Cloning, refolding, purification and preliminary crystallographic analysis of the sensory domain of the *Campylobacter* chemoreceptor for multiple ligands (CcmL)

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A periplasmic sensory domain of the *Campylobacter jejuni* chemoreceptor for multiple ligands (CcmL) has been crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 3350 as a precipitating agent. A complete data set was collected to 1.3 Å resolution using cryocooling conditions and synchrotron radiation. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 42.6$, $b = 138.0$, $c = 49.0$ Å, $\beta = 94.3^\circ$.

1. Introduction

Campylobacter jejuni is one of the most common causes of human bacterial gastroenteritis (Allos, 2001; Butzler, 2004). It colonizes the gastrointestinal tract of poultry and cattle and is transmitted to humans *via* contaminated food (predominantly chicken products) or water (Allos, 2001; Butzler, 2004; Inglis *et al.*, 2005; Wingstrand *et al.*, 2006). *C. jejuni* infections cause fever, abdominal pain and mild to severe diarrhoea (Allos, 2001; Butzler, 2004; Wassenaar & Blaser, 1999). Post-infection complications may include arthritis, reactive myositis and Guillain–Barré syndrome (Schmidt-Ott *et al.*, 2006; Allos, 2001; Friedman *et al.*, 2000). Although the molecular mechanisms involved in its pathogenesis remain poorly understood, several virulence factors, such as chemotaxis, adherence and lipooligosaccharide, have been identified (Young *et al.*, 2007).

Chemotaxis allows motile bacteria to swim in a certain direction in response to extracellular chemical signals. It has been shown to play an important role in initial colonization and development of the disease in animal models (Josenhans & Suerbaum, 2002). *C. jejuni* is attracted to amino acids, organic acids and carbohydrates (Rahman *et al.*, 2014; Hartley-Tassell *et al.*, 2010; Hugdahl *et al.*, 1988). The bacteria sense these chemicals *via* transducer-like proteins (Tlps) located in the bacterial membrane or cytoplasm (Zautner *et al.*, 2012). Interaction between the Tlps and their respective ligands initiates a molecular signal transduction cascade that causes a change in the direction of rotation of flagellar motors.

11 putative Tlps have been identified in *C. jejuni* (Day *et al.*, 2012; Zautner *et al.*, 2012; Parkhill *et al.*, 2000); these chemoreceptors have been classified into three groups according to their predicted domain organization. Tlp1, Tlp2, Tlp3, Tlp4, Tlp7_{mc}, Tlp7_m, Tlp10 and Tlp11 belong to group A, Tlp9 belongs to group B and Tlp5, Tlp6, Tlp7_c and Tlp8 belong to group C (Zautner *et al.*, 2012). Group A Tlps have a similar structure to the methyl-accepting proteins (MCPs) found in

Escherichia coli and to family A transducers of *Halobacterium salinarum* (Zhang *et al.*, 1996). They are predicted to contain an N-terminal transmembrane helix followed by a periplasmic ligand-binding domain, a second transmembrane helix and a C-terminal cytoplasmic signalling domain (Marchant *et al.*, 2002; Zautner *et al.*, 2012). While the signalling domain is highly conserved (and is even identical in the case of Tlp2, Tlp3 and Tlp4), the periplasmic domain exhibits high variability between different Tlps (Parkhill *et al.*, 2000; Marchant *et al.*, 2002).

Four of the group A Tlp receptors of *C. jejuni* (Tlp1, Tlp3, Tlp7 and Tlp11) have been characterized to date. The aspartate receptor Tlp1, also termed CcaA (*Campylobacter* chemoreceptor for aspartate A; Hartley-Tassell *et al.*, 2010), the multiligand-binding receptor Tlp3, also termed CcmL (*Campylobacter* chemoreceptor for multiple ligands; Rahman *et al.*, 2014), and the formic acid receptor Tlp7 (Tareen *et al.*, 2010) are present in most sequenced strains of *C. jejuni*, whereas the galactose receptor Tlp11 is the least common and has only been found in clinical isolates from patients hospitalized owing to *C. jejuni* infection (Day *et al.*, 2012, 2014).

