella regulon are regulated by σ^{28} . Interestingly, a recent microarray study revealed that several non-flagella genes appeared to be also regulated by σ^{54} or σ^{28} promoters.⁵

In previous work, we have established that two of these σ^{28} -regulated non-flagellar genes are virulence factors. One, Cj0859c or FspA, is secreted through the flagella filament. Some alleles of FspA are capable of inducing apoptosis of intestinal epithelial cells.⁴ A mutant in another of the σ^{28} -regulated genes, Cj0977, in *C. jejuni* strain 81–176 invaded INT407 intestinal epithelial cells *in vitro* at levels that were 3-logs lower than the parent strain.⁶ This mutant was also attenuated in a ferret diarrheal disease model.⁶ However, unlike FspA, the Cj0977 protein of wildtype *C. jejuni* was not secreted and the function of the protein in virulence was not obvious.⁶ Cj0977, which was annotated as a conserved, hypothetical protein,⁷ is highly conserved within *Campylobacter* spp., and somewhat conserved within proteobacteria. Cj0977 encodes an acidic protein with a molecular mass of 21.2 KDa, and its expression is dependent on a minimal flagella structure,⁶ consistent with regulation by σ^{28} .

In an effort to gain insight into the structural basis of *C. jejuni* virulence, we set out to solve the crystal structure of Cj0977. The crystal structure reveals that Cj0977 adopts the ‘hot dog’ fold, which is characterized by 6 β -stranded antiparallel β -sheet wrapping around an α -helix. This fold was first observed in the structure of *E. coli* β -hydroxydecanoyl thiol ester dehydratase (FabA).⁸ Since then, this characteristic fold has been found in numerous other enzymes, notably, thioesterases catalyzing diverse acyl-CoA substrates as well as many putative proteins.^{9–16} Unexpectedly, among the experimentally determined structures, the closest structural homolog of Cj0977 was *Bacillus subtilis* FapR, a non-catalytic protein that is involved in transcriptional regulation of fatty acid biosynthesis.¹⁷ The crystal structure suggests that Cj0977 might bind an acyl-CoA derivative, and this interpretation is supported by structure-based site-directed mutagenesis. Furthermore, our functional analysis indicates that the 36 residue C-terminal domain plays a critical role in the activity of Cj0977. A model is proposed as to how Cj0977 could be converted from an inactive to an active form.

Results

Construct design of Cj0977 for structure determination

Initially, N-terminal His-tagged full-length Cj0977 (His-Cj0977) was used for crystallization. The His-Cj0977 protein is highly soluble (routinely concentrated to ~30 mg/ml), and adopts a dimer structure in solution as determined by size exclusion chromatography (Fig. 1A). Although His-Cj0977 crystallized with ammonium sulfate precipitant, His-Cj0977 crystals were recalcitrant to diffraction. Therefore, we sought to construct Cj0977 variants in order to obtain diffracting crystals. When the His-Cj0977 (M_r 23.3 kDa including the His-tag and accessory residues) protein was subjected to limited proteolysis using trypsin, a stable fragment of 21 kDa appeared during the reaction course (Fig. 1B). In addition, as we carefully monitored the stability of the protein solution at 4°C, a smaller fragment of 17 kDa started to appear 2 to 4 weeks after purification, and remained very stable for many months (Fig. 1C). The two fragments of Cj0977, 21 kDa and 17 kDa, share the same N-terminus (Asp4) as analyzed by N-terminal sequencing. The Cj0977 protein appears to be conserved within the proteobacteria, and the most significant homologs are the hypothetical proteins Ws0699 and Hp0420 of *Wolinella succinogenes* and *Helicobacter pylori*, respectively (Fig. 1D). Based on the N-terminal sequence analysis and the sequence alignment, we constructed three Cj0977 variants, referred to as Cj0977_{p21}, Cj0977_{p19}, and Cj0977_{p17} (Fig. 1D) as GST-fusion proteins. The Cj0977 variants are also dimers in solution according to size-exclusion chromatography, as

shown in Fig. 1A for Cj0977_{p17}. While both selenomethionine-derivatized Cj0977_{p21} and Cj0977_{p17} easily crystallized, only a Cj0977_{p17} crystal yielded X-ray diffraction, allowing phasing using the multiple-wavelength anomalous dispersion (MAD) method.

Cj0977_{p17} crystallized in the space group P2₁ with 4 dimers per asymmetric unit and diffracted to a resolution of 2.6 Å at the selenium peak wavelength (Table I). The expected 16 selenium sites in the asymmetric unit were located using the program autoSHARP. Experimental phases to 2.8 Å followed by solvent flipping yielded an electron density map of excellent quality. The present model contains residues 13 – 154 of chains A, C, E, and F, residues 14 – 155 of chain B, residues 12 – 154 of chain D, residues 25 – 154 of chain G, and residues 24 – 153 of chains H.

Molecular architecture of the Cj0977_{p17} subunit and of the functional dimer

The crystal of this study contained eight subunits or four dimers per asymmetric unit. For clarity, the following discussions are based on Chain D of the x-ray coordinate file unless otherwise indicated. The structure of the Cj0977_{p17} subunit reveals a mixed $\alpha + \beta$ ‘hot dog’ fold, according to the naming of the *Escherichia coli* β -hydroxydecanoyl thiol ester dehydratase (FabA), the first structure observed with this fold.⁸ Each subunit consists of six-stranded curved anti-parallel β sheet $\beta 1/\beta 2/\beta 4/\beta 5/\beta 6/\beta 3$ that wraps around a central α -helix ($\alpha 3$) (Fig. 2A). A short α -helix, nearly perpendicular to the central α -helix, is formed at the N terminus, but this helix is irregular among the eight molecules in the asymmetric unit. In addition, a one-turn helix ($\alpha 2$) and a 3_{10} helix form before $\beta 1$ and after $\beta 2$, respectively. Each monomer is a compact structure with dimensions of $\sim 45 \text{ Å} \times 35 \text{ Å} \times 25 \text{ Å}$. Eight molecules in the asymmetric units are superimposed very well, and thus the structural variability among different protomers is observed on the $\alpha 1$ and the loop between the β strands $\beta 4$ and $\beta 5$ (Fig. 3).

