crystallization communications

Preliminary structural studies of the transcriptional regulator CmeR from *Campylobacter jejuni*

Chih-Chia Su,[a] Feng Shi,[b] Ruoyu Gu,[c] Ming Li,[c] Gerry McDermott,[d] Edward W. Yu[a,c]* and Qijing Zhang[b]

[a]Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011, USA, [b]Department of Veterinary Microbiology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA, [c]Department of Physics and Astronomy, Iowa State University, Ames, IA 50011, USA, and [d]Department of Anatomy, School of Medicine, University of California, San Francisco, CA 94143, USA

Correspondence e-mail: ewyu@iastate.edu

In *Campylobacter jejuni*, a Gram-negative bacterial pathogen causing gastro-enteritis in humans, the CmeR regulatory protein controls transcription of the multidrug transporter gene operon *cmeABC*. CmeR belongs to the TetR family of transcriptional regulators. The 210-residue CmeR consists of two functional motifs: an N-terminal DNA-binding domain and a C-terminal ligand-binding domain. It is predicted that the DNA-binding domain interacts directly with target promoters, while the C-terminal motif interacts with inducing ligands (such as bile salts). As an initial step towards confirming this structural model, recombinant CmeR protein containing a 6×His tag at the N-terminus was crystallized. Crystals of ligand-free CmeR belonged to space group $P2_12_12$, with unit-cell parameters $a = 37.4$, $b = 57.6$, $c = 93.3$ Å. Diffraction was observed to at least 2.2 Å at 100 K. Analysis of the detailed CmeR structure is currently in progress.

1. Introduction

Campylobacter jejuni is the leading bacterial cause of foodborne diarrhea in the United States and other developed countries (Friedman *et al.*, 2000). It is also a significant enteric pathogen for young children in developing countries. This Gram-negative enteric organism colonizes the intestinal tracts of animals and has become increasingly resistant to antimicrobials owing to its possession of multidrug efflux transporters and its acquisition of various resistance mechanisms, compromising the effectiveness of antibiotic treatment. According to the genomic sequence of NCTC 11168, *C. jejuni* harbors 13 putative antibiotic efflux transporters of the ATP-binding cassette (ABC), resistance–nodulation–division (RND), multidrug and toxic compound extrusion (MATE), major facilitator (MF) and small multidrug-resistance (SMR) families (Parkhill *et al.*, 2000; Lin, Akiba & Zhang, 2005). At present, CmeABC and CmeDEF, which belong to the RND family, are the only two antibiotic efflux transporters that have been functionally characterized in *Campylobacter* (Akiba *et al.*, 2006; Lin *et al.*, 2002; Pumbwe & Piddock, 2002).

The CmeABC efflux system consists of three members, including an outer membrane channel (CmeC), an inner membrane drug transporter (CmeB) and a periplasmic membrane-fusion protein (CmeA). These three proteins are encoded by a three-gene operon (*cmeABC*) and form an efflux system that extrudes a variety of toxic compounds directly out of *C. jejuni* (Lin *et al.*, 2002). The substrates extruded by CmeABC include commonly used antibiotics (*e.g.* fluoroquinolones, macrolides, ampicillin, tetracycline, chloramphenicol, cefotaxime and rifampin), ions (*e.g.* Co^{2+} and Cu^{2+}) and lipophilic compounds (*e.g.* SDS and various bile salts). Thus, CmeABC contributes significantly to the intrinsic and acquired resistance of *Campylobacter* to structurally diverse antimicrobials (Cagliero *et al.*, 2005; Lin *et al.*, 2002; Luo *et al.*, 2003; Pumbwe & Piddock, 2002). In addition, this efflux system is also essential for *Campylobacter* colonization of the animal intestinal tract by conferring resistance to bile acids (Lin *et al.*, 2003), which are normally present in the animal intestinal tract and have bactericidal effect.