The sensory domain of CcmL, which has been shown to bind multiple ligands including isoleucine, lysine, glucosamine, aspartate, succinic acid, arginine, malic acid, thiamine and sodium deoxycholate, shares no significant sequence similarity to chemoreceptors from non-Epsilonproteobacteria (Rahman *et al.*, 2014; Li *et al.*, 2014). This suggests that the mechanism of ligand recognition by CcmL may be unique to *Campylobacter* spp. and *Campylobacter*-related organisms. Furthermore, a BLAST sequence-similarity search against structures deposited in the Protein Data Bank (PDB) identified no close structural homologues of this domain. However, a Pfam analysis (Finn *et al.*, 2014) of the CcmL primary sequence revealed the presence of a Cache_1 (calcium channels and chemotaxis receptors) motif located between residues 163 and 237 (Anantharaman & Aravind, 2000; Rahman *et al.*, 2014). The Cache motif is a signature of double-PAS sensor domains found in family 1 histidine kinases (Zhang & Hendrickson, 2010) and chemotaxis receptors (Nishiyama *et al.*, 2012). In agreement with this, a search against the PDB using pairwise comparison of profile hidden Markov models implemented on the HHpred server (<http://toolkit.tuebingen.mpg.de/hhpred#>; Söding *et al.*, 2005) identified remote homology at the level of secondary structure (but not sequence) between the sensory domain of CcmL and the double-PAS periplasmic domains of the putative *Vibrio cholerae* chemoreceptor Mlp37 (PDB entry 3c8c; New York SGX Research Center for Structural Genomics, unpublished work) and family 1 histidine kinases (PDB entries 3lib and 3li9; Zhang & Hendrickson, 2010). This permits tentative assignment of the periplasmic domain of CcmL to the recently described novel family of tandem-PAS chemoreceptor sensing domains (Glekas *et al.*, 2010; Nishiyama *et al.*, 2012).

We have recently reported the crystallization of the periplasmic sensory domain of Tlp1 (CcaA) as part of a study to identify the structural features that determine its ligand specificity (Machuca *et al.*, 2015). Tlp1 does not appear to have

Table 1

Macromolecule-production information.

Source organism	<i>C. jejuni</i> serotype O:2 (strain NCTC 11168)
DNA source	Plasmid pGU0816 (Rahman <i>et al.</i> , 2014)
Forward primer	CACCAAAACCTCACTATATGAAAGCAC
Reverse primer	CTCGAGTTAAGCTTTATAAATAGGTTTATTATATAAT
Cloning vector	pET151/D-TOPO
Expression vector	pET151/D-TOPO
Expression host	<i>E. coli</i> strain BL21-CodonPlus (DE3)-RIPL
Complete amino-acid sequence of the construct produced†	MHHHHHGKPIPNPLLGLDSTENLYFQgidpftK-TSLYESTLKNQTDLLKVTQSTVEDFRSTNQSF-TRALEKDIAINLPYQSLITEENIINNVPILKY-YRHSINALNVYGLNNGKVLLSQKSNDKMPERLDDLDIKTKDWYQEALKTNDIFVTPAYLDTV-LKQYVITYSKAIYKDGKIIIGVLGVDIPSEDLQ-NLVAKTPGNTFLFDQKNKIFAATNKELNPSI-DHSPVLNAYKLNNDNFFSYKLNNEERLGACT-KVFAYTACITESADIINKPIYKA

† The TEV cleavage site is underlined and the additional sequence at the N-terminus (cloning artifact) is shown in lower case.

a Cache motif. In this paper, we report the expression, refolding from inclusion bodies, purification and crystallization of the periplasmic domain of CcmL (amino-acid residues 42–291). The determination of the first crystal structure of a double-PAS sensory domain from a characterized chemoreceptor will help us to understand the structural basis of ligand recognition in this novel class of receptor-sensing domains.

2. Materials and methods

2.1. Cloning, protein expression, solubilization of inclusion bodies, protein refolding and purification

The DNA fragment encoding the periplasmic sensory domain of CcmL (CcmL^{peri}; amino-acid residues 42–291; UniProtKB Q0P864; also known as Tlp3 or Cj1564) was amplified from plasmid pGU0816 (Rahman *et al.*, 2014). The PCR reaction was carried out using *Pfu* DNA polymerase (Stratagene) and the primers CACCAAAACCTCACTATATGAAAGCAC (forward) and CTCGAGTTAAGCTTTATAAATAGGTTTATTATATAAT (reverse). The amplified fragment was ligated into the pET151/D-TOPO vector using the TOPO cloning kit (Invitrogen) to produce an expression construct that contained an N-terminal His₆ tag. The correct insertion of the fragment was confirmed by DNA sequencing.