Consistent with the oligomer state of Cj0977_{p17} (and Cj0977) in solution, the crystal structure reveals that individual subunits assemble into dimers in the crystalline lattice. In addition, in considering that the asymmetric unit contains four independent dimers with the same scaffold, the functional quaternary structure of Cj0977 is defined as dimer. The subunit-subunit interface is mainly formed through interactions between the β strand $\beta 3$ and between the central α helix $\alpha 3$ of symmetry-related two subunits (Fig. 2B). Therefore, the two 6-stranded β sheets of each subunit are connected to form a large anti-parallel 12-stranded β sheet in the Cj0977_{p17} dimer. Upon dimer formation, each Cj0977_{p17} monomer buries a surface area of $\sim 1558 \text{ Å}^2$. The conformations of the four dimers in the asymmetric unit are almost identical, reflecting the superposability of 8 protomers (Fig. 3).

Structural comparison with other “hot dog” fold proteins

The ‘hot dog’ fold is characterized by 5 to 7 β -stranded antiparallel β -sheet as the ‘bun’ wrapping around an α -helical sausage. This distinctive fold has been found in various coenzyme A (CoA) derivative binding enzymes as well as many putative proteins. In addition, the hot dog fold appears to form many different quaternary structures. Search for structural homologs using the DALI¹⁸ server returned very high score hits for numerous proteins with this fold (Table II). Among the hot dog fold proteins, the top three structural homologs with known functions are *Bacillus subtilis* FapR, a transcriptional regulator in Gram-positive bacteria, *Thermus thermophilus* phenylacetate thioesterase (TtPaal), and *Arthrobacter* sp. 4-hydroxybenzoyl-CoA thioesterase (ArHBT) (Fig. 4A).^{12,16,17} These three proteins are classified into three distinct subfamilies with a common function of acyl-CoA thioester binding. Like Cj0977_{p17}, the monomer structure of all three proteins consists of the 6-stranded antiparallel β -sheet and a central α -helix. However, while the quaternary structure of FapR is a dimer like Cj0977_{p17}, both of TtPaal and ArHBT adopt tetramers with a similar dimer-dimer

interface. In addition, both TtPaaI and ArHBT, consisting of 136 and 151 amino acid residues per monomer, respectively, are somewhat smaller than Cj0977 (192 amino acid residues), and form a single domain structure with the hot dog fold. By contrast, FapR (188 amino acid residues) is similar to Cj0977 in size, and has an additional helix-turn-helix (HTH) motif at the N terminus (Fig. 4B).¹⁷

In both thioesterases, essential residues for the hydrolysis reaction are the well-conserved HGG triad and Asp48 (in TtPaaI) and Glu73 (in ArHBT) (Fig. 4A & 4C). Of note, Gly40 in TtPaaI and Gly65 in ArHBT, the second residue of HGG triad, are hydrogen-bonded to attacking water and the thioester carbonyl oxygen, respectively, while Asp48 (in TtPaaI) and Glu73 (in ArHBT) function as a nucleophile or general base. None of these key catalytic residues are conserved in Cj0977, indicating that it is unlikely Cj0977 functions as a thioesterase.

Among numerous residues involved in the enzyme-ligand (acyl moiety) contact in TtPaaI, residues Asn33, Gly40, Asp48*, and Ala63* (* indicates amino acid residues of the second subunit) were proposed to participate in the reaction mechanism. These residues correspond to Ala63, Asp71, Asn79*, Ile93*, respectively, in Cj0977. The specificity for the 4-hydroxybenzoate moiety in ArHBT is contributed by residues Gln58, Gly65, Glu73*, Met74*, Thr77*, and Glu78*, corresponding to Ala63, Asp71, Asn79*, Tyr80*, Gln83*, and Ala84*, respectively, in Cj0977 (Fig. 4A & 4C). The dissimilar nature of amino acids involved in the enzyme-ligand contact supports our observation that Cj0977 does not act as a thioesterase.

Putative binding site for acyl-CoA

The hot dog fold structures share a similar binding pocket for acyl-CoA derivatives. Inspection of several acyl-CoA liganded structures indicates that, in terms of recognition sites, acyl-CoA derivatives can be divided into three structural moieties: adenosine 3', 5'-diphosphate, 4-phosphopantetheine, and specificity determining acyl chain (Fig. 5A). For each moiety, mainly two different subunits contribute key residues forming the recognition site or interacting with the ligands. In both TtPaaI and ArHBT structures (both tetramers), the recognition pocket of the 4-phosphopantetheine and acyl moieties is formed by the hot dog fold dimer pair, whereas that of the adenosine 3', 5'-diphosphate moiety involves one subunit from one dimer and another subunit from the second dimer of the tetramer (therefore, three subunits are required for the entire ligand binding site). However, in the FapR structure, which is functionally a dimer like Cj0977, the polar adenosine 3',5'-diphosphate moiety is exposed to the solvent and disordered in the crystal structure. Comparison of the recognition sites for the 4-phosphopantetheine moiety pinpoints key recognition residues and/or sequence motifs including A-(L/V) (from subunit 1) and (F/Y)-(T/F)-R-(P/Q)-(V/L) (from subunit 2), which are positioned in the beginning of β 3 and the loop between the strands β 3 and β 4, respectively (Fig. 4A & Fig. 5A). These amino acid residues correspond to S-V and F-Y-A-P-L of Cj0977, yielding a very similar binding pocket structure for the 4-phosphopantetheine moiety (Fig. 5A).

As for the specificity determining acyl moiety, the crystal structure of the FapR _{Δ 43}-malonyl-CoA complex showed that both the carbon chain size of the malonyl moiety and the presence of a carboxylate group at position 1 are essential for effector-binding specificity.¹⁷ Critical residues of the malonyl moiety binding pocket of FapR include Val119*, Asn115*, Phe99, His108 and Arg106, which correspond to Gln83*, Asn79*, Ala63, Ala72, and Phe70, respectively, in Cj0977 (Fig. 5B). Interestingly, Asn-115, which is hydrogen-bonded to the thioester carbonyl oxygen of the malonyl moiety and largely conserved in the FapR family, is also conserved in Cj0977 (Asn79). However, Arg106, which is essential for the interaction with malonyl carboxylate, is replaced by Phe70 in Cj0977 (Fig. 5B). Taken together, these observations suggested Cj0977 might bind an acyl-CoA derivative, which is important for the Cj0977 function. We next generated Cj0977 mutants (Q83A and F70A) by site-directed mutagenesis and reintroduced the mutated alleles into the Cj0977 mutant strain. The mutant

proteins were stably expressed in *C. jejuni* as determined by immunoblotting with Cj0977-specific antiserum (Fig 6A). No other protein changes were observed by immunoblotting of whole cells with polyclonal antibodies to 81–176 or by silver stain analyses of lipooligosaccharide cores (data not shown). Invasion assays indicated that the strains expressing the mutants with defects in the putative binding site were also defective in invasion, indicating that these sites are essential for virulence (Fig. 6B).