The expression of *cmeABC* is controlled by the transcriptional regulator CmeR (Lin, Akiba, Sahin *et al.*, 2005). The *cmeR* gene is located immediately upstream of the *cmeABC* operon and encodes a 210-residue protein that shares N-terminal sequence and structural
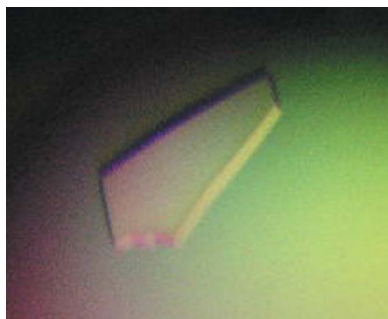
similarities with members of the TetR family of transcriptional repressors (Grkovic *et al.*, 2002; Ramos *et al.*, 2005). Like other members of the TetR family, the N-terminal domain of CmeR contains a predicted DNA-binding helix–turn–helix (HTH) motif, while its C-terminal region contains unique sequences and is expected to be involved in the binding of inducing ligands (Grkovic *et al.*, 2002; Lin, Akiba, Sahin *et al.*, 2005). *cmeR* is transcribed in the same direction as *cmeABC* and the intergenic region between *cmeR* and *cmeA* contains the inverted-repeat (IR) operator site for *cmeABC*. As a transcriptional regulator, CmeR binds directly to the IR operator and represses the transcription of *cmeABC* (Lin, Akiba, Sahin *et al.*, 2005). Deletion of *cmeR* or mutations in the IR operator release the repression, resulting in the overexpression of CmeABC, which in turn leads to the enhanced resistance to multiple antibiotics. In addition, bile compounds, including both conjugated (*e.g.* taurodeoxycholate) and nonconjugated (*e.g.* cholate), induce the expression of *cmeABC* by inhibiting the binding of CmeR to the promoter of *cmeABC* (Lin, Cagliero *et al.*, 2005), suggesting that bile compounds are inducing ligands of CmeR.

The hypothesis is that binding of inducing ligands to the C-terminal domain of CmeR triggers conformational change in the N-terminal DNA-binding region. This change in conformation results in the release of CmeR from its operator DNA and thus allows transcription from its cognate promoter. As an initial step to elucidate the mechanisms that CmeR uses to regulate gene expression, we here report the crystallization and preliminary X-ray diffraction analysis of the CmeR repressor.

2. Materials and methods

2.1. Protein purification and crystallization

Recombinant CmeR was produced in *Escherichia coli* using the pQE30 vector (Qiagen). The cloning, expression and purification procedures have been described previously (Lin *et al.*, 2002, 2003; Lin, Akiba, Sahin *et al.*, 2005). This recombinant CmeR, containing a 6×His tag at the N-terminus, is fully functional in DNA binding *in vitro* and is inducible by bile salts. After purification, the purity of the protein was judged using 10% SDS–PAGE stained with Coomassie Brilliant Blue. The purified protein was extensively dialyzed against buffer containing 10 mM sodium phosphate pH 7.2 and 100 mM NaCl and then concentrated to 10 mg ml^{-1}.

Selenomethionyl-CmeR (SeMet-CmeR) containing a 6×His tag at the N-terminus was overproduced in *E. coli* JM109 cells. Briefly, a
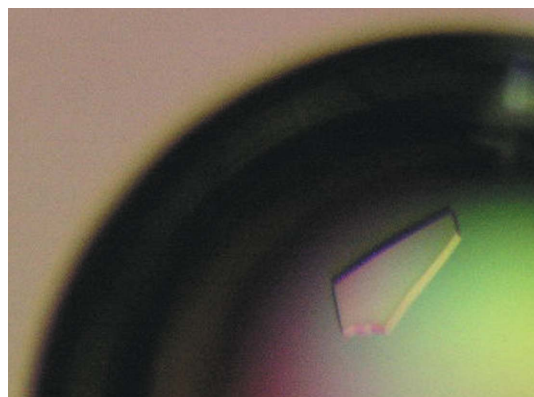

Figure 1
Image of the CmeR crystal. The native crystal was grown using 22% PEG 4000, 0.1 M Tris pH 8.5, 0.2 M MgCl$_2$ and 4% glycerol.