For recombinant protein expression, *E. coli* BL21-CodonPlus(DE3)-RIPL (Stratagene) cells were transformed with the vector and grown at 37°C in LB medium containing 50 µg ml⁻¹ ampicillin until the OD_{600 nm} reached 0.6, at which point expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside. The cells were grown for a further 4 h and harvested by centrifugation at 6000g for 15 min at 4°C. The cell pellet was resuspended in buffer A consisting of 10 mM Tris-HCl pH 8.0 and lysed using an Avestin Emulsi-Flex-C5 high-pressure homogenizer. The lysate was centrifuged at 10 000g for 15 min at 4°C. Analysis of the supernatant and pellet using SDS-PAGE showed that the recombinant

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	15
Composition of reservoir solution	19%(w/v) polyethylene glycol 3350, 100 mM sodium citrate pH 5.0, 200 mM ammonium sulfate

protein was predominantly deposited in inclusion bodies. Details of macromolecule production are summarized in Table 1.

To isolate CcmL^{peri} from inclusion bodies, we used a refolding procedure that we recently developed for the production of a soluble, crystallizable form of the periplasmic sensory domain of the bacterial chemoreceptor TlpC (Liu & Roujeinikova, 2015), with minor modifications. The inclusion-bodies pellet was washed three times with buffer *B* [10 mM Tris-HCl pH 8.0, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 1% Triton X-100] and once in buffer *C* (10 mM Tris-HCl pH 8.0, 0.2 mM PMSF). The inclusion bodies were solubilized in buffer *D* [10 mM Tris-HCl pH 8.0, 8 M urea, 10 mM dithiothreitol (DTT), 0.2 mM PMSF] for 30 min at 4°C with mixing by axial rotation. The mixture was then clarified by centrifugation at 30 000g for 30 min at 4°C and the protein concentration in the supernatant was measured using the Bradford assay (Bradford, 1976). 60 mg of denatured protein was refolded by dilution into 250 ml buffer *E* (3 M urea, 100 mM Tris-HCl pH 8.0, 0.4 M L-arginine monohydrochloride, 20 mM reduced L-glutathione, 2 mM oxidized L-glutathione) followed by a 48 h incubation at 4°C with continuous mixing. The sample was then dialyzed against 5 l buffer *A* for 16 h at 4°C. NaCl, Tris-HCl pH 8.0 and imidazole were added to the protein solution to final concentrations of 500, 20 and 20 mM, respectively, and the sample was loaded

onto a 5 ml HiTrap Chelating HP column (GE Healthcare) pre-washed with buffer *F* (500 mM NaCl, 20 mM Tris-HCl pH 8.0, 20 mM imidazole). The column was washed with 30 column volumes of the same buffer and the protein was eluted with buffer *G* (500 mM NaCl, 20 mM Tris-HCl pH 8.0 and 500 mM imidazole).

The tag was cleaved off with His₆-TEV protease (Invitrogen) whilst dialysing the sample against buffer *H* [150 mM NaCl, 10 mM Tris-HCl pH 8.0, 2 mM DTT, 1%(v/v) glycerol]. NaCl and imidazole were then added to the sample to final concentrations of 500 and 20 mM, respectively, and the TEV protease and uncleaved protein were removed on a HiTrap Chelating HP column. The flowthrough was concentrated to 400 µl in an Amicon Ultra Ultracel 10 kDa cutoff concentrator and passed through a Superdex 200 HiLoad 26/60 gel-filtration column (GE Healthcare) equilibrated with buffer *I* (10 mM Tris-HCl pH 8.0, 150 mM NaCl). The protein purity was judged to be greater than 90% (Fig. 1).