The C-terminal region of Cj0977 is required for invasion of intestinal epithelial cells

Based on the observation that FapR consists of two functional domains, the main structural domain (hot dog fold) and a small N-terminal HTH domain, we hypothesized Cj0977 might employ a similar strategy for its function including the main hot dog domain and a small C-terminal domain. To determine if the C-terminal region of Cj0977 was required for function, a deletion mutant lacking the terminal 36 amino acids was constructed and used to complement the original *C. jejuni* 81–176 mutant in Cj0977.⁶ The truncated protein was expressed in *C. jejuni*, as shown in Fig. 6A (CΔ36), at levels comparable to wildtype 81–176 (WT), but invasion was not restored in the Cj0977 mutant complemented with Cj0977Δ36 (lane CΔ36, Fig. 6B). On the other hand, the Cj0977 mutant clearly restored the invasion activity when complemented with full length Cj0977 (lane C, Fig. 6B).

Discussion

In an effort to understand the mechanism by which Cj0977 contributes to virulence of *C. jejuni* at the molecular level, we first employed Cj0977 primary sequence to search sequence data banks for homologous proteins. A search returned only 45 Blastp hits with 5 levels of alignment scores. The representatives of 5 sequence conservation levels are *Campylobacter* spp. Cj0977 homologs (S=354 bits, E=1e-96, 98% identical/192 residues), *Wolinella succinogenes* Ws0669 (S=90 bits, E=4e-17, 42% identical/116 residues), *Helicobacter pylori* Hp0420 (S=60.5 bits, E=5e-08, 29% identical/131 residues), *Pyrococcus horikoshii* Ph1136 (S=49.7 bits, E=8e-05, 29% identical/115 residues), and *Staphylococcus aureus* fapR (fatty acid and phospholipid biosynthesis Regulator) (S=39.7 bits, E=0.085, 29% identical/115 residues). Of note, *W. succinogenes* Ws0669, *H. pylori* Hp0420, and *P. horikoshii* Ph1136 are somewhat shorter proteins with 155, 142, and 131 residues, respectively. First, we used the full-length Cj0977 protein for crystallography work. Although the full-length protein yielded crystals, they did not diffract beyond 6 Å. Next, based on limited proteolysis as well as survey of protein stability, we designed several truncated versions of Cj0977. Of the constructs, Cj0977_{p17}, which includes residues 5 to 156 and corresponds to a full-length form of Ws0669 or Hp0420, diffracted to 2.6 Å (at the peak wavelength) allowing MAD phasing. Despite no obvious clue from the primary sequence, the 3D structure of Cj0977_{p17} reveals the ‘hot dog’ fold, which is associated with numerous coenzyme A derivative binding oligomeric enzymes.

A DALI search for structural homologs identified over 20 proteins with a Z score of ≥ 8 . The majority of these proteins are acyl-CoA thioesterases with differing substrate specificities from various organisms. Several structures with unknown functions derived from structural genomics projects enlist close structural homologs of Cj0977. Unexpectedly, however, the structure of Cj0977_{p17} was the closest to that of *Bacillus subtilis* FapR with a Z score of 17. FapR is a global transcriptional repressor that controls the expression of many genes involved in fatty acids and phospholipids biosynthesis in *B. subtilis*. Binding of malonyl-CoA, an essential intermediate in fatty acid biosynthesis, to FapR induces conformational changes in the hot dog domain of FapR, which propagates to HTH motifs of FapR dimer, and therefore hinders productive association between FapR and its specific DNA promoter. The hot dog domain of FapR appears to have retained its substrate specificity for malonyl-CoA, but appears to have lost its catalytic ability. Interestingly, both Cj0977 and FapR proteins adopt dimer structures

in crystals as well as in solution. As shown in Figure 4B, these two proteins superimpose well throughout the hot dog fold domain, except that the N-terminal helices orient differently. For the crystallization purpose in both cases, the C-terminal 36 residues portion of Cj0977 is truncated in Cj0977_{p17}, whereas N-terminal 43 residues of FapR are deleted in FapR_{Δ43}. The N-terminal 43 residues domain of FapR represents HTH motif involved in DNA binding. By analogy, although the C-terminal 36 residues portion of Cj0977 was not predicted to contain any known protein domain motifs, it is conceivable that this small domain plays a role in Cj0977 virulence by DNA binding or interaction with other proteins (see below). As shown in Figure 6, the C-terminal 36 amino acid moiety is required for invasion of *C. jejuni* into INT407 cells.

Other significant structural homologs to Cj0977 included *Thermus thermophilus* phenylacetate thioesterase (TtPaal), and *Arthrobacter* sp. 4-hydroxybenzoyl-CoA thioesterase (ArHBT).^{12,16} The Paal thioesterase is involved in bacterial phenylacetic acid catabolic pathway, and HBT catalyzes the hydrolysis of the thioester moiety to yield 4-hydroxybenzoate and CoA. Our careful inspection of the superposed structures indicated some clues as to the function of Cj0977. The liganded forms of all three proteins indicated residues involved in recognition of the common structural moiety of acyl-CoA derivatives, 4-phosphopantetheine. The binding pocket appears to be conserved in Cj0977 with consensus motifs S-V and F-Y-A-P-L (Fig. 5A). However, deducing a function from the specificity determining acyl chain moieties was less obvious. The prominently conserved residues in these subfamily thioesterases, HGG triad and catalytic base Asp or Glu, are completely lacking in Cj0977, indicating an unlikely function of Cj0977 as a thioesterase. While little is known about the biosynthesis pathways of fatty acids and phospholipids in *C. jejuni*, we could identify a few putative thioesterases from the *C. jejuni* genome sequence. To unveil possible functions of Cj0977, we performed enzyme assays using several available acyl-CoA compounds as substrates along with the cloned and purified putative thioesterase enzymes of *C. jejuni*. Thus far, we observed thioesterase activities with one of the putative proteins (Yokoyama *et al.* unpublished result), but, as expected, we could not detect any significant enzyme activity with Cj0977. In considering moderate similarity to known binding pockets of the acyl chain moiety, we also initiated studies to examine binding properties of acyl-CoA compounds to Cj0977. Thus far, we have not found evidence of malonyl-CoA binding to Cj0977, based on solid phase binding assays. Cj0977 contains Phe70 in the place of Arg106 of FapR, which is essential for interacting with malonyl carboxylate, yet Phe70 is indispensable for virulence. Therefore, it appears that the nature of the acyl moiety of the ligand would be a small hydrophobic group. We speculate that Cj0977 may have a similar function as FapR, as a transcriptional regulator sensing an acyl-CoA intermediate of membrane lipid (or fatty acid) biosynthesis *via* the Cj0977 hot dog domain, a possibility that is under investigation.