Table 1
Data collection and crystallographic analysis of CmeR.

Values in parentheses are for the highest resolution shell.

| | Native | SeMet | | |
		Inflection point	Peak	Remote
Data collection				
Wavelength (Å)	0.9795	0.9798	0.9795	0.9662
Space group	$P2_12_12$	$P2_12_12$		
Unit-cell parameters (Å)	$a = 37.4$, $b = 57.6$, $c = 93.3$	$a = 37.3$, $b = 57.5$, $c = 93.0$		
Resolution (Å)	2.24 (2.33–2.24)	2.10 (2.18–2.10)	2.07 (2.28–2.07)	2.07 (2.14–2.07)
Completeness (%)	99.6 (97.7)	97.6 (85.1)	93.4 (84.1)	97.2 (80.3)
Total No. of reflections	297224	168174	179712	179712
No. of unique reflections	10172	12493	14087	12897
R_{sym} (%)	5.5 (26.2)	5.8 (25.0)	5.2 (26.4)	5.1 (27.3)
Average $I/\sigma(I)$	16.6 (4.7)	23.3 (3.8)	22.3 (5.3)	18.2 (3.2)
Phasing				
Se-atom sites		3		
Resolution range of data used (Å)		50–2.80		
Overall figure of merit		0.59		

10 ml LB overnight pre-culture was transferred into 100 ml LB medium containing 100 µg ml^{-1} ampicillin. The cell culture was grown at 310 K and 210 rev min^{-1}. When the OD$_{600}$ value was around 1.2, cells were harvested by centrifugation at 6000 rev min^{-1} for 10 min and then washed two times with 5 ml M9 solution. The cells were resuspended in 10 ml M9 solution and then transferred into 1 l pre-warmed M9 solution containing 100 µg ml^{-1} ampicillin. The cell culture was incubated at 310 K and 210 rev min^{-1}. When the OD$_{600}$ reached 0.4, 100 mg lysine, phenylalanine and threonine, 50 mg isoleucine, leucine and valine and 60 mg L-selenomethionine were added. The culture was induced with 1 mM IPTG after 15 min. Cells were harvested within 4 h and were frozen and stored at 193 K. The purification procedure for the SeMet-CmeR protein was the same as that for native CmeR. The 210-amino-acid CmeR contains three methionines; replacement of these methionine sulfurs with seleniums in the SeMet-CmeR protein was confirmed by MALDI time-of-flight mass spectrometry.

The 6×His CmeR protein was crystallized in 24-well plates using the hanging-drop vapor-diffusion method at 293 K. Initial crystallization screening was performed using commercially available sparse-matrix screens (Jancarik & Kim, 1991) from Hampton Research. Briefly, a 2 µl mixture consisting of 1 µl protein solution (10 mg ml^{-1} CmeR in 10 mM sodium phosphate pH 7.2 and 100 mM NaCl) and 1 µl reservoir solution was equilibrated against 500 µl reservoir solution. Crystals of CmeR appeared within two weeks. After optimization, the best native 6×His CmeR crystals were obtained from reservoir solution containing 22% PEG 4000, 0.1 M Tris pH 8.5, 0.2 M MgCl$_2$ and 4% glycerol. Cryoprotection was achieved by raising the PEG 4000 concentration stepwise to 35% with a 5% increment in each step. Crystals of the SeMet-CmeR protein were grown under the same conditions.

2.2. Data collection and processing

For data collection, a single native crystal was flash-cooled in a cryoprotectant solution containing 35% PEG 4000, 7% glycerol, 0.2 M MgCl$_2$ and 0.1 M Tris buffer pH 8.5 at 100 K. The best diffraction of the native CmeR crystal was obtained to at least 2.2 Å resolution at a cryogenic temperature of 100 K using a synchrotron light source (Table 1). Multiple-wavelength anomalous diffraction (MAD) data were collected from a single SeMet-CmeR crystal to a