2.2. Crystallization

CcmL^{peri} was concentrated to 15 mg ml⁻¹ in buffer *I*, incubated for 30 min with 8 mM isoleucine and centrifuged for 20 min at 13 000g to clarify the solution. Initial screening of crystallization conditions was carried out by the hanging-drop vapour-diffusion method using a Phoenix crystallization robot (Art Robbins Instruments) and the screens JBScreen HTS I and II (Jena Bioscience), The JCSG+ Suite (Qiagen), Crystal Screen HT and PEG/Ion HT (Hampton Research). The droplets in the initial crystallization trials consisted of 200 nl protein solution mixed with 200 nl reservoir solution and were equilibrated against 50 µl reservoir solution in a 96-well Art Robbins CrystalMation Intelli-Plate (Hampton Research). Crystals appeared after 1 d from condition E4 of JBScreen HTS I (Fig. 2), which consisted of 25%(w/v) polyethylene glycol 4000, 100 mM sodium citrate pH 5.6, 200 mM ammonium sulfate. Subsequent refinement of this condition using 2 µl hanging drops suspended over 500 µl reservoir solution

Figure 1
Coomassie Blue-stained 12% SDS-PAGE gel of pure, untagged recombinant CcmL^{peri} used for the crystallization and SEC-MALS experiments. Lane *M* contains molecular-weight marker (labelled in kDa).

Figure 2
Crystals of a putative CcmL^{peri} complex with isoleucine.

yielded an optimal crystallization reservoir solution consisting of 19% (w/v) polyethylene glycol 3350, 100 mM sodium citrate pH 5.0, 200 mM ammonium sulfate (Table 2).

2.3. X-ray diffraction data collection and processing and size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) analysis

The CcmL^{peri} crystal was washed for 1 s in a cryoprotectant solution consisting of 24% (w/v) polyethylene glycol 3350, 100 mM sodium citrate pH 5.0, 200 mM ammonium sulfate, 10% (v/v) glycerol, 10 mM isoleucine and was flash-cooled by plunging it into liquid nitrogen. X-ray data were collected to 1.3 Å resolution on the MX2 beamline at the Australian Synchrotron (Fig. 3). A total of 360 images were collected using a 0.5° oscillation width. The data were processed using *iMosflm* (Battye *et al.*, 2011) and *AIMLESS* (Evans & Murshudov, 2013) from the *CCP4* suite (Winn *et al.*, 2011). Calculation of the self-rotation function was performed using *POLARRFN* (Winn *et al.*, 2011). Statistics for data collection and processing are presented in Table 3.

The absolute hydrated molecular mass of CcmL^{peri} in solution was determined by MALS analysis coupled to size-exclusion chromatography (SEC). CcmL^{peri} was dialysed against buffer *J* (100 mM Tris–HCl pH 8.0, 150 mM NaCl) and concentrated to 100 µM. A 100 µl sample was loaded onto a WTC-030S5 size-exclusion column (Wyatt) pre-equilibrated with buffer *J* flowing at 0.4 ml min^{−1}. The eluate was passed through an in-line DAWN HELEOS light-scattering detector, an Optilab T-rEX differential refractive-index detector

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	MX2 beamline, Australian Synchrotron
Wavelength (Å)	0.95
Temperature (K)	100
Detector	ADSC Quantum 315r CCD
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	<i>P2</i> ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	42.6, 138.0, 49.0
α , β , γ (°)	90.0, 94.3, 90.0
Mosaicity (°)	0.4
Resolution range (Å)	40.0–1.3 (1.32–1.30)
Total No. of reflections	441329 (10462)
No. of unique reflections	128260 (4046)
Completeness (%)	93 (59)
Multiplicity	3.4 (2.6)
$\langle I/\sigma(I) \rangle$	12.2 (2.1)
<i>R</i> _{int}	0.045 (0.295)
Overall <i>B</i> factor from Wilson plot (Å ²)	11.9

(Wyatt) and a quasi-elastic light-scattering detector (Wyatt-QELS, Wyatt). A bovine serum albumin (BSA) standard was used to normalize the MALS detectors. For calculations of the molecular mass and hydrodynamic radius, the light-scattered intensity and the refractive index were analyzed using *ASTRA* 6.0 (Wyatt), with a value for the refractive-index increment (dn/dc) for the protein of 0.185 ml g^{−1}.