Since the first structure with the hot dog fold, many proteins are now known to adopt this characteristic fold. Moreover, this fold is widespread in all three kingdoms of living organisms, eukaryotes, prokaryotes, and archaea.¹⁰ The hot dog fold domain, which can be formed by ~130 amino acid residues, is found to be associated with a wide range of other structural domains. Therefore, proteins containing the hot dog fold can widely vary in size from single domain proteins (~130 residues) to multiple domain proteins (~2800 residues proteins).¹⁰ Of note, among various domain organizations of proteins associated with a hot dog fold, the architecture revealed by Cj0977 is unique in that it contains the N-terminal hot dog domain as the major structural and functional domain and the small C-terminal functional region.

Recently, it has been well appreciated that many gene sequences, especially in eukaryotic genomes, encode large segments (even entire proteins) that lack a well-structured three-dimensional fold.¹⁹ The occurrence of unstructured regions of significant size (>50 residues) is surprisingly common in functional proteins. While the existence of functional unstructured proteins – *e.g.* polypeptide hormones – has been recognized for many years, the functional role

of intrinsically disordered proteins in other crucial areas has only recently been recognized.¹⁹ New examples of functional intrinsically disordered protein domains are constantly emerging. Functions include the regulation of transcription and translation, cellular signal transduction, protein phosphorylation, the storage of small molecules, and regulation of the self-assembly of large multiprotein complexes such as the bacterial flagellum. Many intrinsically disordered proteins undergo transitions to more ordered states or fold into stable secondary or tertiary structures upon binding to their targets – that is, they undergo coupled folding and binding processes. In this regard, we examined the Cj0977 sequence as to disorder propensity using the DISOPRED2 program.²⁰ The result predicted a completely disordered region comprising the C-terminal 36 residues (Fig. 7A), consistent with our experimental result from limited proteolysis and the protein stability test. Together with the requirement of C-terminal 36 residues of Cj0977 for invasion, we propose that this segment of 36 amino acids is unstable and unstructured in solution and undergoes coupled folding and binding to an as yet unidentified cellular *C. jejuni* protein, which might be involved in transcriptional regulation (Fig. 7B). Interestingly, the recently solved structure of mouse acyl-CoA thioesterase 7 shows an additional C-terminal α -helix that packs on the opposite side of the β -sheet of the hot dog fold.²¹ This C-terminal α -helix is unique among experimentally determined hot dog fold proteins. Of note, when we aligned the corresponding regions of the two proteins, the C-terminal 36 residues region of Cj0977 shows a surprising match in terms of sequence length and nature (rich in polar residues), suggesting a possible α -helix structure. Therefore, one can speculate that this C-terminal moiety might acquire structure only after binding to its partner, and an acyl-CoA intermediate of fatty acid biosynthesis might promote or hinder the favorable conformational change of this C-terminal region. Identification of cellular component proteins is currently under investigation by a proteomics approach in our laboratory.

In conclusion, the structure of Cj0977 provides new clues in discovering a novel acyl-CoA effector as well as in the study of this putatively disordered C-terminal region. These studies would indicate a novel linkage between flagella regulation, fatty acid biosynthesis and virulence in *C. jejuni*. Together with the structure of Cj0977 bound to its CoA derivative ligand, the ordered structure promoted by the interaction with a cellular protein partner may aid in crystallization. The structure determination of such complexes would represent a significant step toward the understanding of the structural basis for *C. jejuni* virulence.

Materials and Methods

Cloning, Expression, and Purification

The gene corresponding to Cj0977 of NCTC 11168 was cloned from *C. jejuni* strain 81–176 (CJJ81176_0996) into pET15b and expressed as N-terminal His-tagged full length Cj0977 (His-Cj0977) in BL21(DE3) strain. His-Cj0977 was purified using the 3-step procedure: Ni-NTA affinity column, HiTrapQ anionic exchange column, and a gel filtration column. Typically, bacteria were grown in 1 liter liquid LB media supplemented 100 μ g/ml ampicillin at 37°C. When the culture reached O.D. of ~0.8 at 600 nm, isopropyl- β -D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM, and the culture was grown for 3 more hours at room temperature.

For construction of the fragments Cj0977_{p21}, Cj0977_{p19} and Cj0977_{p17} (defined in Figure 1) as GST fusion proteins, the following primers were designed: pFbam (5' GCA GGA TCC GAT AAT TTT GAA GAA TAT GCA C), pR1xho (5'- GGT CTC GAG TTA TTT TCC ACC CAC AGA GGC C-3'), pR2xho (5'- GGT CTC GAG TTA GGT ACC TTG TTC TTG ATT TTC G-3') and pR3xho (5'- CGG CTC GAG TTA GAG TTT AAA TAT ATG CTC ATC G-3'). PCR was performed using primer pairs of pFbam/pR1xho, pFbam/pR2xho and pFbam/pR3xho for Cj0977_{p21}, Cj0977_{p19} and Cj0977_{p17}, respectively. The constructs were transformed into a Met auxotroph strain (DL41). The SeMet labeled proteins were produced using the well

established protocol in our laboratory.²² After cleavage of the GST-Cj0977_{p17} by the PreScission protease (GE Healthcare), the SeMet Cj0977_{p17} moiety was further purified using an anionic exchange HiTrap Q HP (GE Healthcare) with an AKTA purifier. The proteins were eluted in buffer A (20 mM Tris-HCl pH 8, 50 mM NaCl) with a 0.05 – 1 M NaCl gradient. For the final step including preparative and/or analytical gel filtration, the protein samples were concentrated using a Vivaspin filter, and applied to a Sephacryl™ S-100 HiPrep™ 16/60 column (GE Healthcare) equilibrated in buffer B (20 mM Tris-HCl pH 8.0, 150 mM NaCl) or a Superdex™ 75 10/300 GL High Resolution column equilibrated in buffer C (HEPES pH 7.4, 200 mM NaCl, 5% glycerol, 0.5 mM EDTA).