maximum resolution of 2.1 Å (Table 1). Diffraction data sets from both the native and SeMet-CmeR crystals were taken at the Advanced Light Source (beamline 8.2.2) at cryogenic temperature (100 K) using an ADSC Quantum 315 CCD-based detector. The beam size was 140 × 150 µm. Diffraction data sets were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Using hanging-drop vapor diffusion, we have successfully grown a large quantity of CmeR crystals. The dimensions of the crystals were typically 0.4 × 0.4 × 0.1 mm (Fig. 1). The crystals of 6×His CmeR belonged to space group $P2_12_12$, with unit-cell parameters a = 37.4, b = 57.6, c = 93.3 Å, $\alpha = \beta = \gamma = 90°$.

The SeMet crystal belonged to space group $P2_12_12$, with unit-cell parameters very similar to those of the native crystal (Table 1). The three-wavelength anomalous dispersion (MAD) data were scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Phase calculation was carried out at 2.8 Å resolution using the program *BnP* (Weeks *et al.*, 2005) after finding and refinement of all three selenium sites. The electron-density map obtained was improved by density modification (*DM*) using *RESOLVE* (Terwilliger, 2001). Fig. 2 shows the MAD electron-density map improved by *DM* and the initial C^α protein trace.

The available structures of members of the TetR family of transcriptional repressors, including those of TetR (Hinrichs *et al.*, 1994; Orth *et al.*, 2000), QacR (Schumacher *et al.*, 2001, 2002), CprB (Natsume *et al.*, 2003) and EthR (Dover *et al.*, 2004; Frenois *et al.*,

2004), indicate that all these repressors assemble as dimers. Recently, the preliminary X-ray diffraction data of AcrR (another member of the TetR family) also suggest a dimeric assembly of the repressor (Li *et al.*, 2006). Thus, it is likely that the structure of CmeR is also dimeric.

The *MC* program (Matthews, 1968, 1977) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) suggests that the crystals are likely to contain one CmeR monomer in the asymmetric unit, which gives a Matthews value V_M of 2.0 Å^3 Da^{-1} and a solvent content of 36.9%. By applying the crystallographic symmetry operators, a dimeric arrangement of the protein was found. Analysis of the structure is currently in progress.