3. Results and discussion

Recombinant CcmL^{peri} was expressed with a cleavable N-terminal His₆ tag from the pET151/D-TOPO plasmid in *E. coli* BL21(DE3)-RIPL cells upon induction of T7 polymerase. The expression predominantly resulted in protein deposition in inclusion bodies (approximately 100 mg CcmL^{peri}-His₆ in the form of washed inclusion bodies per litre of culture). CcmL^{peri}-His₆ was isolated from inclusion bodies by following the refolding procedure for the production of a soluble, crystallizable form of periplasmic sensory domains of bacterial chemoreceptors that we have recently reported (Liu & Roujeinikova, 2015). Refolding and purification of protein from the inclusion bodies (including tag removal) resulted in >90% electrophoretic homogeneity based on Coomassie Blue staining of SDS–PAGE gels (Fig. 1). Approximately 3 mg pure untagged CcmL^{peri} was obtained per litre of culture. The recombinant protein used for the preliminary analysis and crystallization comprised amino-acid residues 42–291 of CcmL plus six additional residues from the TEV cleavage site and the vector (GIDPFT). The protein migrated on SDS–PAGE with an apparent molecular weight of 26 kDa, which is close to the value calculated from the amino-acid sequence (28.7 kDa).

To determine the oligomeric state of CcmL^{peri} in solution, multi-angle light-scattering (MALS) analysis coupled to size-exclusion chromatography was carried out. Both in the presence and in the absence of 10 mM isoleucine, CcmL^{peri} eluted as a single symmetrical peak with a polydispersity index value close to 1 (data not shown), indicating that the eluted

Figure 3

A representative 0.5° oscillation image of the data collected using an ADSC Quantum 315r CCD detector at station MX2 at the Australian Synchrotron, Victoria, Australia. The magnified rectangle shows diffraction spots between 1.4 and 1.6 Å resolution.

particles were homogenous with respect to molar mass. The elution volumes with and without isoleucine were almost identical. The derived molecular weight was 27 kDa, indicating that the periplasmic sensory domain of CcmL is monomeric under the tested buffer conditions. Although methyl-accepting chemoreceptor proteins function as dimers and often possess a dimeric sensory domain (Hazelbauer *et al.*, 2008), we note that, like CcaA^{peri}, CcmL^{peri} shows no significant sequence similarity to any of the characterized chemoreceptors and hypothesize that dimerization of CcaA and CcmL may be primarily driven by dimerization of the trans-membrane and cytoplasmic domains. There is a different reported example of a sensory domain of a dimeric methyl-accepting chemoreceptor (specifically, a *Salmonella typhimurium* aspartate chemoreceptor) that has been observed in solution as both monomers and dimers, with dimerization being dependent on protein concentration (Milligan & Koshland, 1993).

Crystals of the putative CcmL^{peri} complex with isoleucine (Fig. 2) were obtained using a sparse-matrix crystallization approach. Analysis of the test data collected from a cryo-cooled CcmL^{peri} crystal using the Australian Synchrotron indicated that these crystals diffracted to at least 1.3 Å resolution (Table 3). Autoindexing using *iMosflm* showed that they belonged to a primitive monoclinic space group, with unit-cell parameters $a = 42.6$, $b = 138.0$, $c = 49.0$ Å, $\beta = 94.3^\circ$. Data analysis using *POINTLESS* from the *CCP4* suite showed systematic absences along the $0k0$ axis, with only reflections with $k = 2n$ present, which suggested that the crystals belonged to space group $P2_1$. Calculations of the Matthews coefficient for two molecules in the asymmetric unit gave a value of $2.5 \text{ Å}^3 \text{ Da}^{-1}$, which lies in the range observed for protein crystals (Matthews, 1977; Kantardjieff & Rupp, 2003). In a self-rotation function calculated using data in the resolution range 10–6 Å with an integration radius of 25 Å, no dominant features that can be confidently assigned to noncrystallographic axes were found in the $\kappa = 90$, 120 or 180° sections. Thus, we are currently unable to determine whether the asymmetric unit contains a symmetric dimer or two independent monomers. A search for heavy-atom derivatives and the determination of the structure using the single- or multiple-wavelength anomalous dispersion method is under way.

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