Construction of a complement of the Cj0977 mutant in which a wildtype Cj0977 gene and its σ^{28} promoter were inserted into an arylsulfatase (*astA*) gene on the chromosome of the mutant has been described.⁶ A complement that lacked the terminal 36 amino acids was constructed as follows. The truncated gene was PCR amplified using primers pg.07.28 (5'-GGTGGTAAGCTTAGAATGGATAGACTCTTTTTCG-3') and pg07.29 (5'-GGAATTCCTTACTTGAGTTTAAATATATGCTCATCGGTGC-3'). These primers introduced HindIII and EcoRI sites, respectively. The resulting fragment was cloned into pBluescript and an *aph3* cassette²³ was inserted at the BamHI site of the resulting plasmid. An XbaI-XhoI fragment, which contained σ^{28} -Cj0977 Δ 36 and *aph3*, was purified, blunted with Klenow and cloned into a unique EcoRV site within the *astA* gene of pRY660.²⁴ This plasmid was used to electroporate the Cj0977::*cat* mutant⁶ with selection on Mueller Hinton agar supplemented with kanamycin (50 μ g/ml). Resulting clones were screened for loss of AstA expression on a chromogenic substrate.²⁴

Site-directed mutagenesis

The plasmid clone originally used to complement the Cj0977 mutant, which contained σ^{28} -Cj0977 and *aph3* for selection inserted within a clone of *astA*,⁶ was subjected to site-directed mutagenesis using QuickChange kits from Stratagene as recommended by the supplier. Primers used were as follows. The primers for Q83A were pg08.35 (5'-CGGCAAATCTATGTAGCAGCAGCCTCTATCAATAAAGAATTTTC-3') and pg08.36 (5'-GAAAATTCTTTATTGATAGAGGCTGCTGCTACATAGTTTGCCG-3'). The primers for F70A were pg08.87 (5'-GCAGATGATCAAGGATTGATTGCTGATGCCTTTATTTTCGCTGC-3') and pg08.88 (5'-GCAGCGAAAATAAAGGCATCAGCAATCAATCCTTGATCATCTGC-3'). Putative mutant clones were sequenced to confirm the desired mutation and the absence of undesired mutations prior to electroporation into the Cj0977::*cat* mutant⁶ with selection on Mueller Hinton agar supplemented with kanamycin (50 μ g/ml). Resulting clones were screened for loss of AstA expression on a chromogenic substrate.²⁴

Limited proteolysis

Analytical protease digestions were performed as described previously²⁵ with modifications. For each 100 μ l reaction, 100 μ g of a pure His-Cj0977 sample was incubated with trypsin (Roche Diagnostics) in 20 mM Tris-HCl (pH 7.5), NaCl 100 mM, and 1 mM EDTA (pH 8). Reactions were performed at ratios of trypsin: Cj0977 (w/w) of 1:50, 1:100, and 1:500 by incubating in a heat block at 37°C up to 3 hrs. At each time point, the aliquots of the reactions were stopped by the addition of 2 $\times$ SDS-PAGE loading buffer followed by boiling 4 min. The samples were analyzed by SDS-PAGE, transferred to a PVDF membrane, and submitted for N-terminal sequencing (Midwest Analytical Inc., St. Louis).

Crystallization and Data Collection

Crystals of SeMet-Cj0977_{p17} were grown in a 1:1 mixture of protein (18 mg/ml in buffer of 20 mM Tris-HCl pH 8.5/100 mM NaCl/5% glycerol) and reservoir solution containing 24–

27% PEG 4K, 0.25 M ammonium acetate and 0.1 M Tris-HCl (pH 7.0–7.4) using the hanging drop vapor diffusion method at 17°C. Crystals (in various shapes) grew to typical dimensions $200 \times 150 \times 50 \mu\text{m}$ within three days. Some crystallization drops in the same condition yielded needle clusters more slowly. Some of these needle crystals were grown very nicely to dimensions of $400 \times 50 \times 10 \mu\text{m}$ within a week. For data collection, SeMet derivatized crystals in various shape and size were treated with cryo-solution containing 20–25 % glycerol or 20 % ethylene glycol as cryo protectant, and directly frozen in liquid N_2 . Multi-wavelength data were collected using a single needle form crystal at 100K (Advanced Photon Source, SBC, 19BM). Each data set was indexed and integrated using HKL3000 and scaled with SCALEPACK²⁶ to 2.80Å, 2.87Å, and 3.2Å for peak, inflection, and remote data, respectively. The SeMet-Cj0977_{p17} crystal belonged to spacegroup $P2_1$ with unit cell dimensions of $a=79.9\text{Å}$, $b=94.5\text{Å}$, $c=81.8\text{Å}$, $\beta=99.3^\circ$ with eight molecules per asymmetric unit.

Structure Determination and Refinement

The Cj0977_{p17} structure was solved by multiple-wavelength anomalous dispersion (MAD) method using selenomethionine containing protein. Se atoms search, initial phasing and density modification were performed using autoSHARP.²⁷ The resulting 2.8Å electron density map was readily interpretable. The initial model was built automatically using RESOLVE and manually using COOT²⁸ and placed 68% of the secondary structural elements with the side chain. From this partial model, molecular replacement was carried out with MOLREP and eight molecules were found in an asymmetric unit with high corr.(t) (> 0.6). The initial refinement was carried out using CNS1.1²⁹ with rigid-body, simulated annealing and restrained individual B -factor refinement by using the data set collected at λ_{peak} . At this step, R -factor was 35% ($R_{\text{free}} = 45\%$) and then several steps of refinement were performed using REFMAC5³⁰ with TLS option³¹ and non-crystallographic symmetry restraints followed by manual adjustment of the model. Coordinates with electron density greater than 3.5σ in $F_o - F_c$ maps were designed as water molecules if the locations were reasonable for hydrogen bonding. A B -factor cutoff of 60.0Å^2 was applied to water molecules, and any water molecules refining to higher values were removed from the model. The final model has an R -factor of 21.3% and free R -factor of 26.9%. The rms deviations from ideal geometry were 0.014Å for bond length and 1.4° for bond angles. Structural analysis of the final model using PDB validation suite indicated that none of the residues are in the disallowed region of the Ramachandran plot, and almost all the residues are in the most favored regions. A summary of the data collection and refinement statistics is given in Table I.