References

Akiba, M., Lin, J., Barton, Y. W. & Zhang, Q. J. (2006). *J. Antimicrob. Chemother.* **57**, 52–60.
Cagliero, C., Mouline, C., Payot, S. & Cloeckaert, A. (2005). *J. Antimicrob. Chemother.* **56**, 948–950.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dover, L. G., Corsino, P. E., Daniels, I. R., Cocklin, S. L., Tatituri, V., Besra, G. S. & Fütterer, K. (2004). *J. Mol. Biol.* **340**, 1095–1105.
Frenois, F., Engohang-Ndong, J., Locht, C., Baulard, A. R. & Villeret, V. (2004). *Mol. Cell*, **16**, 301–307.
Friedman, C. R., Neimann, J., Wegener, H. C. & Tauxe, R. V. (2000). *Campylobacter*, edited by I. Nachamkin & M. J. Blaser, pp. 121–138. Washington DC: ASM Press.
Grkovic, S., Brown, M. H. & Skurray, R. A. (2002). *Microbiol. Mol. Biol. Rev.* **66**, 671–701.
Hinrichs, W., Kisker, C., Duvel, M., Muller, A., Tovar, K., Hillen, W. & Saenger, W. (1994). *Science*, **264**, 418–420.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Li, M., Qiu, X., Su, C.-C., Long, F., Gu, R., McDermott, G. & Yu, E. W. (2006). *Acta Cryst.* F**62**, 1150–1152.
Lin, J., Akiba, M., Sahin, O. & Zhang, Q. (2005). *Antimicrob. Agents Chemother.* **49**, 1067–1075.
Lin, J., Akiba, M. & Zhang, Q. (2005). *Campylobacter: Molecular and Cell Biology*, edited by J. M. Ketley & M. E. Konkel, pp. 205–218. Norwich: Horizon Bioscience.
Lin, J., Cagliero, C., Guo, B., Barton, Y. W., Maurel, M. C., Payot, S. & Zhang, Q. (2005). *J. Bacteriol.* **187**, 7417–7424.
Lin, J., Michel, L. O. & Zhang, Q. (2002). *Antimicrob. Agents Chemother.* **46**, 2124–2131.
Lin, J., Sahin, O., Michel, L. O. & Zhang, Q. (2003). *Infect. Immun.* **71**, 4250–4259.
Luo, N., Sahin, O., Lin, J., Michel, L. O. & Zhang, Q. (2003). *Antimicrob. Agents Chemother.* **47**, 390–394.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Matthews, B. W. (1977). *The Proteins*, edited by H. Neurath & R. L. Hill, Vol. 3, pp. 468–477. New York: Academic Press.
Natsume, R., Ohnishi, Y., Senda, T. & Horinouchi, S. (2003). *J. Mol. Biol.* **336**, 409–419.
Orth, P., Schnappinger, D., Hillen, W., Saenger, W. & Hinrichs, W. (2000). *Nature Struct. Biol.* **7**, 215–219.
Otwinowski, Z. & Minor, M. (1997). *Methods Enzymol.* **276**, 307–326.
Parkhill, J. *et al.* (2000). *Nature (London)*, **403**, 665–668.
Pumbwe, L. & Piddock, L. J. (2002). *FEMS Microbiol. Lett.* **206**, 185–189.
Ramos, J. L., Martinez-Bueno, M., Molina-Henares, A. J., Teran, W., Watanabe, K., Zhang, X. D., Gallegos, M. T., Brennan, R. & Tobes, R. (2005). *Microbiol. Mol. Biol. Rev.* **69**, 326–356.
Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A. & Brennan, R. G. (2001). *Science*, **294**, 2158–2163.
Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A. & Brennan, R. G. (2002). *EMBO J.* **21**, 1210–1218.
Terwilliger, T. C. (2001). *Acta Cryst.* D**57**, 1755–1762.
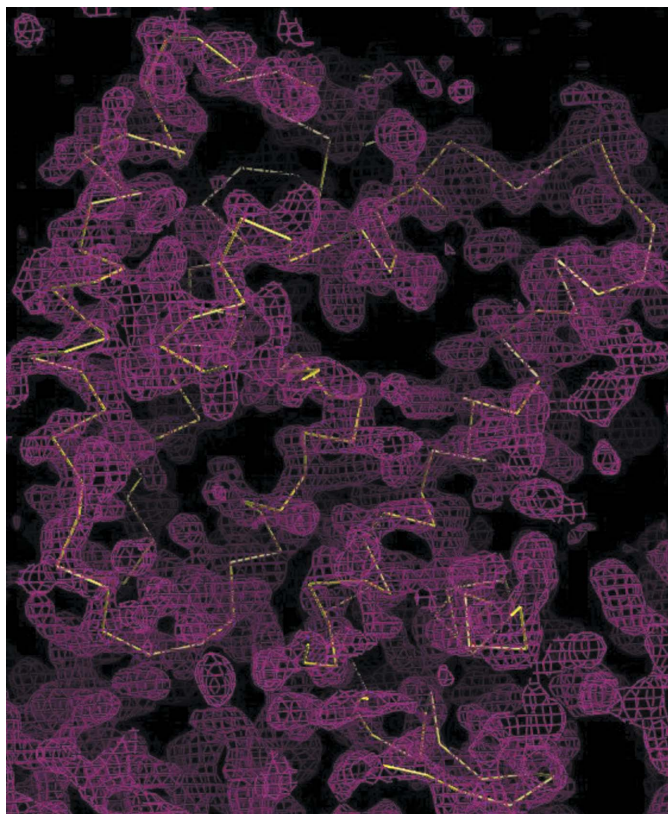Weeks, C. M., Shah, N., Green, M. L., Miller, R. & Furey, H. (2005). *Acta Cryst.* A**61**, C152.

Figure 2
MAD electron-density map of CmeR contoured at 1σ. The C^α plot of one subunit of CmeR is shown in yellow.