SDS-PAGE and immunoblotting

Whole cells of *C. jejuni* were resuspended in solubilization buffer, boiled and electrophoresed on 12.5% SDS-PAGE gels. Following transfer to nitrocellulose, proteins were immunodetected using a rabbit polyclonal antibody to recombinant His-tagged Cj0977 as described previously.⁶

Invasion assays

Invasion assays using INT407 cells were done at an MOI of 20:1 as previously described.^{6, 32, 33} Briefly, the infected monolayer was incubated for 2 h at 37°C in 5% CO_2 /air. The monolayer was washed twice with Hank's balanced salt solution and fresh medium containing gentamicin at 100 $\mu\text{g/ml}$ was added to kill extracellular bacteria. After 2 h incubation the monolayer was washed twice with Hank's balanced salt solution and lysed with 0.01% Triton X-100. Released, intracellular bacteria were enumerated by plate count on Mueller Hinton agar. Invasion was expressed as the percentage of the original inoculum that survived gentamicin killing.³²

Acknowledgements

We thank the staff of beamline 19BM of the Structural Biology Center at APS (Argonne National Laboratory) for help during data collection, Gary Majam for technical assistance and Dr. Joe Eichberg for helpful discussions. This work was supported by the Robert W. Welch Foundation Grant E-1616 and National Institutes of Health Grant AI068943 (to H.J.Y.), and National Institutes of Health Grant AI043559 (to P.G.).

References

1. Guerry P. *Campylobacter* flagella: not just for motility. Trends Microbiol 2007;15:456–461. [PubMed: 17920274]
2. Konkel ME, Klena JD, Rivera-Amill V, Monteville MR, Biswas D, Raphael B, Mickelson J. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. J Bacteriol 2004;186:3296–3303. [PubMed: 15150214]
3. Song YC, Jin S, Louie H, Ng D, Lau R, Zhang Y, Weerasekera R, Al Rashid S, Ward LA, Der SD, Chan VL. FlaC, a protein of *Campylobacter jejuni* TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion. Mol Microbiol 2004;53:541–553. [PubMed: 15228533]
4. Poly F, Ewing C, Goon S, Hickey TE, Rockabrand D, Majam G, Lee L, Phan J, Savarino NJ, Guerry P. Heterogeneity of a *Campylobacter jejuni* protein that is secreted through the flagellar filament. Infect Immun 2007;75:3859–3867. [PubMed: 17517862]
5. Carrillo CD, Taboada E, Nash JH, Lanthier P, Kelly J, Lau PC, Verhulp R, Myktyczuk O, Sy J, Findlay WA, Amoako K, Gomis S, Willson P, Austin JW, Potter A, Babiuk L, Allan B, Szymanski CM. Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by flhA. J Biol Chem 2004;279:20327–20338. [PubMed: 14985343]
6. Goon S, Ewing CP, Lorenzo M, Pattarini D, Majam G, Guerry P. A sigma28-regulated nonflagella gene contributes to virulence of *Campylobacter jejuni* 81–176. Infect Immun 2006;74:769–772. [PubMed: 16369037]
7. Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S, Barrell BG. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 2000;403:665–668. [PubMed: 10688204]
8. Leesong M, Henderson BS, Gillig JR, Schwab JM, Smith JL. Structure of a dehydratase-isomerase from the bacterial pathway for biosynthesis of unsaturated fatty acids: two catalytic activities in one active site. Structure 1996;4:253–264. [PubMed: 8805534]
9. Benning MM, Wesenberg G, Liu R, Taylor KL, Dunaway-Mariano D, Holden HM. The three-dimensional structure of 4-hydroxybenzoyl-CoA thioesterase from *Pseudomonas* sp Strain CBS-3. J Biol Chem 1998;273:33572–33579. [PubMed: 9837940]
10. Dillon SC, Bateman A. The Hotdog fold: wrapping up a superfamily of thioesterases and dehydratases. BMC Bioinformatics 2004;5:109. [PubMed: 15307895]
11. Kimber MS, Martin F, Lu Y, Houston S, Vedadi M, Dharamsi A, Fiebig KM, Schmid M, Rock CO. The structure of (3R)-hydroxyacyl-acyl carrier protein dehydratase (FabZ) from *Pseudomonas aeruginosa*. J Biol Chem 2004;279:52593–52602. [PubMed: 15371447]
12. Kunishima N, Asada Y, Sugahara M, Ishijima J, Nodake Y, Sugahara M, Miyano M, Kuramitsu S, Yokoyama S, Sugahara M. A novel induced-fit reaction mechanism of asymmetric hot dog thioesterase PAAI. J Mol Biol 2005;352:212–228. [PubMed: 16061252]
13. Li J, Derewenda U, Dauter Z, Smith S, Derewenda ZS. Crystal structure of the *Escherichia coli* thioesterase II, a homolog of the human Nef binding enzyme. Nat Struct Biol 2000;7:555–559. [PubMed: 10876240]
14. Song F, Zhuang Z, Finci L, Dunaway-Mariano D, Kniewel R, Buglino JA, Solorzano V, Wu J, Lima CD. Structure, function, and mechanism of the phenylacetate pathway hot dog-fold thioesterase PaaI. J Biol Chem 2006;281:11028–11038. [PubMed: 16464851]

15. Thoden JB, Holden HM, Zhuang Z, Dunaway-Mariano D. X-ray crystallographic analyses of inhibitor and substrate complexes of wild-type and mutant 4-hydroxybenzoyl-CoA thioesterase. *J Biol Chem* 2002;277:27468–27476. [PubMed: 11997398]
16. Thoden JB, Zhuang Z, Dunaway-Mariano D, Holden HM. The structure of 4-hydroxybenzoyl-CoA thioesterase from *Arthrobacter* sp strain SU. *J Biol Chem* 2003;278:43709–43716. [PubMed: 12907670]
17. Schujman GE, Guerin M, Buschiazzi A, Schaeffer F, Llarrull LI, Reh G, Vila AJ, Alzari PM, de Mendoza D. Structural basis of lipid biosynthesis regulation in Gram-positive bacteria. *EMBO J* 2006;25:4074–4083. [PubMed: 16932747]
18. Holm L, Sander C. Dali: a network tool for protein structure comparison. *Trends Biochem Sci* 1995;20:478–480. [PubMed: 8578593]
19. Dyson HJ, Wright PE. Intrinsically unstructured proteins and their functions. *Nat Rev Mol Cell Biol* 2005;6:197–208. [PubMed: 15738986]
20. Ward JJ, McGuffin LJ, Bryson K, Buxton BF, Jones DT. The DISOPRED server for the prediction of protein disorder. *Bioinformatics* 2004;20:2138–2139. [PubMed: 15044227]
21. Forwood JK, Thakur AS, Guncar G, Marfori M, Mouradov D, Meng W, Robinson J, Huber T, Kellie S, Martin JL, Hume DA, Kobe B. Structural basis for recruitment of tandem hotdog domains in acyl-CoA thioesterase 7 and its role in inflammation. *Proc Natl Acad Sci USA* 2007;104:10382–10387. [PubMed: 17563367]
22. Yeo HJ, Cotter SE, Laarmann S, Juehne T, St Geme JW 3rd, Waksman G. Structural basis for host recognition by the *Haemophilus influenzae* Hia autotransporter. *EMBO J* 2004;23:1245–1256. [PubMed: 15029242]
23. Labigne-Roussel A, Courcoux P, Tompkins L. Gene disruption and replacement as a feasible approach for mutagenesis of *Campylobacter jejuni*. *J Bacteriol* 1988;170:1704–1708. [PubMed: 2832375]
24. Yao R, Guerry P. Molecular cloning and site-specific mutagenesis of a gene involved in arylsulfatase production in *Campylobacter jejuni*. *J Bacteriol* 1996;178:3335–3338. [PubMed: 8655516]
25. Yeo HJ, Ziegelin G, Korolev S, Calendar R, Lanka E, Waksman G. Phage P4 origin-binding domain structure reveals a mechanism for regulation of DNA-binding activity by homo- and heterodimerization of winged helix proteins. *Mol Microbiol* 2002;43:855–867. [PubMed: 11929537]
26. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307–326.
27. Vonrhein C, Blanc E, Roversi P, Bricogne G. Automated Structure Solution With autoSHARP. *Methods Mol Biol* 2006;364:215–230. [PubMed: 17172768]
28. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr, sect D* 2004;60:2126–2132. [PubMed: 15572765]
29. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr, sect D* 1998;54:905–921. [PubMed: 9757107]
30. Murshudov GN, Vagin AA, Lebedev A, Wilson KS, Dodson EJ. Efficient anisotropic refinement of macromolecular structures using FFT. *Acta Crystallogr, sect D* 1999;55:247–255. [PubMed: 10089417]
31. Winn MD, Isupov MN, Murshudov GN. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr, sect D* 2001;57:122–133. [PubMed: 11134934]
32. Oelschlaeger TA, Guerry P, Kopecko DJ. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proc Natl Acad Sci USA* 1993;90:6884–6888. [PubMed: 8341714]
33. Yao R, Burr DH, Doig P, Trust TJ, Niu H, Guerry P. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. *Mol Microbiol* 1994;14:883–893. [PubMed: 7715450]

Figure 1.

Purification of Cj0977 and identification of stable domain structures used for crystallization. (A) Analytical gel filtration chromatograph. The purified His-Cj0977 protein eluted as a dimer, with an estimated molecular mass of 50 kDa. For comparison, the elution profile of Cj0977_{p17} is overlaid. The purified Cj0977_{p17} also eluted as a dimer, with an estimated molecular mass of ~35 kDa. The peak positions of molecular standards were indicated as arrows (Conalbumin, 75 kDa; Ovalbumin, 43 kDa; and Carbonic anhydrase, 29 kDa) (B) Limited proteolysis experiments of Cj0977 with trypsin revealed a temporarily stable fragment of 21 kDa. Purified Cj0977 was subjected to the proteolysis reaction in the presence of trypsin (protein:trypsin = 1:50 or 1:100) for indicated time, and the reaction progress was analyzed by SDS-PAGE (15%). The 21 kDa fragment (◀) remained stable for ~2 hrs. (C) The purified Cj0977 samples were checked for stability. After about 4 weeks, the full-length protein solution became a very stable fragment of 17 kDa (◀). Lanes 1 to 4 correspond to 1 day-, 2 week-, 4 week-, and 6 week-old sample, respectively. Molecular masses are shown in the left of gels. (D) Sequence alignment of Cj0977 with its homologs, *Wolinella succinogenes* Ws0669 and *Helicobacter pylori* Hp0420. Amino acid residues included in Cj0977_{p21}, Cj0977_{p19}, and Cj0977_{p17} are denoted as Cjp21, Cjp19, Cjp17, respectively.

Figure 2. Crystal Structure of Cj0977_{p17}. **(A)** Ribbon diagrams of the Cj0977_{p17} monomer structure. The secondary structures are labeled. N and C denote the N and C termini of Cj0977_{p17}, respectively. The central helix (α3) is highlighted in red. **(B)** Ribbon diagrams of the Cj0977_{p17} dimer structure. Two subunits are shown in green and yellow. The subunit in green of the top panels is shown in the same orientation as in (A).

Figure 3. Superposition of 8 protomers in the asymmetric unit. The overlay of 8 protomers was carried out with respect to the protomer D. The view is in the same orientation as in Figure 2A. The variable region within the core hotdog fold, loop between $\beta 4$ and $\beta 5$, is highlighted in orange circle.

Figure 4.

(A) Secondary structure of Cj0977_{p17} and structure-based sequence alignment. Secondary structure elements of Cj0977_{p17} are shown at the top of the sequences with β strands and helices denoted as arrows and cylinders (central helix $\alpha 3$ in red), respectively. BsFapR, *Bacillus subtilis* FapR; TtPaaI, *Thermus thermophilus* PaaI; and ArtTE, *Arthrobacter* sp. 4-hydroxybenzoyl-CoA thioesterase. Green and blue shadings highlight residues identical to Cj0977 sequence and identical residues among hot fold proteins but Cj0977, respectively. Key residues involved in ligand recognition and/or catalysis are highlighted by red boxes. (B) Superposition of Cj0977_{p17} and FapR $\Delta 43$. Two structures in dimers were superimposed using the program of the CCP4 suite with the Cj0977_{p17} structure in green and yellow (residues 13–

154) and the FapR_{Δ43} structure in blue (residues 51–186). The loop region in orange circles corresponds to the variable loop region within Cj0977 protomers as shown in Figure 3. The region indicated in red circles corresponds to the ligand binding site in FapR_{Δ43}. (C) Structural features of the thioesterase active sites. Left, superposition of TtPaaI (in complex with hexanoyl-CoA, PDB 1wn3) and ArHBT (in complex with CoA and hydroxybenzoic acid, PDB 1q4s), Right, superposition of TtPaaI and Cj0977. Key amino acid residues are labeled (TtPaaI, gray; ArHBT, cyan; and Cj0977, black underlined) and highlighted in stick representation. TtPaaI (gray), ArHBT (blue), Cj0977 (yellow and green) are in ribbon diagrams. The ligands are highlighted with pink (CoA and hydroxybenzoic acid) and light pink (hexanoyl-CoA) bonds.

Figure 5. Putative binding site for acyl-CoA. **(A)** Acyl-CoA molecule and ligand recognition scheme. Shown are conserved residues critical for recognition of the 4-phosphopantetheine moiety. The corresponding residues in Cj0977 are indicated in blue. **(B)** Close up view of the putative binding site with conserved features between Cj0977_{p17} and FapR_{Δ43} (in complex with malonyl-CoA). Key amino acid residues are labeled (Cj0977 residues underlined) and highlighted in stick presentation. Cj0977 (yellow and green ribbons) residues in green, FapR_{Δ43} (gray ribbons) residues in cyan, and malonyl-CoA in pink.

Figure 6. Invasion of *C. jejuni* strains into INT407 cells. (A)

Expression of Cj0977 and Cj0977 mutant proteins in *C. jejuni* cells. Whole cells of *C. jejuni* were electrophoresed on 12.5% SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with rabbit polyclonal antiserum to recombinant Cj0977.⁶ WT, wildtype 81–176; Q83A, Cj0977_Q83A; F70A, Cj0977_F70A; M, the Cj0977 mutant⁶; C, the Cj0977 mutant complemented with the full length Cj0977 gene inserted into *astA*; CΔ36, the Cj0977 mutant complemented with a copy of Cj0977 deleted for the codons encoding the terminal 36 amino acids. **(B)** INT407 cells were infected with wildtype 81–176, the Cj0977 mutants, and mutant complemented with either the full length or deleted form of Cj0977. Invasion was monitored as described in Materials and Methods. The results are from three independent assays run in duplicate. The *P* values between the invasion levels of the Cj0977 mutant and Cj0977Δ36 compared to wildtype were <0.001 and the *P* values between Cj0977_F70A and Cj0977_Q83A compared to wildtype were <0.01. The differences between wildtype and the mutant complemented with the full-length Cj0977 (C) were not statistically significant (*P* >0.05).

Figure 7. Domain architecture and proposed model of coupled folding and binding of Cj0977. (A) Top, domain organization of Cj0977 showing the main structural and functional domain determined in this study and C_{TAIL} (residues 157–192). Bottom, Disordered profile plot generated by the DISOPRED2 Disorder Prediction Server.³² (B) The hotdog fold domain of Cj0977 binds as yet unidentified CoA derivative ligands (triangles). Ligand binding may facilitate the initial transition from 'disorder to order' state of the C-terminal region (C_{TAIL}, depicted as black strings) of Cj0977. By interacting with cellular protein(s), the C_{TAIL} moiety is now folded and competent for a regulator such as binding to DNA or other proteins. 'C-ter' denotes the C terminus of Cj0977.

Table I

Statistics from crystallographic analysis.

Data Collection	Peak	Inflection	Remote
Wavelength (Å)	0.979291	0.979450	0.971620
Space group			
Cell parameters (<i>a</i> , <i>b</i> , <i>c</i> =Å, β=deg.)		<i>P</i> 2 ₁ , <i>a</i> =79.88, <i>b</i> =94.54, <i>c</i> =81.82, β=99.32	
Resolution (Å)	50-2.6	50-2.87	50-3.2
Reflections (Total/Unique)	216,635/36,505	95,846/28,241	59,422/19,966
Completeness ^a (%)	95.6 (68.3)	96.5 (86.2)	94.3 (86.0)
<i>R</i> _{merge} ^{a,b} (%)	9.8 (33.9)	8.4 (33.0)	10.5 (28.2)
<1/σ(<i>I</i>)>	7.1	6.2	4.4
Redundancy ^a	6.43 (2.5)	3.5 (2.3)	3.1 (2.3)
Structure Refinement			
Resolution (Å)	50-2.6		
No. reflections (working/test)	34,092/1,790		
<i>F</i> /σ(<i>F</i>)	>0		
<i>R</i> ^c (%) / <i>R</i> _{free} ^d (%)	21.3/26.9		
Total number of atoms ^e	8,728		
Protein molecules	8		
Average B factor, Å ²	33.9		
rms deviation			
Bonds (Å)	0.014		
Angles (°)	1.433		
Ramachandran plot (%)			
Most favored regions	89.1		
Additional allowed regions	9.6		
Generously allowed regions	1.3		

^aThe values in parentheses correspond to statistics for the outermost-resolution shell.^b $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where *I*_{*i*} is the scaled intensity of the *i*th measurement, <*I*> is the mean value for that reflection.^c $R \text{ factor} = \sum ||F_O| - |F_C|| / \sum |F_O|$, where *F*_O and *F*_C are the observed and calculated structure factor amplitudes, respectively.^d*R*_{free} = *R* factor calculated with 5% of the total reflections chosen randomly and omitted from the refinement.^eincludes 8 water molecules.

Table II

Representative structural homologs of Cj0977

Protein: activity	PDB	Z/r.m.s.d.	Organism	4° structure	References
FapR: transcription factor	2f3x	17/2.2	<i>B. subtilis</i>	dimer	17
Paal	1jly	15.8/1.9	<i>T. thermophilus</i>	tetramer	12
Thioesterase	1q4s	13.8/2.4	<i>Arthrobacter</i> sp.	tetramer	16
FabZ: dehydratase	1u1z	12.6/2.7	<i>P. aeruginosa</i>	hexamer	11
Thioesterase II	1c8u	12.3/2.8	<i>E. coli</i>	double hot-dogs (dimer)	13
FabA: dehydratase-isomerase	1mka	11.2/3.0	<i>E. coli</i>	dimer	8
Hydrolase	1bvq	9.8/2.2	<i>Pseudomonas</i> sp.	tetramer	9